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The quaking I pathway and regulation of alternative splicing in myelinating glia

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The quaking I pathway and regulation of alternative splicing in myelinating glia

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An abstract of
a dissertation submitted to the Faculty of the
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Abstract

The quaking I pathway and regulation of alternative splicing in myelinating glia

By Mariana Dalit Mandler

Splicing, exon ligation and intron removal from pre-mRNA, is fundamental for protein production in eukaryotic cells. Alternative splicing (AS), the differential inclusion of exons to increase proteome diversity, occurs for 95% of expressed mammalian genes. AS is rigorously regulated during normal cell growth and development, particularly in the central nervous system (CNS). Dysregulation of AS is observed in several types of cancer, and is implicated in psychiatric disorders and neurodegenerative diseases (NDDs). Specifically, numerous genes expressed in oligodendroglia (OLs), the myelinating glia of the CNS, undergo extensive regulation by AS. However, the underlying mechanisms are poorly understood. The selective RNA-binding protein quaking I (QKI) is critical for AS in OLs. Three main QKI isoforms display differential nuclear-cytoplasmic distribution, nuclear QKI-5, and cytoplasmic QKI-6 and 7, and play distinct functions. QKI deficiency in OLs results in severe AS defects. However, how QKI controls AS remains elusive. Additionally, mechanism(s) that determine QKI isoform expression are unknown.

I found that QKI deficiency results in abnormal upregulation of splicing factors (SFs) in OLs that cause aberrant AS, which are rescued by the cytoplasmic QKI-6. QKI-6 targets the mRNAs of SFs directly or likely involving microRNAs, and inhibits mRNA translation, which contributes to the developmental downregulation of SFs during OL differentiation. Some QKI-target SFs are also implicated in cancer and NDDs.

During differentiation of OL progenitor cells, QKI mRNA and protein isoforms are differentially up-regulated, suggesting translation regulation of QKI isoform expression during OL differentiation. In contrast, QKI mRNA isoforms display different profiles in glial and neuronal progenitors, suggesting regulation of QKI mRNA biogenesis and/or stability during neural lineage specification. Finally, I identified a role for the SF FOX2 in controlling QKI-7 mRNA levels in OLs.

These studies unveiled novel pathways controlling AS in myelinating glia. I provided the first evidence that QKI-6 is a major regulator of SF expression dictating AS in OLs. Furthermore, I uncovered potential mechanisms that underlie QKI isoform expression during neuron-glia cell fate specification and OL differentiation. Future investigation will define the role of QKI in cancer and brain disorders that harbor QKI deficiency and AS abnormalities.

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List of Abbreviations

A2BP1: ataxin 2-binding protein 1

aa: amino acid

AD: Alzheimer's disease

AKT: protein kinase B

ALS: amyotrophic lateral sclerosis

AMP: adenosine monophosphate

AON: antisense oligonucleotide

APA: alternative poly-adenylation

AS: alternative splicing

ATXN2: ataxin-2

Bcl2: B-cell lymphoma 2

Capzb: Capping Protein (Actin Filament) Muscle Z-Line, Beta

CD45: cluster of differentiation 45

Cdk2: cyclin-dependent kinase 2

CFTR: cystic fibrosis transmembrane regulator

CMT: Charcot-Marie tooth disease

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

CNS: central nervous system

c-src: v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog

Ct: cycle threshold

CTCF: CCCTC-Binding Factor (Zinc Finger Protein)

DISC1: disrupted in schizophrenia 1

DMD: duchenne muscular dystrophy

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

DTT: dithiothreitol

ECL: enhanced chemiluminescence

EGFR: epidermal growth factor receptor

eIF5α: eukaryotic translation initiation factor 5α

EMSA: electrophoretic mobility shift assay

ENU: N-Ethyl-N-Nitrosourea

ENY2: enhancer of yellow 2 homolog

ErbB4: V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 4

ESE: exon splicing enhancer

ESS: exon splicing suppressor

FBS: fetal bovine serum

FRAP: fluorescence recovery after photobleaching

FTLD: frontotemporal lobar degeneration

GABA: gamma-aminobutyric acid

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GBM: glioblastoma multiforme

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

hESC: human embryonic stem cells

hnRNP: heterogeneous nuclear ribonucleoprotein

Ig: immunoglobulin

IR: insulin receptor

ISE: intron splicing enhancer

ISS: intron splicing suppressor

KEGG: Kyoto Encyclopedia of Genes and Genomes

KH: hnRNP K homology

L-MAG: large myelin-associated glycoprotein

MADD: MAP-kinase activating death domain protein

MAG: myelin-associated glycoprotein

MBP: myelin basic protein

MBNL1: muscleblind-like splicing regulator 1

MDM: mouse double minute

miRNA: micro ribonucleic acid

MOG: myelin oligodendrocyte glycoprotein

MPZL1: myelin protein zero-like 1

mRNA: messenger ribonucleic acid

MS: multiple sclerosis

NADH: reduced form of nicotinamide adenine dinucleotide

NDD: neurodegenerative disease

NMD: nonsense-mediated decay

NMR: nuclear magnetic resonance

NLS: nuclear localization signal

Nova: neuro-oncological ventral antigen 1

NRS: negative regulator of splicing

NSC: neural stem cell

nSR100: neural-specific serine-arginine-related protein 100

OL: oligodendroglia

Olig1: oligodendroglia-specific transcription factor 1

OPC: oligodendroglia progenitor cell

OPN: optic nerve

ORF: open reading frame

PCR: polymerase chain reaction

PD: Parkinson's disease

pEGFPC2: plasmid enhanced green fluorescent protein C-terminus

PI-3 kinase: phosphoinositide-3 kinase

PKM2: pyruvate kinase M2 isoform

PLP: proteolipid protein

PMD: Pelizaeus-Merzbacher disease

PNS: peripheral nervous system

pre-mRNA: pre-messenger ribonucleic acid

PSD-95: post-synaptic density protein 95

PSF: polypyrimidine tract binding protein associated splicing factor

PTBP: polypyrimidine tract binding protein

PZR: protein-zero related

QKI: quaking I

qk^v/qk^v: quakingviable mutant mouse

QRE: quaking response element

qRT-PCR: quantitative real time polymerase chain reaction

RBD: RNA-binding domain

RBFOX: RNA binding protein, fox-1 homolog (C. elegans)

REST: repressor element 1 (RE-1) silencing transcription factor

RFP: red fluorescent protein

RGG box: arginine-glycine-glycine box

RS: arginine-serine domain

RT-PCR: reverse transcriptase polymerase chain reaction

SCA: spinocerebellar ataxia

SCN: sciatic nerve

SCZ: schizophrenia

Seq: sequencing

SF: splicing factor

SH3: src-homology 3

SHP-2: protein-tyrosine phosphotase, non-receptor type 11

shRNA: short hairpin ribonucleic acid

siRNA: small-interfering ribonucleic acid

S-MAG: small myelin-associated glycoprotein

SMA: spinal muscular atrophy

SMN: survival of motor neuron

SNP: single nucleotide polymorphism

snRNA: small nuclear ribonucleic acid

snRNP: small nuclear ribonucleic particles

SOX6: SRY (Sex Determining Region Y)-Box 6

SR: serine-arginine rich protein

SRE: splicing regulatory elements

SRRM4: serine-arginine repetitive matrix 4

SRSF1: serine-arginine splicing factor 1

SWC: schwann cell

SV40: simian virus 40

TDP-43: tar DNA-binding protein 43

U2AF: U2-associated factor

UAP1: UDP-N-Acetylglucosamine pyrophosphorylase 1

UTR: untranslated region

UV-CLIP: ultraviolet cross-linking immunoprecipitation

wt/qk^{v}: non-phenotypic heterozygote control mouse

ZASP/LBD3: Z-band alternatively spliced PDZ-motif protein/LIM domain binding 3

Chapter 1: Introduction to Dissertation

Gene transcription gives rise to precursor messenger RNAs (pre-mRNAs) that are processed into mature mRNAs, which are then exported to the cytoplasm as translation templates for protein production. Splicing, a processing step defined by the ligation of exons and removal of introns from pre-mRNAs, is vital for producing functional proteins in eukaryotes, and is mediated by the spliceosome to target degenerate sequences that are present at every defined intron from pre-mRNA transcripts. Alternative splicing (AS) allows for the differential combination of exons to generate multiple mature mRNAs from one expressed gene, which greatly increases proteome diversity to orchestrate cell growth and development during eukaryotic gene expression (Nilsen and Graveley 2010). In mammals, at least 95% of genes undergo AS when expressed (Wang et al. 2008). Many studies have revealed specific functions for splice variants that often play opposing roles in cells, and can be differentially expressed in distinct cell types (Kelemen et al. 2013). The integration of high-throughput sequencing technologies over the last decade has provided incredible capabilities to elucidate AS events and regulation (Buratti, Baralle and Baralle 2013). In contrast to constitutive splicing, which can be defined as the splicing events that always occur, AS is extensively regulated by splicing factors (SFs), in a target-specific and cell-type specific manner, that can recruit or redirect components of the spliceosome and determine alternative inclusion or exclusion of sequence (Matera and Wang 2014, Schwartz et al. 2008). Target-specific AS regulation can be coordinated by interplay between multiple SFs depending on the location of consensus binding sites in the pre-mRNA (Huelga et al. 2012, Ke and Chasin 2011). Information obtained from high-throughput studies has led to identification of modes of regulation by which different SFs control AS in an attempt to define a splicing code (Elliott 2014). However,

pathways dictating the expression of SFs that in turn control AS in cell-type specific manners or during cell differentiation still remain elusive. Highlighting the physiological importance of this process, dysregulation of AS has been implicated in the pathogenesis of several diseases, such as cancer and neurodegenerative diseases (NDDs) (Mills and Janitz 2012, Tazi, Bakkour and Stamm 2009). Delineating the underlying mechanisms that regulate AS will help to reveal the causes of dysregulation, and may consequently be useful in establishing novel therapeutic avenues against disease pathogenesis.

High-throughput sequencing technologies have helped determine that the highest number of alternative mRNA transcripts is expressed in the mammalian brain (Licatalosi and Darnell 2010). Regulation of AS in the mammalian central nervous system (CNS) is especially critical for the differentiation, development and function of neural cell types (Fogel et al. 2012, Grabowski 2011, Merkin et al. 2012, Wang et al. 2012). In particular, almost every gene that is important for the development and function of oligodendroglia (OLs), which are the myelin-producing cells of the CNS, undergoes AS when expressed (Wang et al. 2012, Wang, Dimova and Cambi 2007, Wu et al. 2002, Zhao et al. 2010). Dysregulated expression of OL-specific splice variants can lead to failures in proper myelin formation and maintenance (Hardy 1998, Wang, Dimova and Cambi 2007). Loss of myelin is the main factor that causes neurodegeneration in multiple sclerosis (Bankston, Mandler and Feng 2013, Franklin et al. 2012). In addition, improper myelination is largely implicated in the pathogenesis of NDDs and psychiatric diseases (Bankston, Mandler and Feng 2013, Tkachev et al. 2003). However, the underlying mechanisms regulating AS in myelinating glia are unknown. **This dissertation focuses on identifying the factors and defining the pathways that control AS in OLs.**

1.1 The discovery and function of alternative splicing (AS)

1.1.1 The splicing process and core machinery in eukaryotic gene expression

The pre-messenger ribonucleic acid (pre-mRNA) produced during transcription of genes by RNA polymerase II must be properly processed to make mature protein-coding messenger RNA (mRNA) before eventual export into the cytoplasm and translation into protein (Fasken and Corbett 2009). In eukaryotes, most genes contain long stretches of non-coding sequences (introns) that separate the coding sequences (exons), which were recognized in the mid to late 1970s (Berget, Moore and Sharp 1977, Gilbert 1978). When a gene is transcribed, these introns are initially present in the emerging pre-mRNAs, but are excised and absent from the fully processed mature mRNAs (Matera and Wang 2014). The removal of introns is the mechanism the cell uses to ensure that the adjoined exons provide an open reading frame amenable to translation into the correct amino acid sequence found in functional proteins (Cepko et al. 1981, Gilbert 1978, Le Hir, Nott and Moore 2003).

The process of the removal of introns and ligation of exons from pre-mRNA is called splicing, which is carried out by a large dynamic multisubunit complex called the spliceosome (Will and Luhrmann 2011). The spliceosome is composed of 5 small nuclear ribonucleic particles (snRNPs) that bind to degenerate sequences in pre-mRNAs within defined introns. These snRNPs are comprised of U1, U2, U4, U5 or U6 small nuclear ribonucleic acids (snRNAs) and their associated proteins (Matera and Wang 2014, Will and Luhrmann 2011). The abundant non-coding snRNAs, which are transcribed by RNA polymerase II, primarily function in the nucleus where they catalyze splicing (Matera and Wang 2014). Spliceosome assembly begins with the binding of U1 snRNA to the 5’

splice site, which is the exon/intron boundary at the 5' end of the intron as shown in Figure 1-1. U2 associated factor (U2AF) binds to the polypyrimidine tract at the 3' splice site, which is the intron/exon boundary at the 3' end of the intron, and the other snRNPs are recruited to help catalyze the splicing events (House and Lynch 2008, Matera and Wang 2014). Splicing occurs by two transesterification reactions. The first occurs when the 2' OH of an adenosine at a branch point within the intron performs a nucleophilic attack on the first nucleotide in the 5' end of the intron, usually a guanosine (Moore and Sharp 1993). This results in a 5' to 2' phosphodiester bond between the 5' end of the intron and the 2' OH of the branch point to form a lariat structure and free the 5' exon (Matera and Wang 2014, Moore and Sharp 1993). Following the first step the spliceosome catalyzes the second transesterification reaction that is dependent on U5 snRNP (Matera and Wang 2014, Moore and Sharp 1993). The 3' OH at the 3' end of the free upstream exon performs a nucleophilic attack on the 5' OH of the first nucleotide on the downstream exon (Moore and Sharp 1993, Will and Luhrmann 2011). The final products include the ligation of upstream and downstream exons and release of the intron lariat structure (Black 2003, House and Lynch 2008, Matera and Wang 2014).

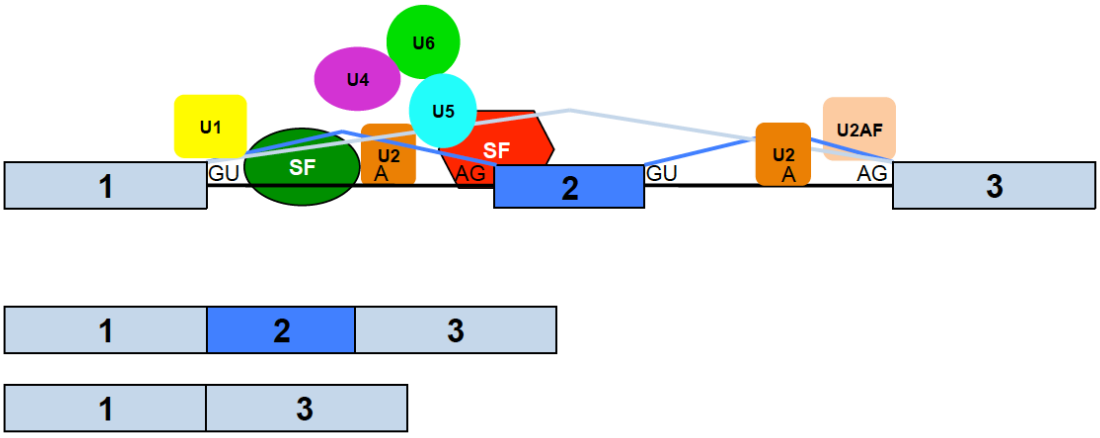
1.1.2 AS fosters proteome diversity in higher eukaryotes

Exons that are always present in mRNA produced from pre-mRNA splicing are referred to as constitutive exons (Black 2003, Will and Luhrmann 2011). On the other hand, a large subset of exons may or may not always be included in certain species of the mature mRNA produced from that given pre-mRNA, which are called alternative exons. The spliceosome is capable of assembling at every intron in a pre-mRNA transcript, but

other regulatory splicing factors (SFs) can interfere and redirect the spliceosome to splice together different combinations of pre-mRNA sequence, which are called alternative splice variants (Will and Luhrmann 2011). As a result, one gene can produce multiple mRNA isoforms by the molecular process called alternative splicing (AS) of the pre-mRNA (Figure 1-1). The multiple mRNAs formed can then be translated to produce different protein isoforms that contain distinct domains and often function differentially in the cell (Birzele, Csaba and Zimmer 2008, Kelemen et al. 2013). In addition, inclusion of alternative sequence can purposefully cause the open reading frame (ORF) to become out of frame, which could introduce a pre-mature stop codon, subjecting the mRNA to nonsense-mediated decay (NMD), and resulting in reduced expression.

Figure 1-1: The spliceosome can be redirected by SFs to cause AS and form multiple mRNAs. The pre-mRNA diagram is represented by rectangles to denote constitutive exons (grey), the alternative (blue) cassette exon and lines to denote introns. The 5' splice site is labeled with a GU, the branch point with a A and the 3' splice site with an AG, to illustrate the degenerate splice sites targeted by components of the spliceosome (U1, U2, U2AF, U4, U5, U6). Regulatory splicing factors (labeled as SF) can bind specific target sequences within the pre-mRNA to regulate accessibility of spliceosomal components to the splice sites, and inhibit (red) or promote (green) usage of splice sites and inclusion of an alternative exon. As a result, two potential splicing events can occur, denoted by the color-coded exon-adjoining lines, to form two potential mRNAs as shown below, resulting in inclusion or exclusion of the cassette exon.

Figure 1-1

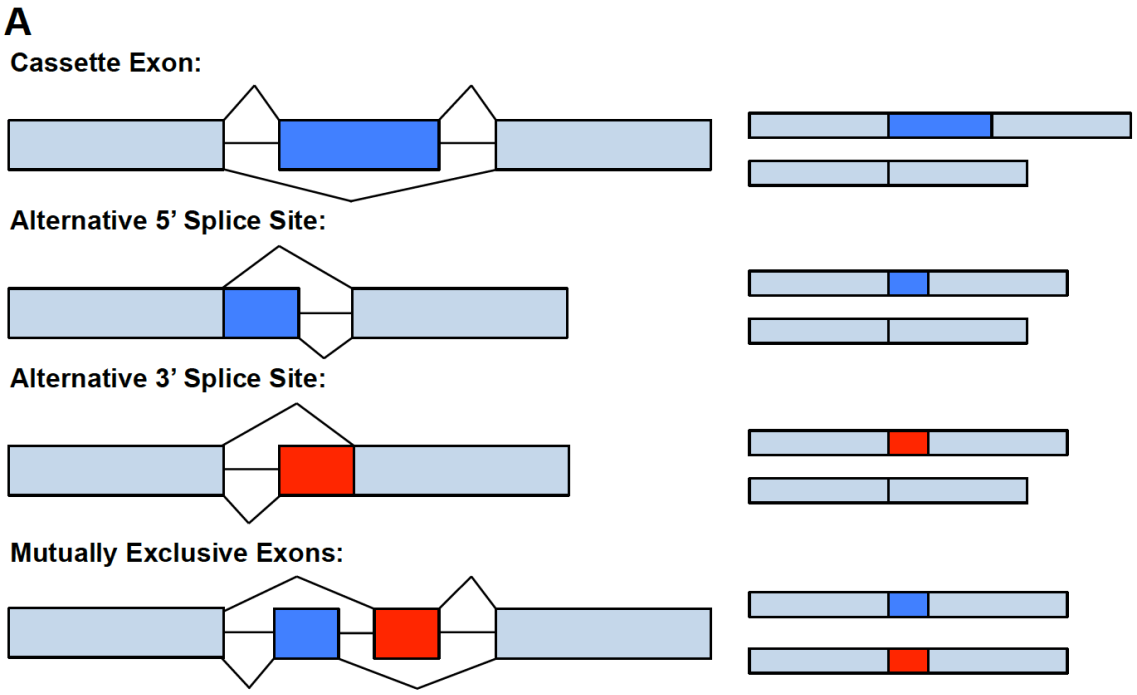


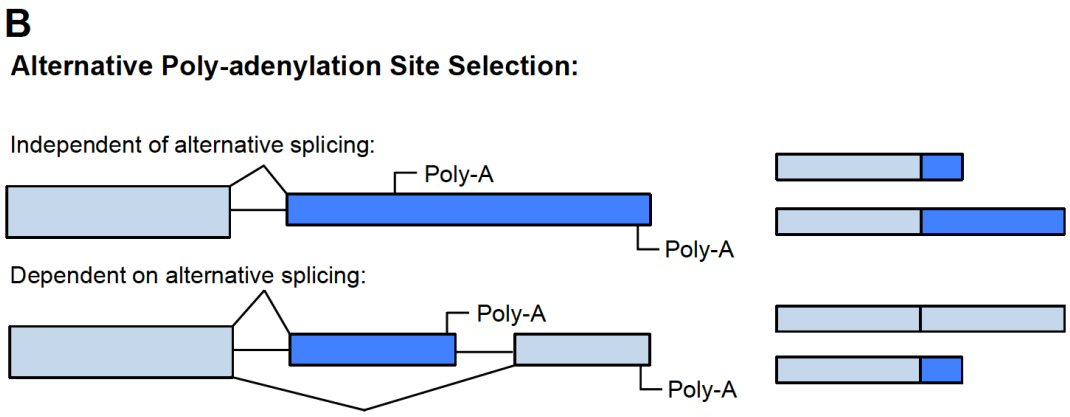
AS can occur in multiple ways as illustrated in Figure 1-2A. The most commonly observed type of AS occurs by the inclusion or exclusion of cassette exons, represented also by the example illustrated in Figure 1-1, which accounts for about 40% of all AS events in humans and mice (Gamazon and Stranger 2014). Additionally, alternative 5' or 3' splice sites within exonic sequence can be utilized to allow inclusion of the adjacent upstream or downstream sequence, respectively (Gamazon and Stranger 2014). Other types of AS observed less frequently are the alternative inclusion of mutually exclusive exons, coordinated cassette exons, the retention of an intron, or alternative first and last exons (Gamazon and Stranger 2014).

AS can occur at any part of the pre-mRNA in which the spliceosome can associate, thus it is not restricted to coding sequence but can also occur in 5' and 3' untranslated regions (UTRs) of a pre-mRNA (Elkon, Ugalde and Agami 2013, Hughes 2006). However, AS is less common in 3' UTRs due to a smaller presence of intronic sequence, but is often linked to use of alternative poly-adenylation (APA) sites which can contribute to regulation of gene expression (Shabalina et al. 2014). APA can lead to unique sequence in coding regions and UTRs that could be targeted by distinct regulatory trans-acting factors, such as microRNAs (miRNAs) or RNA-binding proteins (Elkon, Ugalde and Agami 2013). Spliceosome component U1 snRNP binding to a 5' splice site can mask usage of a downstream alternative poly-A site, without necessarily affecting splicing (Berg et al. 2012). Additionally, splicing can mediate removal of sequence that contains APA sites (Elkon, Ugalde and Agami 2013). Thus, APA can be dependent or independent of AS, and result in distinct 3' UTR sequences in mature mRNAs that can lead to differential mRNA fates (Figure 1-2B) (Elkon, Ugalde and Agami 2013).

Figure 1-2: Major types of pre-mRNA processing governed by AS and/or APA. (A) The pre-mRNA diagrams are represented by rectangles to denote constitutive exons (grey), alternative (blue, red) exons and lines to denote introns. The major types of different pre-mRNA AS events that can occur are indicated and labeled. The subsequently produced mRNA products are shown to the right of each diagram. (B) Alternative poly-adenylation can also result in formation of multiple mRNAs by mechanisms dependent on AS or independent of AS.

Figure 1-2


A
Cassette Exon:
Alternative 5' Splice Site:
Alternative 3' Splice Site:
Mutually Exclusive Exons:


B
Alternative Poly-adenylation Site Selection:
Independent of alternative splicing:
Poly-A
Poly-A
Dependent on alternative splicing:
Poly-A
Poly-A

Two independent groups first observed AS in adenoviruses (Berget, Moore and Sharp 1977, Chow et al. 1977). They both identified discontinuity within primary transcripts transcribed from adenovirus DNA to produce multiple mRNAs. One of the first records identifying AS in the mammalian genome was in 1981, from expression of the gene encoding calcitonin (Leff, Rosenfeld and Evans 1986, Rosenfeld et al. 1981, Rosenfeld et al. 1982). AS has since been observed in all eukaryotes (Black 2003). Interestingly, the number of AS events increases from invertebrates to vertebrates suggesting that use of new alternative exons could be a contributing factor to drive genetic evolution (Kelemen et al. 2013, Kim et al. 2007). One striking and supporting piece of evidence is the relative absence of introns in prokaryotes, compared to the enormity of intronic sequence found in eukaryotes (Light and Elofsson 2013). In fact, an increase in the number of introns was observed at the time metazoa originated, which prompted the need for complex regulation of intron removal in metazoan gene expression (Light and Elofsson 2013, Rogozin et al. 2012). The presence of introns is thought to influence most steps involved in the regulation of RNA metabolism, from biogenesis to decay (Le Hir, Nott and Moore 2003), potentially explaining its prevalence in more complex organisms. Walter Gilbert proposed in 1978 that the evolutionary rise of introns could have occurred as a means to allow easier diversification of gene expression by overriding the need for complete gene duplication (Gilbert 1978).

It is now well known that 95% of multi-exonic genes expressed in mammals undergo alternative inclusion or exclusion of introns, which provides extensive diversification of gene expression (Wang et al. 2008). Some AS events are conserved between species, but are often observed to be remarkably complex in higher organisms

(Kelemen et al. 2013). Evolutionary studies reveal that conservation of AS events diverged between species about 300 million years ago, before tissue-specific divergence, which occurred about 6 million years ago (Merkin et al. 2012). AS plays an important role in the development of different tissue-types, as it largely contributes to differences in gene expression between terminally differentiated cell types (Merkin et al. 2012, Wang et al. 2008). Interestingly, tissue-specific AS events are largely conserved in the brain, heart, skeletal muscle and testes among different species (Merkin et al. 2012). However, the underlying molecular mechanisms regulating AS in different cell types, especially during cell differentiation, are still poorly understood. The efforts to begin to understand the regulation of AS over the last several decades and to determine a splicing code are briefly summarized in the following sections.

1.1.3 Regulation of AS to control gene expression

In order to ensure that the correct protein isoforms are encoded by distinct mRNAs expressed from each gene in the right cell at the right time, the process of AS must be highly regulated (Braunschweig et al. 2013). Regulation of AS is largely influenced by a coordination between cis-acting elements and trans-acting/auxiliary SFs (Wang and Burge 2008). Cis-acting elements are specific consensus sequences, also known as splicing regulatory elements (SREs) present in the pre-mRNA that can coordinate AS (Wang and Burge 2008). SREs are not limited to the splice sites but include the regulatory sequence that can be present anywhere in introns or exons (Ke and Chasin 2011). These sequence elements are often referred to as intronic splicing enhancers (ISEs), intronic splicing suppressors (ISSs), exonic splicing enhancers (ESEs)

or intronic splicing suppressors (ISSs), to define their location and specific roles in regulating the inclusion of a given exon (Wang and Burge 2008).

SREs often consist of a consensus sequence that is targeted by a specific trans-acting SF, and can be weak or strong depending on how similar the sequence is to the defined consensus for the given SF (Wang and Burge 2008). In addition, the sequence elements alone can affect assembly of the spliceosome at alternative exons by forming specific secondary structures (Wang and Burge 2008). SFs are most often documented as interacting proteins, RNA-binding proteins or protein/RNA complexes that bind to the cis-acting elements in the pre-mRNA (Matlin, Clark and Smith 2005). The binding and coordination between different trans-acting factors and their respective cis-acting elements is thought to be critical for defining introns and exons (Ke and Chasin 2011). Two extensively studied families of trans-acting SFs are the heterogeneous nuclear ribonucleoproteins (hnRNPs) and the SR proteins, named after their serine-arginine rich amino acid composition. The number of hnRNP and SR proteins increases with the complexity of AS in higher organisms, many of which are ubiquitously expressed (Busch and Hertel 2012, Nilsen and Graveley 2010).

SR proteins were identified in the mid 1980s, with the characterization of the first SR protein ASF/SF2 by two independent groups (Fu and Manley 1987, Ge and Manley 1990, Krainer, Conway and Kozak 1990, Krainer and Maniatis 1985, Manley and Tacke 1996). Fu and Manley identified ASF, while Krainer and Maniatis identified SF2, later realizing the cDNAs encoding these proteins were identical (Fu and Manley 1987, Krainer and Maniatis 1985, Manley and Tacke 1996). Identified later were multiple members of the SR protein family, such as SC-35, SRp20, and SRp40, which were

recently renamed to SRSF1 to 12 (Manley and Krainer 2010). SR proteins can play essential roles in splicing, as well as regulatory roles in AS (Manley and Tacke 1996). SR proteins are distinguished by their N-terminal RNP-type RNA-binding domains (RBD) and C-terminal arginine-serine rich (RS) domains (Manley and Tacke 1996). The RS domain, which can recruit specific components of the spliceosome to mediate splicing, is thought to function as a splicing enhancer (Manley and Tacke 1996). SR proteins are predominantly localized to the nucleus via the importin transportin-SR, which targets phosphorylated RS domains (Manley and Tacke 1996, Twyffels, Gueydan and Kruys 2011). However, SR proteins can also shuttle to the cytoplasm in an RNA-dependent manner, to regulate mRNA stability and translation (Twyffels, Gueydan and Kruys 2011).

hnRNPs were initially defined as RNA-binding proteins that can bind to heterogeneous ribonucleic acids (hnRNAs) but are distinct from the other RNP complexes, such as snRNPs (Dreyfuss et al. 1993). All hnRNPs contain at least one or more RBDs of three different types, the RBD/RRM/RNP-CS motif, the RGG box, and the K-Homology (KH) motif (Weighardt, Biamonti and Riva 1996). The RBD/RRM/RNP-CS motif is 90 amino acids long and contains a highly conserved octapeptide (RNP1) and hexapeptide (RNP2) that can directly interact with RNA (Weighardt, Biamonti and Riva 1996). The RGG box consists of Arg-Gly-Gly repeats that binds RNA (Weighardt, Biamonti and Riva 1996). The KH motif is 45 amino acids long and was initially found in hnRNP K to bind RNA (Weighardt, Biamonti and Riva 1996). In higher eukaryotes, hnRNPs are among the most abundant nuclear proteins, and are labeled alphabetically from A1 to U, with the exception of a few letters (Dreyfuss et

al. 1993, Weighardt, Biamonti and Riva 1996). Most hnRNPs can shuttle between the nucleus and cytoplasm, often when bound to a target mRNA, and can have functional roles in the cytoplasm. hnRNP C and U are two exceptions that contain SV40 T antigen and nucleoplasm-type nuclear localization signals, which restrict these proteins to the nucleus (Dreyfuss et al. 1993). Many hnRNPs have alternate names, such as hnRNP I, which is also known at polypyrimidine tract binding protein (PTBP1) (Dreyfuss et al. 1993). Several of the hnRNPs are grouped into sub-families based on similarities in protein structure and amino acid sequence identity; such as the A/B proteins comprised of hnRNP A1, A2, and B1 (Dreyfuss et al. 1993). The A/B proteins all contain 2 RNP-motif RBDs and a glycine-rich auxiliary domain at the C terminus (Dreyfuss et al. 1993). Early studies on splicing regulation by these nuclear RNA-binding proteins suggested that the roles for hnRNPs were to inhibit inclusion of alternative exons, whereas SR proteins promote inclusion of alternative exons by recruiting spliceosomal machinery. However, to this day a number of examples indicate splicing regulation by hnRNPs results in exon inclusion. The currently accepted model for regulation of AS depends largely on the specific trans-acting factor, the location of the targeted sequences and the interplay/interactions between other SFs and the spliceosome (Figure 1-1) (Ke and Chasin 2011, Wang et al. 2012).

The interplay between cis and trans-acting factors can promote or block accessibility of components of spliceosome (snRNPs) to splice sites within a pre-mRNA, and/or can affect the interactions between the snRNPs that are required to catalyze intron removal/exon ligation (Figure 1-1) (House and Lynch 2008). Inclusion or exclusion of an exon can be largely dependent on the location of the cis-acting sequence element that is

targeted by the trans-acting SF (Wang et al. 2012). In addition, the expression and presence of the SF, and often the coordination between different SFs is required for such regulation to determine the composition of the mature mRNA (Wang and Burge 2008). As a result, the degenerate canonical splice sites that are targeted by the spliceosome can be ubiquitously found at intron/exon boundaries in all pre-mRNA sequence, however the unique cis-acting sequence elements that can be targeted by different SFs to determine splice site selection in distinct pre-mRNAs render the regulation of AS target-specific. Thus, the presence of distinct sequence elements is critical for regulation of AS.

AS events are tightly regulated during cell differentiation, but the underlying molecular changes that lead to distinct splice variant expression between an undifferentiated cell and a differentiated cell, are not fully understood. There are many examples in the literature showing the changes in AS within a cell type, such as the CD45 AS by hnRNPLL during the transition between naïve and activated T-cells (Oberdoerffer et al. 2008). Also, the expression of certain SFs, such as polypyrimidine tract binding proteins 1 and 2 (PTBP1 and PTBP2), are tightly controlled during differentiation of neural stem cells (NSCs), and cell-type specification (Grabowski 2011, Licatalosi et al. 2012). Splice variant expression of a gene encoding the selective RNA-binding protein quaking I (QKI), which will be discussed in subsequent sections, is critical for the differentiation of oligodendroglia (OL), the myelin-producing cells of the central nervous system (CNS) (Bockbrader and Feng 2008, Larocque et al. 2009, Larocque et al. 2005). The regulated expression and/or localization of RNA-binding proteins and SFs contribute largely to the remodeling of gene expression patterns during cell differentiation.

1.1.4 Dysregulation of AS implicated in disease

Incorrect AS can occur by the dysregulation of expression or localization of SFs and/or gene mutations in cis-acting elements. Mutations that cause defects in splicing of critical genes have been identified in different diseases. About 12% of 2,000 mutations affect splicing of the cystic fibrosis transmembrane regulator (CFTR) in the autosomal recessive disorder cystic fibrosis. A monosymptomatic form of cystic fibrosis is marked by a direct defect of inclusion or exclusion of exon 9, which may be due to abnormal expression or regulation by trans-acting SFs, such as hnRNP A1 that was shown to regulate AS of exon 9 in a human CFTR minigene (Gamazon and Stranger 2014, Pagani et al. 2000). Thus, the role for AS regulation relies on the correct interplay between the cis and trans-acting factors.

Duchenne muscular dystrophy (DMD) is one of the most common X-linked inherited diseases caused by mutations in the dystrophin gene (Ahn and Kunkel 1993). AS regulation of dystrophin gene expression is extensive, considering the size of the gene, which is 2.5 Mb, the number of exons, and the sheer number of distinct protein isoforms produced (Sadoulet-Puccio and Kunkel 1996). Regulation of dystrophin gene expression relies on alternative usage of 7 possible promoters, as well as AS of 6 different exons resulting in distinct C-terminal amino acids (Sadoulet-Puccio and Kunkel 1996). AS of dystrophin results in differential expression of splice variants between tissue types (Sadoulet-Puccio and Kunkel 1996). A high percent of the mutations in the dystrophin gene are deletions detectable by PCR, or mutations that cause frame-shift leading to NMD, however smaller mutations were identified that disrupted splice sites and led to dysregulated dystrophin isoform expression (Im et al. 1996, Sadoulet-Puccio

and Kunkel 1996). Several studies from different groups over the last decade have investigated therapeutics by using antisense oligonucleotides (AONs) to direct expression of the correct splice variants (Nelson et al. 2009). The novel treatments for DMD have pioneered new therapeutic venues, which could prove to be useful for potentially correcting AS defects *in vivo* for other genetic diseases.

The number of links identified between defects in AS is increasing as high-throughput sequencing becomes more prevalent in the analyses of diseases. The benefit of high-throughput studies is to allow investigators to make connections between different proteins and gene expression patterns that may not have otherwise been identified. Integrating high-throughput data and molecular validation to delineate the specific underlying regulatory mechanisms that control AS through development and between different cell types will help us understand what may be going awry in disease. Interestingly, some SFs are highly expressed during cell proliferation, such as hnRNP F/H, and are developmentally downregulated (Wang, Dimova and Cambi 2007). A recent high-throughput study suggested hnRNP F/H promote expression of proliferation-inducing genes (Wang et al. 2012). Thus, abnormal overexpression of hnRNPs could lead to aberrant cell proliferation and result in tumorigenesis (Han, Li and Zhang 2013). Dysregulation of AS has also been implicated in CNS disorders, including psychiatric diseases and neurodegenerative disease (NDDs) (Bentmann, Haass and Dormann 2013, Morikawa and Manabe 2010). Delineating the mechanisms that underlie normal AS regulation in the central nervous system (CNS) is crucial to begin to understand the dysregulation and abnormalities observed in disease.

1.2 AS in the nervous system

1.2.1 AS in the development and function of neuronal cell types

Regulation of AS has been extensively studied in the development and maintenance of the CNS. Gene expression and the AS events that occur in the brain are some of the most highly conserved between different species and through evolution (Merkin et al. 2012). In fact, the brain harbors the most complex AS events and the highest number of alternative mRNA transcripts (Licatalosi and Darnell 2010, Pan et al. 2008, Raj et al. 2011). Numerous examples illustrate the importance for proper regulation of AS in normal brain development, focusing extensively on the differentiation and function of two classes of neuronal cell types, neurons and glia. A critical step in the development of the nervous system is the differentiation of neural stem cells (NSCs) to mature neural cell types. NSCs become neurons or glia depending on the signaling pathways initiated to promote specific gene expression patterns, which determine cell fate (Azim et al. 2014). Changes in AS contribute to the observed differences in transcriptome profiles, which likely play critical roles in establishing the regulation of gene expression to determine cell fate, and also in the later stages of cell differentiation and maturation (Yeo et al. 2008).

A well-established example illustrating the regulation of AS during neuronal differentiation from NSCs in a global manner is initiated by a microRNA expressed specifically in the brain. Expression of microRNA 124 (miR-124) is upregulated and maintained during brain development, and has been shown to promote neuronal differentiation and neurite outgrowth in cultured Neuro2a cells, a neuroblastoma cell line (Makeyev et al. 2007). The underlying mechanism by which miR-124 promotes neuronal

differentiation is through its role in inhibiting expression of the SF polypyrimidine tract binding protein 1 (PTBP1). PTBP1 regulates AS in non-neuronal cells, and was shown to inhibit expression of neuron-specific transcripts (Makeyev et al. 2007, Xue et al. 2009). Interestingly, PTBP1 functions to regulate AS of the neuronal-specific paralog PTBP2 (nPTB), by inhibiting inclusion of exon 10 in PTBP2 pre-mRNA, which introduces an in-frame premature stop codon, and leads to NMD (Boutz et al. 2007, Grabowski 2011, Makeyev et al. 2007). miR-124 inhibits PTBP1 expression by targeting its mRNA at the 3' UTR, leading to reduced mRNA levels (Grabowski 2011, Makeyev et al. 2007). This reduction in PTBP1 expression, in concert with a stimulatory effect by nSR100, allows for inclusion of exon 10 from PTBP2 pre-mRNA and PTBP2 expression (Grabowski 2011). A high-throughput study found that PTBP2 was essential for survival of neurons in postnatal mouse brain development (Licatalosi et al. 2012). PTBP2 inhibits expression of alternative exons that are present in adult neurons, and the lack of PTBP2 in PTBP2-null mice leads to premature neurogenesis, a reduction in the number of progenitors, and abnormal neuronal polarity (Licatalosi et al. 2012). PTBP1 and PTBP2 also share overlapping targets, such as post-synaptic density protein 95 (PSD-95), and prevent pre-mature expression in early stages of neuronal development (Zheng et al. 2012).

AS regulates expression of genes important for neuronal signaling. Dendrites projecting from the neuronal cell body receive signals from axons of other neurons in a synapse, mediating signaling through different kinds of receptors, which can bind neurotransmitters to regulate neuronal activity. Pre-mRNAs for many of these receptors are alternatively spliced, such as serotonin receptors (Lee and Irizarry 2003). One isoform of a serotonin receptor that has a shorter C-terminus is thought to respond better to an

agonist (Lee and Irizarry 2003). For some receptors, such as the NMDA, dopamine, and GABA receptors, localization within the neuron is dependent on AS due to the differential effects of alternative protein domains (Lee and Irizarry 2003).

Evidence over the last two decades clearly indicates the critical roles for AS in neuronal development and function. In addition, the importance for AS is becoming increasingly recognized in various glia lineages. A more recent report by Thomsen et al. suggests the functional importance for AS regulation in the development of astrocytes, a glial cell type of the CNS. The glial fibrillary acidic protein (GFAP) is a protein component of intermediate filaments in astrocytes, which play important roles in cytoskeleton formation and signaling (Thomsen et al. 2013). GFAP expression is developmentally upregulated during astrocyte differentiation (Thomsen et al. 2013). Processing of GFAP pre-mRNA can result in 8 different isoforms resulting from AS and/or APA (Thomsen et al. 2013). Two isoforms have differential localization in primary astrocytes GFAPα, and GFAPδ, in which GFAPα is more highly concentrated in astrocytic protrusions, suggesting distinct functional roles for these isoforms in astrocytes (Thomsen et al. 2013). Multiple studies have also reported on the importance for regulation of AS in the function of microglia, among other cell types in the nervous system (Malik et al. 2013). Additionally, almost all of the genes expressed that are critical for the differentiation and function of oligodendroglia (OLs), the myelin-producing cells of the CNS, undergo extensively regulated AS, which is covered in more detail in 1.2.2.

AS clearly contributes to defining transcriptome profiles, which can differ extensively between different neural cell types. However, the underlying mechanisms

that result in cell-type specific AS are not fully understood. Some SFs were found to be predominantly or solely expressed in certain cell types, such as neurons that were found to express neural-specific SFs (e.g. Nova1/2, and nPTB). Many of the downstream functional targets of these SFs play critical roles in neuronal migration, neurite outgrowth and synapse formation. RBFOX1, also known as A2BP1, is a neuronal-specific SF that was shown to control AS of a large set of targets implicated in the differentiation and maturation of neurons, which is critical for brain development (Fogel et al. 2012). One neural-specific SR protein, nSR100/SRRM4, was found to promote inclusion of about 11% of alternative exons specifically expressed in the brain (Raj et al. 2011). A target of nSR100 is the gene encoding the repressor element 1 (RE-1) silencing transcription factor (REST) (Raj et al. 2011). REST normally represses expression of genes that promote neurogenesis. nSR100 was found to promote inclusion of an alternative exon in the REST transcript to produce an isoform called REST4, which has diminished activity, and thus allows for the expression of neurogenesis-promoting genes (Raj et al. 2011). Whether and how other glial cell types employ independent mechanisms to differentially control AS and gene expression is a critical question to be addressed in this dissertation.

1.2.2 Functional roles for AS in myelinating glia

AS has been extensively studied in the myelinating glia, i.e. OLs and Schwann cells (SWCs), which are functionally similar cell types responsible for the development and function of the CNS and the PNS, respectively. The major responsibility for these two glial cell types is to produce and maintain a fatty insulation of neuronal axons, called the myelin sheath. Myelin is a crucial component of both CNS and PNS, which ensures

quick and efficient information flow across long neuronal axons by means of saltatory conduction between gaps in myelin called the nodes of Ranvier (White and Kramer-Albers 2014). Aside from insulating neuronal axons, myelin is thought to play roles in neuroprotection from inflammation and oxidative stress (Bankston, Mandler and Feng 2013). Loss of myelin is linked to multiple different neurodegenerative diseases (NDDs), along with classical demyelinating diseases such as multiple sclerosis and amyotrophic lateral sclerosis (ALS) (Bankston, Mandler and Feng 2013). Neurons also elicit signals to promote OL proliferation, differentiation and survival (Simons and Trajkovic 2006). As a result, the interactions between neurons and glia are important for normal brain function (Simons and Trajkovic 2006).

Myelin production requires the proper differentiation, migration, and maturation of myelinating glia. OL differentiation begins with the proliferation of bipolar OL progenitor cells (OPCs) that is initiated in the ventricular region of the brain in early stages of embryogenesis, and is followed by proliferation of OPCs in the subventricular zone in late embryogenesis (Butts, Houde and Mehmet 2008). Specific markers that are expressed at each stage can be used to track OL differentiation, as well as changes in morphology. A2B5, as well as Olig1 and Olig2, are markers for early OPCs. OPCs are differentiated to immature OLs and express the early differentiation marker O4 (Butts, Houde and Mehmet 2008). Galactocerebroside and 2’3’-cyclic nucleotide-3’-phosphodiesterase (CNP) are markers for immature OLs that then begin to form multiple primary, secondary and tertiary branches (Butts, Houde and Mehmet 2008). OLs mature once they come in contact with neuronal axons and begin to produce myelin by expressing myelin proteins, such as myelin basic protein (MBP), proteolipid protein

(PLP), myelin OL glycoprotein (MOG), and myelin-associated glycoprotein (MAG) (Butts, Houde and Mehmet 2008). In mice, myelin formation peaks at day 20 in brain development and is maintained through adulthood (Zhao et al. 2006).

The differentiation of OLs and SWCs, and the development of myelin, is highly dependent on regulated AS of myelin-specific genes. The expression of major myelin proteins MBP, PLP and MAG was shown to be regulated by AS during OL differentiation (Campagnoni and Macklin 1988). To this day, the functional roles of the different splice variants produced from AS of pre-mRNA transcripts encoding these proteins have been the focus of much investigation.

One of the most abundant proteins expressed in OLs and SWCs that comprises at least 30% of all myelin proteins is MBP (Roach et al. 1983). MBP is deposited into the myelin sheath and plays critical structural roles in the adhesion between cytosolic surfaces of the plasma membrane layers of multilamellar compact myelin and maintains a neutral charge between each layer (Boggs 2006). Lack of MBP is known to cause extensive neurological defects (Roach et al. 1983). The gene encoding MBP is over 30 kilobases in length (de Ferra et al. 1985). Early studies in the 1980s identified four MBP protein isoforms (21.5, 18.5, 17, and 14 kDa) that came from expression of one gene (de Ferra et al. 1985), followed later by identification of a fifth isoform (Newman, Kitamura and Campagnoni 1987). The MBP pre-mRNA undergoes AS of multiple exons, which is predicted to form 8 distinct MBP isoforms in mice (Newman, Kitamura and Campagnoni 1987). The 21.5 and 17 kDa MBP isoforms contain 26 amino acids by inclusion of exon 6, which can result in nuclear localization. The 18.5 kDa isoform is predominant in adult myelin in humans and is highly conserved in mammals (Harauz, Ladizhansky and Boggs

2009). The 18.5 and 21.5 kDa MBP isoforms were shown to interact with SH3-domain containing proteins and cytoskeletal proteins, with the functional capability to attach these proteins to the lipid membrane (Smith et al. 2012). The different MBP isoforms display distinct subcellular localization. Live-cell imaging and FRAP analysis showed that isoforms encoded by transcripts with exon 2 inclusion are dynamically translocated between the nucleus and cytoplasm, movement that is likely important for OL proliferation (Ozgen et al. 2013). The distinct functions and subcellular localizations of the MBP isoforms depend on correct alterative splicing of the MBP pre-mRNA, rendering the regulation of AS critical for normal MBP expression and myelin development.

PLP is a myelin structural protein that is abundantly expressed in OLs and SWCs. Alternative 5' splice site usage in exon 3 of the pre-mRNA expressed from the *PLP* gene leads to the formation of two isoforms that are both transmembrane proteins, PLP and DM20 (Nave et al. 1987). An upstream 5' splice site within the full exon 3 sequence defines exon 3a, which is spliced with the downstream exon 4 to form DM20. The splicing event results in exclusion of sequence that leaves out a hydrophilic cytosolic domain from the translated protein (Nave et al. 1987). Use of a distal 5' splice site to form exon 3b downstream of the first alternative splice site, allows for inclusion of 105 nucleotides of additional sequence to form PLP, which contains the cytosolic domain in the protein (Nave et al. 1987). DM20 is expressed at low levels in OL progenitor cells (Wang et al. 2006). During mouse brain development PLP expression and the ratio of PLP to DM20 levels is markedly upregulated, rendering PLP the predominant isoform in adult (Nave et al. 1987, Wang et al. 2006). Mutations that led to splicing abnormalities in

PLP pre-mRNA and reduced PLP levels resulted in axonal degeneration, which is observed in Pelizaeus-Merzbacher disease (PMD) (Hobson et al. 2002, Wang et al. 2006). DM20 alone cannot fully replace PLP function since mice expressing only DM20 display morphological abnormalities and increased cytoplasmic inclusions that could lead to axonal degeneration in adult mice (Sporkel et al. 2002, Stecca et al. 2000).

MAG is another major myelin structural protein expressed specifically in OLs and SWCs that is enriched in the periaxonal myelin membranes and functions in the interactions made between glia and neuronal axons in both the CNS and PNS (Quarles 2007). Expression of MAG protein occurs early in myelination with a suggested role of MAG in the glia-axon interactions that are necessary to form myelin. Having five immunoglobulin (Ig)-like extracellular domains qualifies MAG as a member of the Ig superfamily (Quarles 2007). MAG is a type I transmembrane protein that functions in cell adhesion, the initiation of myelin formation, signal transduction, maintenance of myelin structure at the periaxonal space, and inhibition of neurite outgrowth to prevent abnormal myelination (Minuk and Braun 1996).

AS of MAG pre-mRNA is another example of the differential regulation of AS during cell differentiation and maturation, even between two functionally similar cell types of the CNS and the PNS. MAG exists as two isoforms that are derived by AS of the exon 12 in the MAG pre-mRNA. The longer mRNA isoform, which includes exon 12 introduces an early stop codon and results in the translation of a smaller protein, called S-MAG (Lai et al. 1987). Exclusion of exon 12, which forms a shorter mRNA renders the first in-frame stop codon to be further downstream and results in the formation of a larger protein, called L-MAG (Lai et al. 1987). The extracellular and transmembrane domains

of S-MAG and L-MAG are identical, but due to the AS event that results in translation of unique amino acids, S-MAG and L-MAG differ in their C-terminal cytoplasmic domains, which likely underlies their functions in dictating distinct signaling pathways (Quarles 2007). Expression of the MAG isoforms is developmentally regulated (Quarles 2007, Schachner and Bartsch 2000). In OLs, L-MAG is the earliest expressed isoform, whereas S-MAG expression increases at later stages in myelin development. L-MAG was shown to be important for proper CNS myelination, but can be spared for PNS myelination (Fujita et al. 1998). In SWCs, S-MAG is the predominant isoform at any stage in the development of the PNS. The function of L-MAG was suggested to be involved in internode formation, and there may be a reduced need for it in PNS myelination as fewer internodes exist in the myelinated axons of the PNS (Quarles 2007).

Besides myelin structural genes, the pre-mRNAs for genes encoding for proteins that play roles in other facets critical for OLs, such as differentiation, apoptosis, and signaling are also alternatively spliced. The selective RNA-binding protein Quaking I (QKI) is necessary and sufficient for OL differentiation, and undergoes AS regulation of the 3' terminal coding exons (Chen et al. 2007). The regulation of QKI isoform expression and functional importance are reviewed in section 1.4. AS of genes expressing the Bcl2-related protein family, which plays a role in regulating cell apoptosis, is differentially regulated during OL differentiation (Itoh, Itoh and Pleasure 2003).

Cell signaling between neurons and glia also depends highly on the regulation of AS. Genes expressed to encode for the neuregulin family of proteins undergo extensive AS (Carteron, Ferrer-Montiel and Cabedo 2006). One neural-specific splice variant of neuregulin 3 was shown to be involved in OL survival by activating the PI-3 kinase

pathway likely through the erbB4 receptor to phosphorylate AKT, which is a kinase involved in promoting cell survival (Carteron, Ferrer-Montiel and Cabedo 2006). This example illustrates that AS regulation is not only critical to gene expression in one cell type, but also for the signaling pathways controlled by interactions between different splice variants expressed in two communicating cell types, such as neurons and glia. Additionally, AS of the erbB4 receptor of neuregulins is tightly regulated in neurons and OLs, and important for its function in cell signaling (Veikkolainen et al. 2011).

Despite the critical role of AS for the normal function and development of myelin in the nervous system, the underlying molecular mechanisms controlling AS in myelinating glia are not well understood. A well-studied mouse model identified in the 1960s (Sidman, Dickie and Appel 1964) is deficient of QKI specifically in myelinating glia (Hardy et al. 1996, Lu et al. 2003). The homozygous recessive mutation in this mouse is a 1.2 Mb deletion in the promoter region upstream of the *qkI* gene that results in reduced transcription of qkI specifically in myelinating glia and consequent hypomyelination (Ebersole, Rho and Artzt 1992, Sidman, Dickie and Appel 1964). Due to minimal expression of QKI that allows for viability and a severe tremor phenotype, the mutant mouse was named *quakingviable* (qk^{v}/qk^{v}) (Ebersole, Rho and Artzt 1992). A molecular consequence of reduced QKI expression in this mouse model is aberrant expression of alternative isoforms from myelin-specific genes (Zhao et al. 2010, Zhu et al. 2012). Reintroducing QKI specifically in the OLs rescued the AS defects, suggesting a major role for QKI in the regulation of AS in OLs. However, the underlying mechanisms remain elusive, which is a major focus of this dissertation. The characterization and function of QKI will be described in detail in section 1.4.

1.2.3 Dysregulation of AS in CNS/PNS disorders

The critical roles of AS in the normal development and function of the nervous system highlight the importance for its proper regulation. Further adding to the need for precise regulation of AS, several brain diseases have been linked with defects in AS (Licatalosi and Darnell 2006). Discussed here are examples of NDDs, demyelinating disorders and gliomas that display defects in AS.

Dysregulation in AS is implicated in several NDDs, such as Alzheimer's disease (AD), Parkinson's disease (PD), and frontotemporal lobar dementia (FTLD) (Licatalosi and Darnell 2006). Some have AS defects of genes associated with the disease, such as dysregulation of AS of tau in AD (Qian and Liu 2014). A recent study found components of the U1 snRNP sequestered into cytoplasmic tangles, which is linked with a number of defects in RNA processing and the accumulation of unspliced RNA species in AD (Bai et al. 2013). Other NDDs are caused by defects in RNA-binding proteins that are known to regulate AS, such as TDP-43 and Fus in ALS and FTLD (Honda et al. 2013).

PMD is an X-linked disorder in which patients have mutations in the gene expressing PLP that result in hypomyelination. Analysis of the genetic defects in patients with PMD revealed improper ratios of PLP to DM20 (Regis et al. 2009), suggesting mutations in the gene affect AS to cause this disease. Also, a splicing enhancer of the PLP isoform is deleted in a mild form of PMD (Wang et al. 2008). Charcot-Marie-Tooth disease (CMT) is another hereditary demyelinating disorder of the PNS. Genetic analysis identified mutations in genes associated with CMT that could cause splicing abnormalities (Hayashi et al. 2013). Analysis of mRNA levels from patients with CMT

revealed abnormal expression of alternative splice variants, suggesting potential defects in AS associated with this disease (Hayashi et al. 2013).

Defects in AS have been implicated in different types of gliomas. Abnormally upregulated expression of different SFs was observed in pilocytic astrocytomas, low-grade astrocytomas, and glioblastoma multiforme (GBM), which correlates with AS of pyruvate kinase pre-mRNA to express the isoform that promotes tumor progression (PKM2) (David et al. 2010, Han, Li and Zhang 2013). GBMs have mutations in signaling pathways controlled by growth factors receptors, such as epidermal growth factor receptor (EGFR), which is upregulated in at least half of GBM-stricken patients (Babic et al. 2013, Cancer Genome Atlas Research 2008). A constitutively activated mutant of EGFR, EGFRvIII, upregulates expression of hnRNP A1, which leads to gene expression patterns that promote proliferation and tumorigenesis (Babic et al. 2013). Mouse double minute 4 (MDM4) is structurally related to MDM2, which is a negative regulator of tumor suppressor p53, and is thought to function also in repressing p53 function (Wang et al. 2013). Inhibition of p53 function leads to abnormal cell cycle progression and is associated with almost all types of cancer (Golubovskaya and Cance 2013). MDM4 is overexpressed in many cancers, including glioma (Wang et al. 2013). In several cases now hnRNPs are found to be highly abundant in proliferating cells, and reduced during differentiation (Lefave et al. 2011, Wang et al. 2012). Abnormalities in elevated hnRNP expression could likely have a significant contribution to tumorigenesis (Han, Li and Zhang 2013).

Schizophrenia (SCZ) and other related psychiatric disorders have extensive defects in AS of many disease-risk genes, such as dopamine receptors, GABA receptors,

erbB4, and disrupted in schizophrenia 1 (DISC1) (Morikawa and Manabe 2010). Neuronal functions that largely depend on correct AS events that are dysregulated in SCZ, depression, and bipolar disease include dopamine, GABA, and glutamate signaling, as well as brain development and maturation (Morikawa and Manabe 2010). Interactions between neuregulin 1 and erbB4, both of which undergo AS, are disrupted in SCZ (Veikkolainen et al. 2011). More recent literature has investigated a role for the long non-coding RNA Gomafu in SCZ (Barry et al. 2014). Gomafu is implicated in AS regulation of SCZ-risk isoforms of DISC1 and erbB4, and is downregulated in post-mortem brains from SCZ patients (Barry et al. 2014). Interestingly, Gomafu was shown to interact with RNA-binding proteins SRSF1 and QKI, and QKI is also dysregulated in SCZ (Aberg et al. 2006, Barry et al. 2014). However, the functional role for Gomafu in affecting QKI is not well understood. Given the numerous examples of dysregulated AS in different CNS diseases, delineating the underlying mechanisms and pathways that control AS in the CNS will be crucial to determine the deficiencies or abnormalities that can cause or contribute to disease pathogenesis.

1.3 Roles for splicing factors hnRNP F and H in oligodendroglia (OLs)

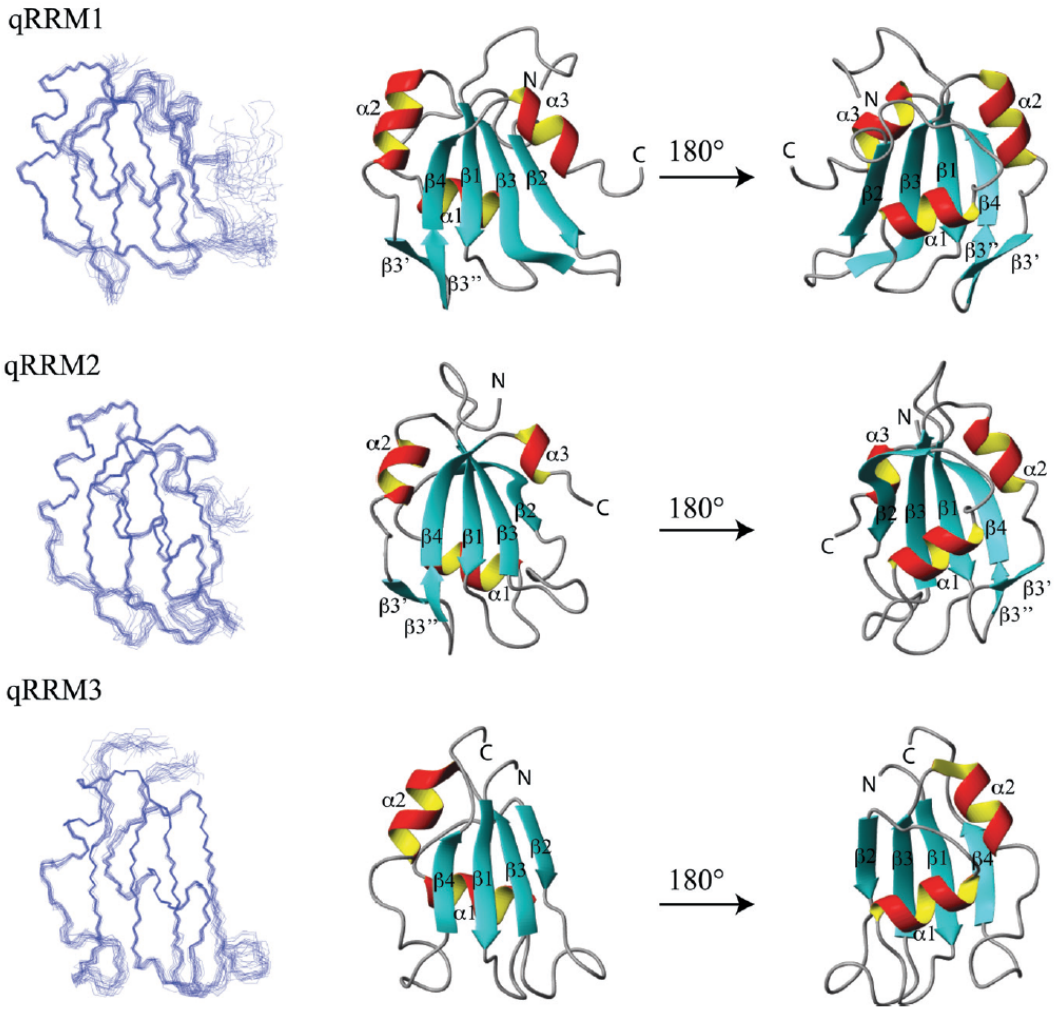
1.3.1 hnRNP F and H are functional orthologues that regulate RNA metabolism

Two SFs identified as functional homologues are hnRNP F and hnRNP H (hnRNP F/H), both are members of the hnRNP H family of proteins (Caputi and Zahler 2001, Honore et al. 1995). hnRNP F/H are highly abundant proteins expressed in most cell types. The two proteins are expressed from different genes but share 78% sequence identity, have similar immunological reactivity and are shown to play overlapping roles

in RNA metabolism (Honore et al. 1995, Matunis, Xing and Dreyfuss 1994). hnRNP F/H have three repeated RNA-binding domains that are highly similar known as quasi RNA recognition motifs (qRRMs) and two glycine-rich auxiliary domains (Caputi and Zahler 2001). The structures of the three qRRMs in hnRNP F were previously solved by using nuclear magnetic resonance (NMR) and similar to classical RRMs have a compact $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ fold (Figure 1-3) (Dominguez and Allain 2006). hnRNP F/H were originally purified from HeLa cells using poly(rG) affinity chromatography, and were shown to have a strong specificity for poly(rG) sequence (Matunis, Xing and Dreyfuss 1994). The sequence GGGA is sufficient for *in vitro* binding to hnRNP F/H (Caputi and Zahler 2001). The strength of binding to G-runs positively correlates with an increasing number of guanines in a row but is also independently affected by the strength of 5' splice sites in the target pre-mRNA (Xiao et al. 2009). hnRNP F/H are concentrated in the nucleus to regulate splicing, and previously shown to be shuttled to the nucleus by interacting with transportin 1 (Van Dusen et al. 2010). However, hnRNP F/H have been detected in the cytoplasm with different reported roles in mRNA export and localization (Kim et al. 2005, White et al. 2012).

Figure 1-3: Structures of three qRRM domains in hnRNP F solved by NMR. Previously solved structures of three qRRM domains found in hnRNP F by the Allain lab (Dominguez and Allain 2006). An overlay of 20 different final structural analyses identified the $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ fold. MOLMOL was used to generate the displayed ribbon structures using in two orientations upon shifting 180° (Dominguez and Allain 2006, Koradi, Billeter and Wuthrich 1996).

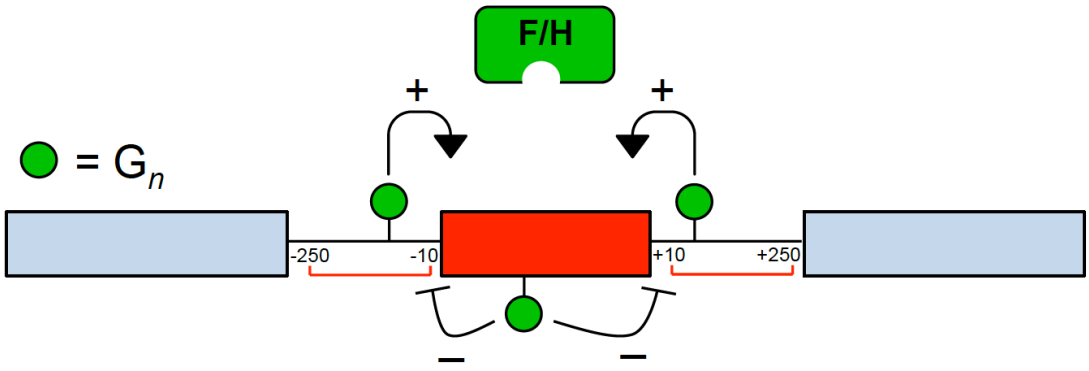
Figure 1-3



The mode of regulation by hnRNP H was established by high-throughput CLIP-seq analysis to globally assess binding, along with RNA-seq upon knockdown of hnRNP H to assess the AS regulation by hnRNP H manipulation (Figure 1-4) (Katz et al. 2010). The siRNA used to target hnRNP H mRNA also targets hnRNP F mRNA. This study, along with a previous study published by the same group, reported on hundreds of hnRNP F/H targets in HEK293T cells (Xiao et al. 2009). When targeting intronic sequence upstream or downstream of an alternative exon, hnRNP F/H were most often shown to promote inclusion of the alternative exon. However, hnRNP F/H can also bind to G-runs within exonic sequence, and block exon inclusion (Katz et al. 2010). High-throughput studies can offer extensive information to determine genome-wide trends in the modes of regulation by a SF. However, exceptions have been identified for specific targets and between different cell types, often depending on coordinated or antagonistic effects by other interacting factors (Huelga et al. 2012). Specific targets of hnRNP F/H that are differentially affected in different glial cell types were identified in myelinating glia (Mandler, Ku and Feng 2014) and are presented in Chapter 2.

Figure 1-4: Proposed mode of regulation by hnRNP F/H in regulating AS. The data from RNA-seq analysis upon hnRNP F/H knockdown combined with data from CLIP-seq analysis, performed by Katz et al. 2010, established a mode of regulation by hnRNP F/H. The pre-mRNA diagram is illustrated as described in Figures 1-1 and 1-2. hnRNP F/H (green box) target G-runs (green ball and stem) either in introns within 250 bp upstream or downstream of the alternative exon to promote inclusion, or target the exon itself and promote exclusion of the alternative exon.

Figure 1-4



1.3.2 hnRNP F/H can regulate AS in a cell-type specific manner

The hnRNP H targets identified by high-throughput analysis on HEK293T cells (Xiao et al. 2009) and the targets identified in a recent global analysis of hnRNP F/H performed in OL progenitor cell line (OPC) oli-neu (Wang et al. 2012), display little overlap, suggesting hnRNP F/H-dependent AS may be controlled by distinct mechanisms in different cell types (Mandler, Ku and Feng 2014). One of the best-studied cell-type specific targets of hnRNP F/H in myelinating glia is AS regulation of PLP pre-mRNA, which was investigated thoroughly by the Cambi lab (Wang, Dimova and Cambi 2007, Wang et al. 2008, Wang et al. 2011). This group identified two cis-acting elements regulating AS of the PLP pre-mRNA. In addition to a 19 bp ISE identified in the intron downstream of exon 3b promoting exon 3b inclusion, a G-run element functioning as an ESE for DM20 within exon 3b was identified by linker scanning to be critical for usage of the DM20 5' splice site and exon 3b exclusion (Wang, Dimova and Cambi 2007). Mutating both the G-run ESE of DM20 and the ISE of PLP increased the PLP/DM20 ratio. Replacing both regulatory elements with the 19 bp ISE decreased PLP/DM20, but replacing the elements with the G-run ESEs also decreased PLP/DM20, suggesting the location of the sequence element within exon 3b is a strong enhancer for regulation of AS (Wang, Dimova and Cambi 2007).

The authors analyzed 5' splice site usage by siRNA knockdown of hnRNP F/H and found that knockdown of hnRNP H alone led to an increase in the PLP/DM20 ratio (Wang, Dimova and Cambi 2007). Although, hnRNP F knockdown (KD) alone did not change the PLP/DM20 ratio, the knockdown of both hnRNP F/H lead to an even further increase in PLP/DM20 as compared to hnRNP H knockdown alone, suggesting both

hnRNP F/H could be important for promoting DM20 5' splice site usage (Wang, Dimova and Cambi 2007). The authors concluded that the 19 bp ISE is a positive regulator of PLP, and a G-run ESE is a positive regulator of DM20 (Wang, Dimova and Cambi 2007). When mutating G-run ESE, PLP increases, but knocking down hnRNP F/H leads to even more abundant PLP, suggesting hnRNP F/H are enhancers of DM20 expression, as well as repressors of PLP expression. The same group later identified that both hnRNP F and H regulate the PLP/DM20 ratio by recruiting the U1 snRNP to the DM20 5' splice site (Wang and Cambi 2009).

hnRNP F/H was shown to be involved in regulating other cell-type specific splicing events. One such example is the first functional identification of hnRNP F in a complex that regulates splicing of c-src N1 exon in a neural-specific manner, despite the fact that hnRNP F expression is not neural-specific (Min, Chan and Black 1995). Likely, this is due to the coordination with a neural specific factor that also regulates c-src N1 exon splicing. Originally, negligible amounts of hnRNP H were identified in the neural-specific splicing complex; however later reports identified hnRNP H as a part of this splicing complex (Chou et al. 1999). This study also showed that hnRNP F and H can interact *in vitro*, but whether they form heterodimers *in vivo* to regulate splicing has yet to be fully understood (Chou et al. 1999). Abnormally high levels of hnRNP F/H were also shown to differentially affect AS between OLs and SWCs (Mandler, Ku and Feng 2014), which is presented in Chapter 2.

hnRNP F/H and other members of the hnRNP H family do not always function together to regulate AS of the same targets (Caputi and Zahler 2001). An early account of an hnRNP H-specific target was the negative regulator of splicing (NRS) element in Rous

sarcoma virus (Fogel and McNally 2000). hnRNP H was shown to bind to the NRS by UV-crosslinking and electromobility shift assays (EMSAs), whereas hnRNP F did not promote supershifting of the element (Fogel and McNally 2000). hnRNPs can coordinate or antagonize each other to regulate AS of downstream targets (Huelga et al. 2012), such as is the case for the insulin receptor (IR) gene. hnRNP F, hnRNP H, hnRNP A1, and SRSF1 were shown to bind to the pre-mRNA for IR. hnRNP A1 can bind to the consensus sequence AGGGA and inhibits inclusion, whereas hnRNP F promotes inclusion and hnRNP H has no effect (Talukdar et al. 2011). This study also compared AS of IR between HepG2 cells and HEK293 cells, and proposed the differences were likely due to the relative expression levels of the hnRNPs (Talukdar et al. 2011).

1.3.3 Functions for hnRNP F/H in OL differentiation

The role for hnRNP F/H in OLs has been thoroughly studied. hnRNP F/H were shown to regulate AS and expression of myelin-specific genes such as PLP (Wang, Dimova and Cambi 2007), and were implicated in controlling OL differentiation (Wang et al. 2012). Additionally, hnRNP F was shown to play a role in post-transcriptional regulation of MBP expression, which is critical for normal myelin development (White et al. 2012). Genome-wide transcriptomic analysis was performed on the OL progenitor cell line oli-neu by Affymetrix exon array to examine overall gene expression, as well as specific exon usage following siRNA-mediated knockdown of hnRNP F/H. hnRNP F/H knockdown in oli-neu cells affected splicing of 250 exons with a fold change ≥ 1.5 and a p-value ≤ 0.05, and 1700 exons with a fold change ≥ 1.2 and a p-value ≤ 0.05 (Wang et al. 2012). hnRNP F/H manipulation affected all different types of AS events, such as

alternative 5' or 3' splice site usage, intron retention, and cassette exon. Aside from the largest group of unknown internal splicing events, the fraction of exons affected was highest for usage of alternative terminal exons (Wang et al. 2012). For many of the examples shown, AS was most affected upon simultaneous knockdown of both hnRNP F and hnRNP H. However, in some cases specific knockdown of either hnRNP F or hnRNP H resulted in a change of alternative exon usage without further enhancement using an siRNA targeting both hnRNP F/H (Wang et al. 2012).

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of affected genes upon knockdown of hnRNP F/H showed several pathways that are linked to regulation of cell-cycle progression, such as the mTOR pathway (Wang et al. 2012). Gene expression and AS of cell cycle regulators including cdk2 and SOX6, which is a negative regulator of OL differentiation, were both reduced or affected upon hnRNP F/H knockdown in oli-neu cells (Wang et al. 2012). The reduction of gene expression of cdk2 and Sox6 during OL differentiation *in vivo* by sorting of OPCs, pre-OLs, and differentiated OLs correlated with the known developmental downregulation of hnRNP F/H expression (Wang et al. 2012). This study provided the first clue of a functional role for hnRNP F/H in promoting expression of proliferation-inducing genes and inhibiting expression of differentiation-inducing genes to negatively regulate OL differentiation.

1.3.4 Dysregulation of hnRNP F/H in disease

Several AS targets of hnRNP F/H are dysregulated in different diseases. In agreement with the idea that hnRNP F/H promotes expression of proliferation-inducing genes, abnormally abundant hnRNP F/H expression could contribute to cancer and

tumorigenesis. hnRNP F regulates AS of tumor-associated NADH oxidase (Tang et al. 2011). hnRNP H is upregulated in human colon and head and neck cancers (Rauch et al. 2011). hnRNP H was also implicated in neurodegeneration. However, less has been reported on its specific functional role aside from being a sequestered RNA-binding protein. Hexanucleotide repeats of GGGGCC, targeted directly by hnRNP H, were identified in the NDDs ALS and FTD (Lee et al. 2013). Abnormal expansion of this sequence in ALS and FTD can lead to formation of RNAs with secondary structures of G quadruplexes that can be targeted by hnRNPs in a conformation-dependent manner, including hnRNP F (Haeusler et al. 2014).

hnRNP H also regulates AS of IG20/MADD, which is an adaptor protein involved in apoptosis (Lefave et al. 2011, Xiao et al. 2009). Two AS events occur to form 4 isoforms by alternative 5' splice site usage in exon 13 and inclusion/exclusion of exon 16 (Lefave et al. 2011). Alternative exon 16 is included in IG20 and excluded in MADD. IG20 triggers apoptosis, while MADD promotes cell survival, thus AS can lead to opposite fates of a particular cell (Lefave et al. 2011). AS of exon 16 is altered in several GBM tissue samples, which abnormally reduced inclusion of exon 16 and expressed higher levels of the anti-apoptotic form MADD, likely preventing the death of abnormal cells and contributing to tumorigenesis (Lefave et al. 2011). Also, hnRNP H is abnormally upregulated in GBM (Lefave et al. 2011). Knockdown of hnRNP H in glioma cell lines led to increased exon 16 inclusion and increased cell death, which was rescued with co-expression of an isoform-specific siRNA to IG20 (Lefave et al. 2011). These results suggest that hnRNP H contributes to the tumorigenesis of GBMs by regulating AS events that control the balance between cell survival and cell death in glioma cell lines.

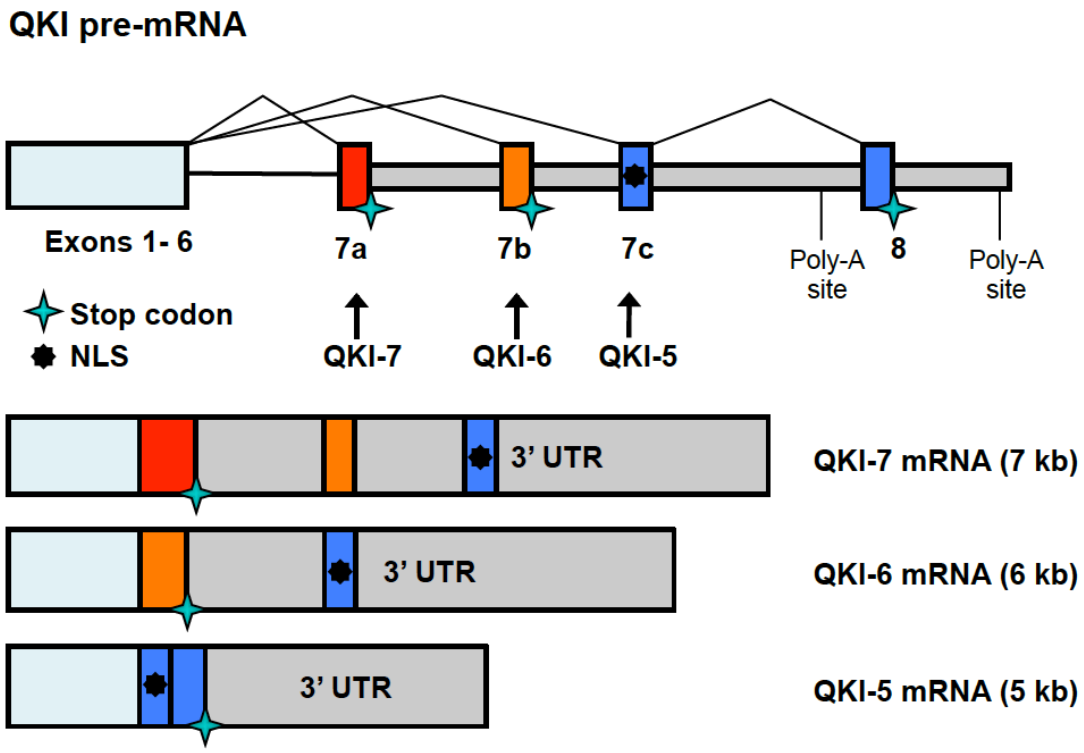
1.4 Function and importance of selective RNA-binding protein quaking I (QKI)

1.4.1 Regulation of QKI isoform expression

The selective RNA-binding protein quaking I **(**QKI) plays critical roles in the differentiation of myelinating glia and the development of myelin (Chen et al. 2007, Larocque et al. 2009, Larocque et al. 2005, Zhao et al. 2006). Three main QKI isoforms regulate multiple facets of RNA metabolism from splicing in the nucleus to mRNA stability and translation in the cytoplasm (Larocque et al. 2005, Li et al. 2000, Wu et al. 2002, Zhao et al. 2010). The isoforms QKI-5, QKI-6, and QKI-7, are produced by AS of the terminal coding exons in QKI pre-mRNA, and are named by the size of the resultant mature mRNAs (Bockbrader and Feng 2008). The *qkI* gene structure, primary coding sequence, and splicing pattern are highly conserved between humans and rodents (Kondo et al. 1999). Exons 1-6 are common between all isoforms, whereas selection of downstream exons 7a, 7b and 7c results in formation of QKI-7, QKI-6, and QKI-5, respectively (Figure 1-5). Splicing to exons 7a and 7b are final splicing events to form QKI-7 and QKI-6, as no additional 5' splice sites are present after these exons. Both QKI-6 and QKI-7 utilize the same poly-adenylation site, thus the sequence representing exon 7b and 7c is included in the 3' UTR of QKI-7 mRNA. As a result, QKI-6 and QKI-7 mRNAs share most of their 3'UTR sequences, with the exception of the unique region in QKI-7 3'UTR upstream of exon 7b sequence. Selection of exon 7c and a subsequent constitutive splicing event to a downstream exon 8, removes the sequence containing the first poly-A site to form QKI-5 mRNA. As a result, an APA site is utilized downstream of exon 8 to form QKI-5 mRNA. Thus, biogenesis of the different QKI isoforms depends on proper regulation of AS and of APA site selection.

Figure 1-5: AS of the 3' end of QKI pre-mRNA. The pre-mRNA diagram is illustrated to show common exons 1-6 (light grey), the connecting intron and downstream exons 7a (red), 7b (orange), 7c and 8 (blue). The grey rectangle refers to the sequence that either becomes 3' UTR upon splicing to the upstream respective exon, or is spliced out. The blue diamonds denote the stop codons and the nuclear localization signal is represented by a black star. The AS events are illustrated with lines adjoining the exons, and the subsequent mRNA products that are produced are illustrated below, named after the sizes of the respective mRNAs. The two poly-A sites are demarcated; the first poly-A site is utilized for QKI-6 and QKI-7 expression, whereas the poly-A site downstream of exon 8 is utilized for QKI-5.

Figure 1-5


QKI pre-mRNA
Exons 1- 6
7a
7b
7c
Poly-A site
8
Poly-A site
Stop codon
NLS
QKI-7
QKI-6
QKI-5
3' UTR
QKI-7 mRNA (7 kb)
3' UTR
QKI-6 mRNA (6 kb)
3' UTR
QKI-5 mRNA (5 kb)

QKI expression differs between neural cell types. In neural progenitor cells expression of all three isoforms is detected, but during cell fate specification, neurons no longer express detectable amounts of QKI, whereas all three QKI isoforms are maintained in glia (Hardy 1998). However the relative levels of QKI isoforms differ between astrocytes and OLs (Hardy et al. 1996). In fact QKI-5 is abundant in most neural cell types, whereas in mature myelinating glia QKI-6 is the predominant isoform (Lu et al. 2003, Zhao et al. 2006). In the qk^{v}/qk^{v} mouse, despite reduced expression of all three QKI isoforms, the deficiency in QKI-6 and QKI-7 is thought to be the reason for defects in OL differentiation (Larocque et al. 2005). QKI-6 is preferentially reduced suggesting a more prominent functional role in OL differentiation and CNS myelination (Zhao et al. 2006). QKI-7 is implicated in later stages of myelin development but the specific function is less well understood (Wu et al. 2001, Zhang et al. 2003). The role for QKI-7 has been studied more in astrocytes and is thought to function in interferon-related inflammatory pathways (Jiang et al. 2010). In fibroblasts and primary rat OL cell culture, cytoplasmic QKI-7 overexpression was shown to promote apoptosis, further indicating the proper balance of QKI isoform expression is likely important for cell survival (Pilotte, Larocque and Richard 2001).

Expression of the QKI isoforms in myelinating glia is developmentally regulated. QKI-5 is the earliest expressed isoform, is abundant in proliferating cells, and is essential for viability due to its role in vascular development during embryogenesis (Bohnsack et al. 2006). Northern blot of total brain showed QKI-5 mRNA levels are abundant in the first two weeks of postnatal development but then decrease, while QKI-6 and QKI-7 mRNA levels increase during active myelination in mouse brain development, peak at

postnatal day 14, and are maintained through adulthood (Hardy et al. 1996, Lauriat et al. 2008). QKI-6 and QKI-7 isoforms were shown to block proliferation and promote differentiation of OLs and SWCs (Larocque et al. 2009, Larocque et al. 2005). All three QKI isoforms can promote differentiation of OL progenitor cells in culture, suggesting distinct roles for the QKI isoforms at different stages of OL differentiation and myelin development (Chen et al. 2007).

QKI isoform expression is tightly regulated in neuron-glia cell fate specification and during differentiation of myelinating glia (Hardy 1998, Larocque et al. 2009, Larocque et al. 2005). However, what exactly regulates QKI isoform expression is unknown. Specifically, whether QKI isoform mRNA expression levels are dependent strictly on AS, or could also be regulated by APA remains undetermined. In addition, whether other steps in RNA metabolism, such as mRNA stability and mRNA translation, are involved in regulating QKI isoform expression still remains elusive. Furthermore, whether distinct molecular mechanisms underlie QKI isoform expression between different stages of cell differentiation, and/or between different cell types, is an intriguing question to be addressed. The experimental progress in understanding the regulation of QKI isoform expression is addressed in Chapter 4.

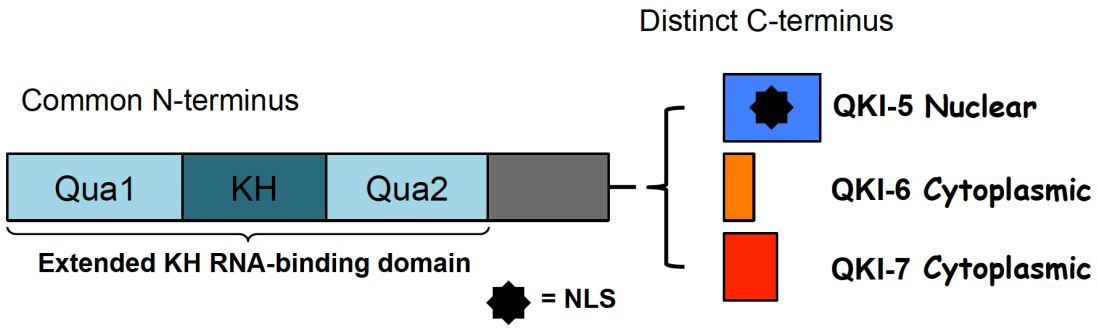
1.4.2 Functional dependence of the QKI isoforms on sub-cellular localization

All three QKI protein isoforms share a common RNA-binding domain that is an extended hnRNP K homology (KH) domain at the N-terminus. As illustrated in Figure 1-6, the extended KH domain is comprised of three subdomains, the first is responsible for protein dimerization (QUA1), the second contains the core RNA-binding KH domain,

and the third domain is responsible for RNA-binding stabilization (QUA2). QKI binds RNAs through a quaking response element (QRE) with a consensus sequence ACUAAY-N_{1-20}-UAAY determined by SELEX (Galarneau and Richard 2005), PAR-CLIP (Hafner et al. 2010) and SILAC (Scheibe et al. 2012). The *in vitro* mutational analysis suggests the additional UAAY sequences could strengthen the binding interaction with QKI for certain targets, such as MBP (Galarneau and Richard 2005, Teplova et al. 2013). However, there are examples of RNA targets identified by PAR-CLIP that contain just the core sequence ACUAAY (Hafner et al. 2010, Scheibe et al. 2012). These studies identified an extensive list of over a thousand QKI mRNA targets. All three QKI isoforms bind RNA *in vitro* with similar affinity (Galarneau and Richard 2005). However, the *in vivo* mRNA ligands for each QKI isoform have not yet been identified. Individual studies elucidated specific functional targets of QKI to regulate mRNA processing, stability and translation, several of which are critical for OL differentiation, such as MBP, $p27^{kip1}$, and hnRNP A1 (Larocque et al. 2005, Larocque et al. 2002, Li et al. 2000, Zhao et al. 2010).

Figure 1-6: Protein domains of the QKI isoforms. The diagram shows the extended KH RNA-binding domain as the N-terminal common region of all three QKI isoforms, which includes Qua1, KH and Qua2. As shown, the C-terminal amino acids differ between QKI-5 (blue), QKI-6 (orange) and QKI-7 (red). QKI-5 contains a nuclear localization signal (NLS) denoted by a black star, which is absent from QKI-6 and QKI-7. As a result, QKI-5 is the nuclear isoform, and QKI-6 and QKI-7 are predominantly cytoplasmic isoforms.

Figure 1-6


Distinct C-terminus
Common N-terminus
Qua1
KH
Qua2
QKI-5 Nuclear
QKI-6 Cytoplasmic
QKI-7 Cytoplasmic
Extended KH RNA-binding domain
= NLS

The C-terminal amino acids differ between all three QKI protein isoforms, with QKI-6 having the shortest (Figure 1-6). QKI-5 C-terminus is the longest, and it contains a non-canonical nuclear localization signal (NLS), resulting in nuclear localization (Wu et al. 1999). QKI-6 and QKI-7 lack this NLS and are considered cytoplasmic isoforms (Wu et al. 1999). QKI-5 localizes predominantly in the nucleus at steady state and is considered to be the nuclear isoform, despite the evidence of heterodimerization between QKI isoforms that can result in nuclear-cytoplasmic shuttling (Pilotte, Larocque and Richard 2001, Wu et al. 1999). As the QKI isoforms share exons 1-6 that are translated to form the common RNA-binding domain, QKI isoforms can all bind the same RNAs. However, their distinct C-terminal domains that determine nuclear-cytoplasmic localization likely confers to each of their specific roles in regulating RNA metabolism at the different post-transcriptional steps, such as pre-mRNA processing, mRNA stability, and translation. Previous studies have suggested a role for QKI-5 in regulating AS in other cell types where QKI-6 is not the major isoform (Hall et al. 2013, Wu et al. 2002). Interestingly, the AS defects observed in OLs of the qk^{v}/qk^{v} mouse are completely rescued when a QKI-6 transgene is specifically expressed in OLs, without increasing nuclear levels of QKI-5 (Zhao et al. 2010). As a result, QKI-6 is thought to play a crucial role in regulating AS in myelinating glia (Mandler, Ku and Feng 2014, Zhao et al. 2010), which is the focus of Chapters 2 and 3 of this dissertation.

1.4.3 Alterations of QKI expression in human disease

Regulating the metabolism of target RNAs defines specific roles for the QKI isoforms that may be contributing factors towards a larger scheme of cellular events with

dysregulation likely resulting in disease. Indeed, the QKI isoforms have been implicated in a number of different cellular processes. QKI-5 is thought to be involved in embryogenesis due to its function in blood vessel formation and vascular remodeling (Noveroske et al. 2002). All three QKI isoforms were proposed to play roles in glial cell fate determination (Hardy 1998). Overexpression of QKI-7 causes apoptosis, thus a balanced expression of isoforms is important for preventing cell death (Pilotte, Larocque and Richard 2001). QKI-6 plays crucial roles in the formation of myelin, as transgenic expression of QKI-6 alone rescues the hypomyelination phenotype of the qk^{v}/qk^{v} mice (Zhao et al. 2006). QKI protein is aberrantly detected in cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients (Menon et al. 2011). In addition, abnormal QKI expression was observed in several diseases, including mental disorders, ataxia, and different gliomas (Chenard and Richard 2008). Many of these diseases are augmented by defects in the function and development of myelin, which might involve QKI dysregulation (Bankston, Mandler and Feng 2013). In addition to myelin-related disorders, QKI deficiency is also implicated in various types of tumors (Bian et al. 2012, Novikov et al. 2011, Yang et al. 2010, Zhao et al. 2014, Zong et al. 2014). Discussed here are specific cases in which QKI dysfunction is linked to disease. Future studies will attempt to define the underlying molecular mechanisms regulated by QKI to better understand disease pathogenesis.

The dysfunction of myelin and OL are contributing factors towards pathological development of psychiatric diseases, including schizophrenia (SCZ), bipolar disorder, and major depression (Sokolov 2007, Tkachev et al. 2003). SCZ patients were found to have morphological changes in white matter (Chenard and Richard 2008). Along with the

reduction of myelin-specific genes downstream of QKI, such as MAG and PLP (Aberg et al. 2006), mRNA expression of QKI isoforms is reduced in SCZ (Haroutunian et al. 2006) and in major depression (Klempan et al. 2009). One study identified preferential reduction of QKI-7 in the pre-frontal cortex of SCZ patients (Aberg et al. 2006). Another study found QKI-7 levels were responded well to treatment with the anti-psychotic drug haloperidol, suggesting a more prominent role for QKI-7 as compared to other QKI isoforms in the SCZ pathogenesis (Jiang et al. 2009). Additionally, a SNP that could potentially disrupt normal QKI isoform expression was identified in the *qkI* gene between exons 7b and 7c, which co-segregates with SCZ patients in a large cohort (Aberg et al. 2006). These studies not only implicate QKI in psychiatric disease, but also highlight the importance for the tight regulation of QKI isoform expression.

Two of the QKI mutant mouse models that harbor QKI deficiency, the qk^{v}/qk^{v} and the ENU-induced mutation qk^{e5}/qk^{e5}, are marked by swelling of Purkinje cell axons, leading to neurodegeneration, which is a hallmark of several types of ataxia (Chenard and Richard 2008). From over 23 different types of ataxia that share similar interacting protein pathways, proteomic studies identified at least 29 different proteins that interact with QKI, including a genetic modifier of spinocerebellar ataxia type 1 (Chenard and Richard 2008). Interestingly, aside from QKI protein interactions expression of Ataxin2 alternative splice variants is dysregulated in the qk^{v}/qk^{v} mutant, suggesting a role for QKI in regulating expression of Ataxin2 (Mandler, Ku and Feng 2014). The exact function of Ataxin2 splice variants is not known. However, poly-glutamine expansion in ataxin2 causes spinocerebellar ataxia type 2 and contributes to pathogenesis of amyotrophic lateral sclerosis (ALS), as well as other NDDs (Ross et al. 2011).

In addition to CNS disorders, QKI abnormality is of interest to the cancer field. Multiple studies have suggested QKI as a tumor suppressor in differ types of cancer (Chen et al. 2012). A recent study investigated the role for QKI in lung cancer and found that reduced QKI expression correlated with increased severity of prognosis (Zong et al. 2014). Interestingly, squamous cell carcinomas and adenocarcinomas marked by reduced QKI-5 had abnormal AS events of several downstream targets (Zong et al. 2014). QKI-5 directly competes with SF1 to regulate AS of NUMB, which is involved in the Notch signaling pathway (Zong et al. 2014). The proposed model suggests that QKI-5 suppresses inclusion of exon 12 in NUMB pre-mRNA and blocks tumorigenesis (Zong et al. 2014). Dysregulated QKI expression is also implicated in prostate, colon, colorectal, and gastric cancers (Bian et al. 2012, Ji et al. 2013, Jiang et al. 2010, Yang et al. 2010, Zhao et al. 2014). Several studies have identified reduced QKI levels or abnormal isoform expression in gliomas, including GBM, astrocytomas, and different glioma cell lines, which correlates with abnormally upregulated expression of hnRNPs in cancer, some of which are known targets of QKI, such as hnRNP A1 and hnRNP H (Dery et al. 2011, Han, Li and Zhang 2013, Lefave et al. 2011, Li et al. 2002, Zong et al. 2014).

The normal regulation of QKI isoform expression is likely a crucial component to prevent tumorigenesis and the onset or pathogenesis of several myelin-associated brain diseases. The following chapters discuss the functional role of QKI in regulating AS of downstream targets in myelinating glia. Reported in Chapter 4 are the potential mechanisms involved in the regulation of QKI isoform expression at different stages of OL differentiation to ensure proper regulation of downstream pathways that control OL differentiation and normal myelin development.

1.5 Summary

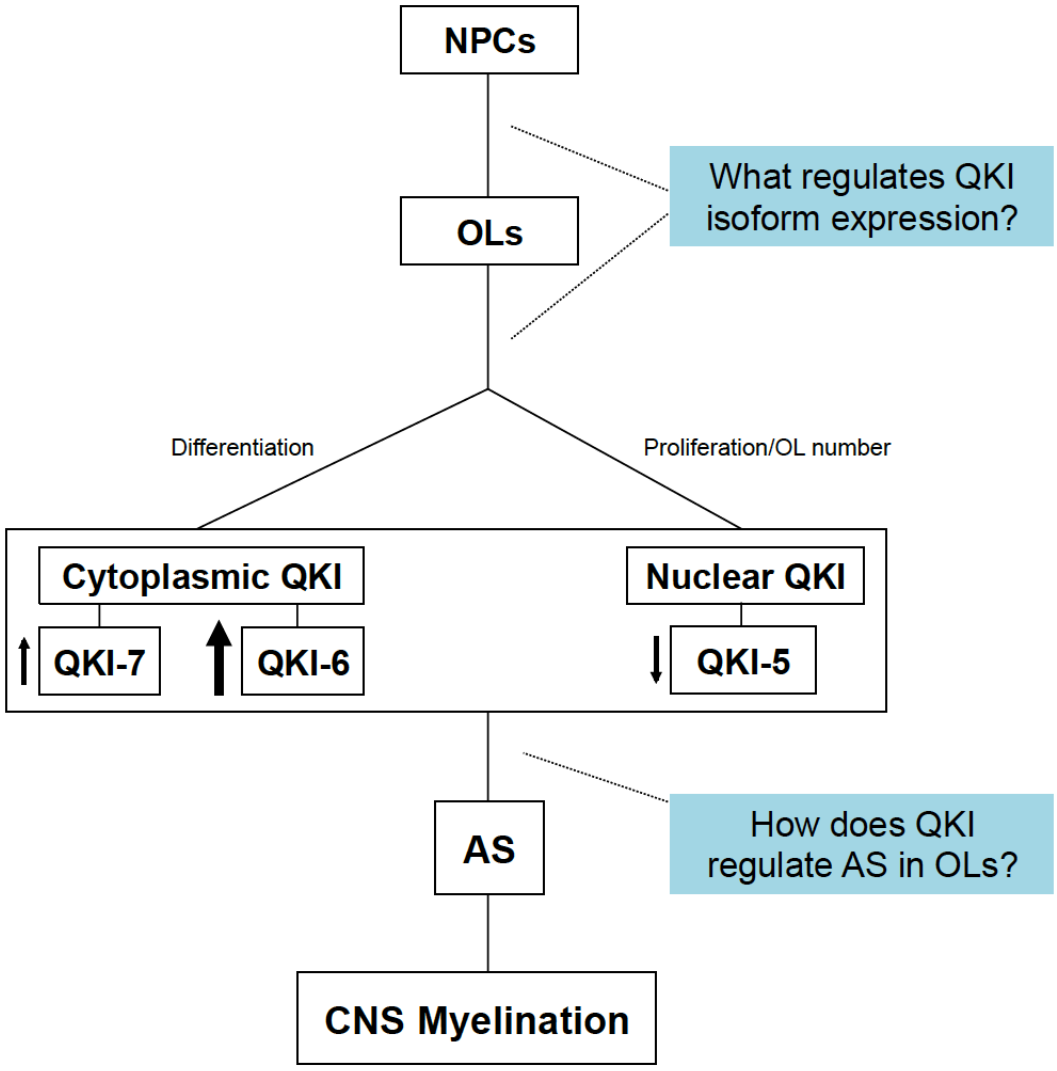
In this dissertation, I used a combination of genetic *in vivo* mouse and cell culture model systems to understand the mechanisms that underlie AS in OLs. I focused specifically on the selective RNA-binding protein quaking I (QKI), as deficiency of QKI in OLs leads to severe dysregulation of AS in the qk^v/qk^v mutant mouse (Mandler, Ku and Feng 2014, Zhao et al. 2006). The unknowns that are addressed in this dissertation are modeled in Figure 1-7. In Chapter 2 and 3 of this dissertation, I asked: **How does QKI control AS in myelinating glia?** In Chapter 2, I identified a novel pathway in which the cytoplasmic isoform QKI-6 controls AS by regulating expression of SFs hnRNP F/H. I showed that QKI deficiency leads to dysregulation of hnRNP F/H-dependent AS, which is differentially affected in the myelinating glia of the CNS and PNS. In Chapter 3 of this dissertation I expanded the study to address: **Does QKI deficiency affect expression of other SFs?** I identified several SFs that play critical roles in development and function of the brain that are abnormally expressed in the brain stem of the qk^v/qk^v mutant, and are rescued by OL-specific QKI-6 expression.

The expression of QKI isoforms is tightly regulated during development and differs between cell types (Hardy 1998). During neuron-glia lineage establishment in neural progenitors, nuclear QKI-5 is the major detected isoform (Hardy 1998). In contrast to the developmental decline of QKI expression in the neuronal lineage, during OL lineage specification QKI expression is up-regulated (Hardy 1998). Moreover, during later stages of OL differentiation and myelin development the nuclear and cytoplasmic QKI isoforms are reciprocally regulated, with reduced QKI-5 and increased QKI-6/QKI-7 expression (Hardy et al. 1996, Larocque et al. 2005, Lauriat et al. 2008). These

isoforms perform distinct functions in OL and myelin development. The nuclear QKI-5 is thought to maintain OLs in a proliferative state (Larocque et al. 2002, Wu et al. 2002). QKI-6 and QKI-7 can drive OL differentiation (Larocque et al. 2005, Li et al. 2000, Zhao et al. 2010). However, what controls QKI isoform expression during OL differentiation and between different cell types is poorly understood. In Chapter 4 of this dissertation: **I investigated the underlying mechanisms controlling QKI isoform expression in neuronal and OL progenitor cell lines.** I found that during early OL differentiation all three QKI mRNAs are upregulated. However QKI-6 protein is preferentially upregulated, indicating the mRNA levels do not recapitulate protein levels, and suggesting differential mRNA translation might control QKI isoform expression. However, the QKI isoform mRNAs display distinct expression patterns in neuronal and OL cell lines, suggesting alternative processing of QKI pre-mRNA. AS alone or in cooperation with APA may be an important mechanism that governs QKI isoform expression in different cell types. Consistent with this view, I presented data suggesting the SF FOX2 regulates AS of the QKI pre-mRNA in OLs. I further discussed potential mechanisms that could contribute to differential regulation of QKI isoform expression between different cell types or stages of cell differentiation.

Figure 1-7: The QKI pathway and regulation of AS in OLs. QKI is expressed in neural progenitor cells (NPCs), with QKI-5 being the most abundant isoform. Expression of QKI isoforms is maintained during OL cell fate specification. The upregulation of cytoplasmic QKI isoforms drives OL differentiation, whereas nuclear QKI maintains and a proliferating pool of OL progenitor cells. Alternative splicing (AS) is extensively regulated during OL differentiation and CNS myelination, and is dysregulated in a QKI deficient mouse. Balanced expression of these isoforms and proper regulation of AS is critical for CNS myelination. However, what regulates QKI isoform expression and how QKI regulates AS in OLs are unknowns addressed in this dissertation.

Figure 1-7


NPCs
OLs
What regulates QKI isoform expression?
Differentiation
Proliferation/OL number
Cytoplasmic QKI
Nuclear QKI
QKI-7
QKI-6
QKI-5
AS
How does QKI regulate AS in OLs?
CNS Myelination

Chapter 2: A cytoplasmic quaking I isoform regulates the hnRNP F/H-dependent alternative splicing pathway in myelinating glia

This chapter is published as:

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2.1 Introduction

Alternative splicing (AS) allows production of multiple mRNAs from a single gene that often encode distinct protein isoforms to perform paradoxically opposite functions (Nilsen and Graveley 2010). More than 90% of mammalian genes are regulated by AS, which greatly increases proteomic diversity (Kornblihtt et al. 2013). Inclusion or exclusion of an alternative exon is achieved by interactions between canonical splicing factors and specific sequences in the pre-mRNA, which in turn control the recruitment of the splicing machinery (Lin and Fu 2007, Martinez-Contreras et al. 2007). The majority of splicing factors are ubiquitously expressed (Nilsen and Graveley 2010), yet alternatively spliced mRNA isoforms are often differentially regulated during development and in different cell types (Kamma, Portman and Dreyfuss 1995, Woodley and Valcarcel 2002). How generic splicing factors achieve differential regulation of AS is an intriguing question that remains largely unanswered. In oligodendrocytes (OLs) and Schwann cells (SWCs), two types of functionally related glial cells that form myelin membranes to enable nerve conductance in the central and peripheral nervous systems (CNS and PNS), respectively, numerous transcripts are subjected to regulation by AS during myelin development (Bockbrader and Feng 2008, Hardy 1998). Dysregulation of AS in OLs causes human neurological diseases, as seen in the aberrant AS of proteolipid protein (PLP) pre-mRNA in a familial form of Pelizaeus–Merzbacher disease (Regis et al. 2009). In addition, severe dysregulation of AS is caused by deficiency of the quaking I (QKI) RNA-binding protein in myelinating glia of the homozygous quakingviable (qk^v/qk^v) mutant mouse, represented by the pre-mRNAs of PLP and myelin associated glycoprotein (MAG) (Wu et al. 2002, Zhao et al. 2010, Zhu et al. 2012).

Three QKI protein isoforms are derived from AS, named QKI-5, 6 and 7 (Bockbrader and Feng 2008). All QKI isoforms share the N-terminal K-homology (KH) domain for binding RNA whereas the distinct C-termini determine isoform-specific nuclear-cytoplasmic distribution (Chen et al. 2007, Hardy 1998, Hardy et al. 1996). QKI-5 predominantly localizes in the nucleus at steady state (Hardy et al. 1996, Larocque et al. 2002, Wu et al. 1999). In contrast, QKI-6 and QKI-7 are largely cytoplasmic (Hardy et al. 1996, Larocque et al. 2005, Wu et al. 1999, Zhao et al. 2006, Zhao et al. 2010). The nuclear isoform QKI-5 is expressed in most cell types, and was previously shown to regulate AS of pre-mRNA and control mRNA nuclear export, whereas the cytoplasmic isoforms QKI-6 and QKI-7 govern stability and translation of their bound mRNAs (Bockbrader and Feng 2008, Hall et al. 2013, Li et al. 2000, Lu et al. 2003, Saccomanno et al. 1999, Wu et al. 2002, Wu et al. 1999, Zhao et al. 2006, Zhao et al. 2010). Interestingly, QKI-6 is the most abundant isoform in myelinating glia (Lu et al. 2003, Zhao et al. 2006). Although all QKI isoforms are reduced in OLs of the qk^{v}/qk^{v} mutant (Hardy et al. 1996, Lu et al. 2003), OL-specific expression of cytoplasmic QKI-6 alone rescues AS abnormalities in the qk^{v}/qk^{v} brain without increasing nuclear QKI-5 levels (Zhao et al. 2010). Thus, QKI-6 must control an undefined post-transcriptional cascade, which in turn governs AS during myelin development.

Heterologous nuclear ribonucleoproteins (hnRNPs) F and H are functionally orthologous splicing factors, both of which target polyguanine (G-run) sequences within or surrounding alternative exons in pre-mRNAs to regulate AS (Caputi and Zahler 2001, Katz et al. 2010, Wang, Dimova and Cambi 2007). During differentiation of OL progenitor cells (OPCs) and CNS myelination, both hnRNP F and H are markedly down-

regulated, which in turn regulates AS of numerous pre-mRNAs (Wang et al. 2012), including the PLP pre-mRNA known to be affected by QKI deficiency (Wu et al. 2002, Zhu et al. 2012). However, molecular mechanisms that regulate hnRNP F/H expression still remain elusive. Moreover, the functional AS targets of hnRNP F/H in myelin development are largely unknown.

We report that the cytoplasmic QKI-6 acts upstream of hnRNP F/H to regulate AS in myelinating glia. Deficiency of QKI in OLs and SWCs of the qk^{v}/qk^{v} mutant mice results in aberrant over-production of both hnRNP F and hnRNP H. Such dysregulation of hnRNP F/H in OLs can be completely rescued by transgenic expression of the cytoplasmic QKI-6 alone. Furthermore, we identified a pool of functional hnRNP F/H targets *in vivo*, which are differentially affected in OLs and SWCs of the qk^{v}/qk^{v} mutant mice. These findings reveal that cytoplasmic QKI-6 and hnRNP F/H form a novel regulatory pathway to control AS in myelinating glia.

2.2 Results

2.2.1 QKI-6 deficiency in OLs is responsible for aberrant over-expression of hnRNP F/H proteins without affecting their mRNAs

Our previous studies showed that QKI-6 is necessary for normal AS of the PLP pre-mRNA specifically expressed in OLs, which is a well-characterized target of hnRNP F/H (Wang, Dimova and Cambi 2007, Wang et al. 2011, Zhu et al. 2012). This raises a question whether QKI-6 may control AS through regulating hnRNP F and/or hnRNP H. Consistent with this idea, putative QREs are found in the 3′UTRs of hnRNP F and hnRNP H mRNAs, which are conserved in mouse, rat and human (Figure 2-1). To test

whether QKI-6 may interact with hnRNP F/H mRNAs in myelinating OLs *in vivo*, we performed UV-crosslinking immunoprecipitation (CLIP) to isolate mRNA complexes formed with cytoplasmic QKI-6 from the brain of transgenic mice that express FLAG-QKI-6 specifically in OLs (Zhao et al. 2006). Successful isolation of FLAG-QKI-6 was indicated by immunoblot (Figure 2-2A, top panel) and the co-immunoprecipitated RNA was subjected to RT-PCR using hnRNP F- and hnRNP H-specific primer sets that target the 3′UTRs at or near the putative QREs. hnRNP H mRNA was clearly co-immunoprecipitated with FLAG-QKI-6 from OLs in the brain (Figure 2-2A, bottom panel). In contrast, despite abundant expression, hnRNP F mRNA was not co-isolated with FLAG-QKI-6. CLIP-RT-PCR also demonstrated that endogenous human hnRNP H mRNA, but not hnRNP F mRNA, was co-isolated with FLAG-QKI-6 expressed in the human OL cell line HOG (Figure 2-2B), which was attenuated without UV crosslinking (Figure 2-3). In addition, fusing the 3′UTR of mouse hnRNP H to a reporter mRNA enabled an interaction with FLAG-QKI-6 in transfected HOG cells, which was impaired when the predicted bipartite QRE in the 3′UTR was entirely deleted (ΔQRE, Figure 2-2C). Moreover, when co-transfected into HOG cells, QKI-6 significantly suppressed expression of luciferase from the reporter that carries the hnRNP H 3′UTR, whereas deleting the putative QRE reduced the effect by QKI-6 (Figure 2-2D). Together, these data suggest that cytoplasmic QKI-6 selectively interacts with mouse and human hnRNP H mRNA, and negatively regulates hnRNP H in cells of the OL lineage by targeting the conserved QRE in the 3′UTR.

Figure 2-1: Putative quaking response elements (QRE) are conserved between rodents and human. The putative QREs are underlined in the 3' untranslated region (3'UTR) of mouse, rat and human hnRNP H and hnRNP F mRNAs.

Figure 2-1

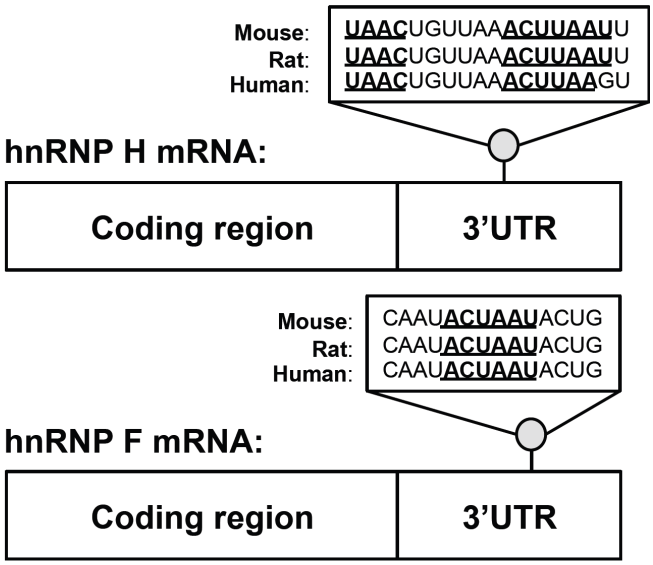

Mouse: UAACUGUUAAACUUAAUU
Rat: UAACUGUUAAACUUAAUU
Human: UAACUGUUAAACUUAAGU
hnRNP H mRNA:
Coding region
3'UTR
Mouse: CAAUACUAAUACUG
Rat: CAAUACUAAUACUG
Human: CAAUACUAAUACUG
hnRNP F mRNA:
Coding region
3'UTR

Figure 2-2: QKI-6 binds hnRNP H mRNA in a QRE-dependent manner, but not hnRNP F mRNA. (A) UV-crosslinking immunoprecipitation (CLIP) for co-isolation of mRNAs with FLAG-tagged QKI-6 expressed in OLs of the brain stem in the FLAG-QKI-6 transgenic mouse. Top panels: immunoblot detection of FLAG-QKI-6 in INPUT and CLIP complexes. The housekeeping protein elF5α is detected in INPUT but absent from CLIP. Bottom panels: RT-PCR detects hnRNP H mRNA by CLIP, but not hnRNP F mRNA. (B) CLIP of the human OL cell line HOG transfected with FLAG-QKI-6 plasmid (QKI-6) or pcDNA vector (Pc). Immunoblot indicates successful isolation of FLAG-QKI-6 (top panels) and RT-PCR indicates co-IP of hnRNP H mRNA, but not hnRNP F mRNA (bottom panels). (C) CLIP of HOG cells co-expressing FLAG-QKI-6 with a reporter either fused with mouse wild-type 3′UTR of hnRNP H (H-3′UTR) or a mutant 3′UTR that lacks the putative QRE (ΔQ). Similar amount of FLAG-QKI-6 is isolated from each lysate (top panel). The absence of the QRE diminished the ability for co-IP of the reporter mRNA with FLAG-QKI-6 by RT-PCR using primers specific for the reporter coding region, whereas endogenous hnRNP H mRNA still co-immunoprecipitated with FLAG-QKI-6 (bottom panel). Replicates from two independent transfections with the wild-type hnRNP H 3′UTR were subjected to CLIP in parallel, one of which was processed without reverse transcriptase (RT) as a negative control. All CLIP experiments were performed in the IP buffer containing 0.01% SDS. (D) Luciferase activity from hnRNP H reporters, wild-type (full) or mutant (ΔQRE) in HOG cells transfected by FLAG-QKI-6 construct or the pcDNA control vector. The firefly luciferase activity is normalized to the activity of co-expressed Renilla luciferase. Results are graphically displayed as percent of pcDNA control.

Figure 2-2

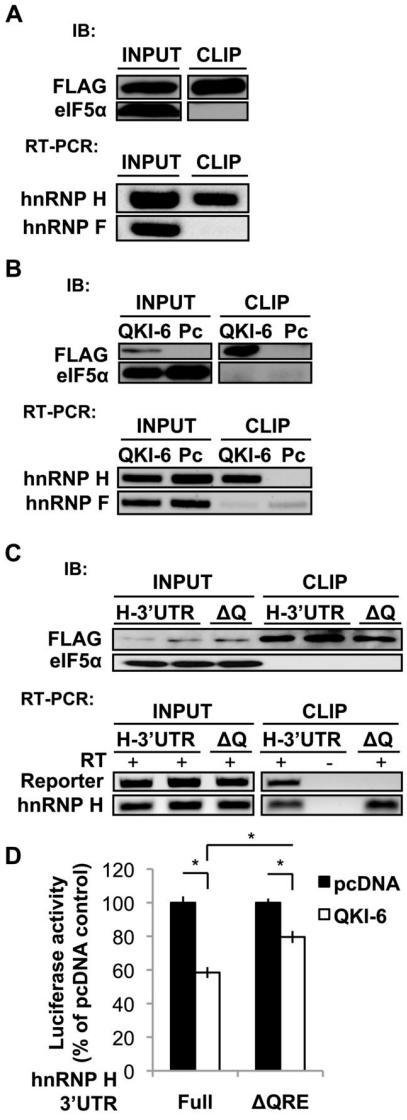
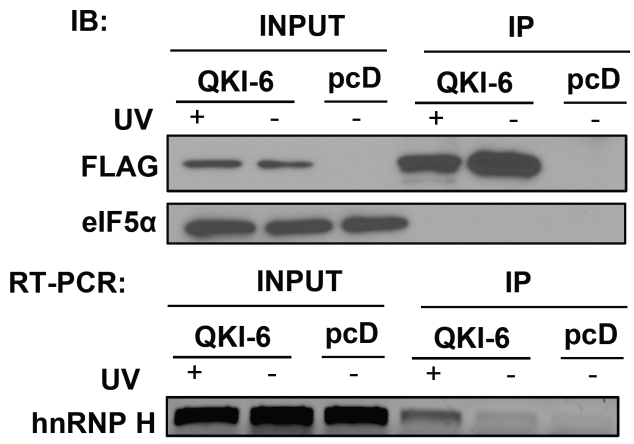

A
IB:
INPUT
CLIP
FLAG
eIF5α
RT-PCR:
INPUT
CLIP
hnRNP H
hnRNP F
B
IB:
INPUT
CLIP
QKI-6 Pc
QKI-6 Pc
FLAG
eIF5α
RT-PCR:
INPUT
CLIP
QKI-6 Pc
QKI-6 Pc
hnRNP H
hnRNP F
C
IB:
INPUT
CLIP
H-3'UTR
ΔQ
H-3'UTR
ΔQ
FLAG
eIF5α
RT-PCR:
INPUT
CLIP
H-3'UTR
ΔQ
H-3'UTR
ΔQ
RT
+ + + + - +
Reporter
hnRNP H
D
Luciferase activity (% of pcDNA control)
120
100
80
60
40
20
0
pcDNA
QKI-6
hnRNP H 3'UTR
Full
ΔQRE

Figure 2-3: Co-immunoprecipitation with QKI-6 by the hnRNP H mRNA under denaturing conditions is attenuated without UV crosslinking. Immunoprecipitation from HOG cells transfected with FLAG-QKI-6 or pcDNA control was performed in the presence of 0.5% SDS, with or without UV crosslinking as indicated on top of the corresponding lanes. Before immunoprecipitation, cell lysates were subjected to ultracentrifugation at 77,000 xg for 20 min at 4°C. Immunoblots of IPs were probed with anti-FLAG antibody to detect FLAG-QKI-6 and anti-eIF5α antibody for loading control (top). hnRNP H mRNA in the input and IP was detected by RT-PCR (bottom).

Figure 2-3

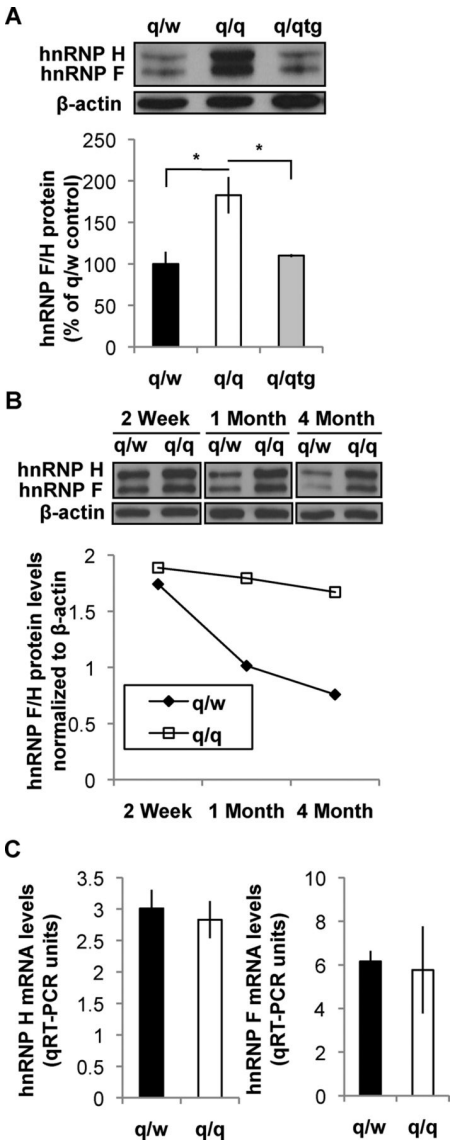

IB:
INPUT
IP
QKI-6
pcD
QKI-6
pcD
UV
+
-
-
+
-
-
FLAG
eIF5α
RT-PCR:
INPUT
IP
QKI-6
pcD
QKI-6
pcD
UV
+
-
-
+
-
-
hnRNP H

We next examined whether and how QKI-6 regulates expression of hnRNP H during CNS myelin development. hnRNP H is broadly expressed in many cell types (Caputi and Zahler 2001, Nilsen and Graveley 2010) whereas QKI deficiency in the CNS of the qk^v/qk^v mutant mice is restricted to OLs (Lu et al. 2003). Therefore, we performed immunoblot analysis to quantify hnRNP H protein in the OPNs highly enriched of OLs. An antibody that detects both hnRNP F and H, which display distinct sizes on SDS-PAGE, was used. Surprisingly, both hnRNP F and H were aberrantly increased approximately 2-fold in the qk^v/qk^v OPNs as compared to that in the non-phenotypic heterozygous qk^v/wt control (Figure 2-4A), despite the lack of interaction between QKI-6 and the hnRNP F mRNA (Figure 2-2). Moreover, transgenic expression of FLAG-QKI-6 alone specifically in qk^v/qk^v OLs completely reversed hnRNP F/H proteins to normal levels. These results clearly indicate that both hnRNP F and hnRNP H are functional targets of QKI-6 in OLs. We further showed that both hnRNP F and H were down-regulated in the OPNs during normal myelin development whereas QKI deficiency in the qk^v/qk^v OLs impaired such regulation (Figure 2-4B). However, despite the significantly elevated expression of hnRNP F/H proteins in qk^v/qk^v OPNs, the steady-state levels of the hnRNP F/H mRNAs remain normal (Figure 2-4C). In addition, a detectable increase in polyribosome association was observed for both the hnRNP H and hnRNP F mRNAs in the brain stem of qk^v/qk^v as compared to that in the qk^v/wt littermate control based on linear sucrose gradient fractionation followed by qRT-PCR (data not shown). Consistent with the known function of QKI-6 in suppressing translation of its bound mRNAs (Saccomanno et al. 1999, Zhao et al. 2010), these results suggest that QKI-6 may directly

repress translation of its bound hnRNP H mRNA and indirectly repress translation of the hnRNP F mRNA through yet undefined mechanisms.

Figure 2-4: QKI-6 deficiency results in over-expression of hnRNP F/H in OLs during CNS myelin development without affecting the abundance of hnRNP F/H mRNAs. (A) Immunoblot of hnRNP F/H in adult (4–5 months) OPNs derived from the heterozygous qk^v (q/w) non-phenotypic control, homozygous qk^v mutant (q/q), and q/qtg mice that express FLAG-QKI-6 in OLs. β-actin was a loading control for quantification of hnRNP F/H. Percent changes of hnRNP F/H protein levels relative to that in q/w are graphically displayed in the bottom panel. (B) Immunoblot of hnRNP F/H in q/q and q/w OPNs during myelin development at the indicated postnatal age. Signal density of hnRNP F/H was normalized to β-actin and graphically displayed in the bottom panel. (C) qRT-PCR quantification of hnRNP H and F mRNAs isolated from adult OPNs of q/w and q/q mice normalized to β-actin mRNA readings by ΔΔCt.

Figure 2-4


A
q/w
q/q
q/qtg
hnRNP H
hnRNP F
β-actin
hnRNP F/H protein (% of q/w control)
250
200
150
100
50
0
*
*
q/w
q/q
q/qtg
B
2 Week
1 Month
4 Month
q/w
q/q
hnRNP H
hnRNP F
β-actin
hnRNP F/H protein levels normalized to β-actin
2
1.5
1
0.5
0
q/w
q/q
2 Week
1 Month
4 Month
C
hnRNP H mRNA levels (qRT-PCR units)
3.5
3
2.5
2
1.5
1
0.5
0
q/w
q/q
hnRNP F mRNA levels (qRT-PCR units)
10
8
6
4
2
0
q/w
q/q

2.2.2 hnRNP F/H targets G-run elements to regulate inclusion of the alternative exon in the MAG pre-mRNA, which is dysregulated in the qk^v/qk^v mutant

We next asked whether hnRNP F/H might control AS of pre-mRNAs that are dysregulated in the qk^v/qk^v OLs. Inclusion of MAG Exon 12 is aberrantly increased in qk^v/qk^v OLs (Zhao et al. 2010), which is rescued by the FLAG-QKI-6 transgene (Zhao et al. 2010, Zhao et al. 2006). Noticeably, long stretches of G-runs, analogous to the consensus sequence targeted by hnRNP F/H in numerous pre-mRNAs (McCullough and Berget 1997, Wang, Dimova and Cambi 2007, Wang et al. 2011), are found in the introns within 250 nt of both the 3′ and 5′ splice sites that define Exon 12 in the mouse, rat and human MAG gene (Figure 2-5), all within optimal distance known for regulation by hnRNP F/H (Katz et al. 2010). To test whether hnRNP F/H may regulate AS of endogenous MAG pre-mRNA that harbor G-runs flanking Exon 12 (Figure 2-6A), we knocked down hnRNP F and hnRNP H simultaneously in the rat OL cell line CG4 (Figure 2-6B) using a previously validated siRNA (Wang, Dimova and Cambi 2007). As a result, inclusion of Exon 12 was significantly reduced based on semi-quantitative RT-PCR that detects both MAG mRNA isoforms with a single primer set (Figure 2-6C). Knockdown hnRNP F/H in CG4 cells in a parallel experiment also significantly affected AS of the PLP pre-mRNA (Figure 2-6D), which is a well-characterized target of hnRNP F/H (Wang et al. 2012, Wang, Dimova and Cambi 2007, Wang et al. 2011).

Figure 2-5: G-run motifs are identified surrounding MAG Exon 12 in rodents and human. Intronic G-runs are present within 250 base pairs upstream and downstream of the alternative Exon 12 in human, mouse, and rat MAG pre-mRNA.

Figure 2-5

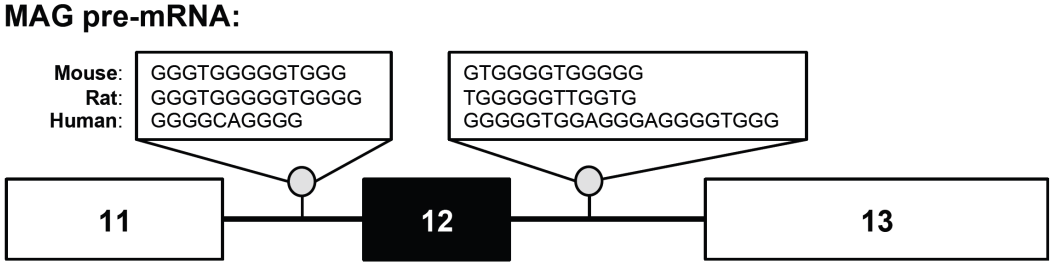
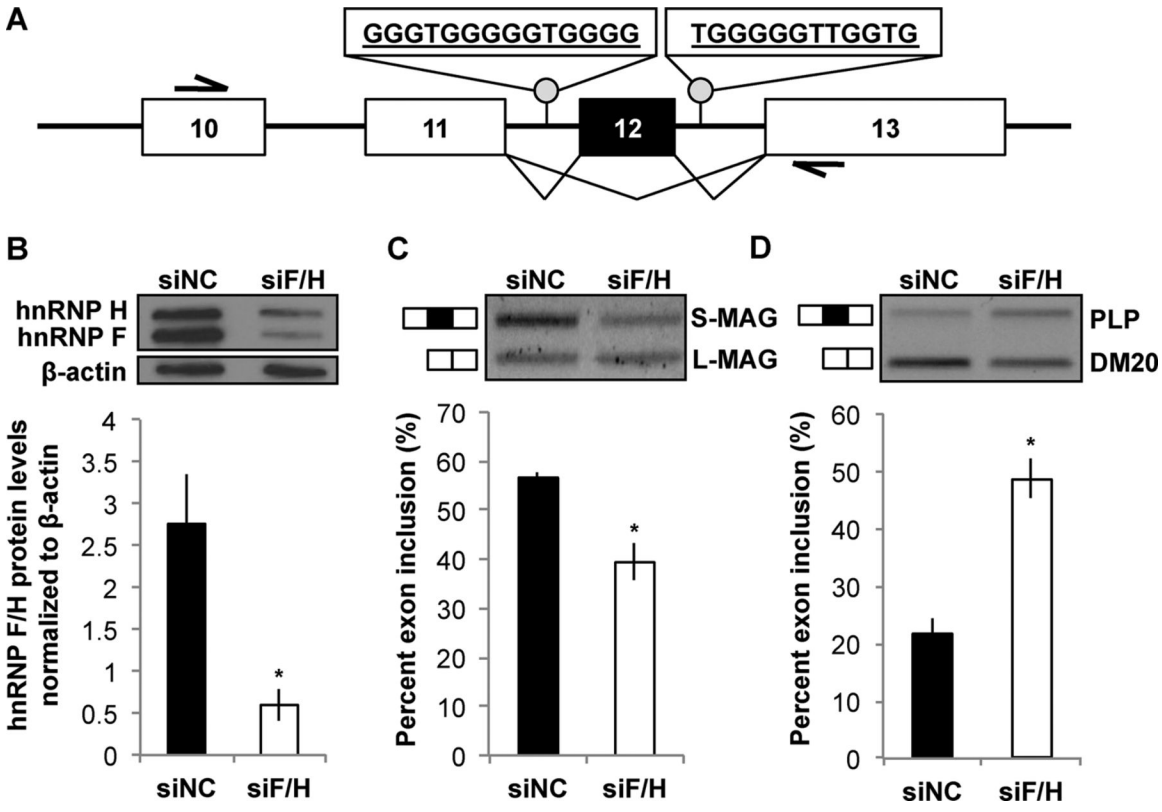

MAG pre-mRNA:
Mouse: GGGTGGGGGTGGG
Rat: GGGTGGGGGTGGGG
Human: GGGGCAGGGG
GTGGGGTGGGGG
TGGGGGTTGGTG
GGGGGTGGAGGGAGGGGTGGG
11
12
13

Figure 2-6: hnRNP F/H promotes inclusion of Exon 12 in MAG pre-mRNA. (A) Schematic of inclusion or exclusion of the alternative Exon 12 (marked black) in MAG pre-mRNA. Exons are boxed/numbered, introns are displayed as lines, and intronic G-run (rat) sequences are indicated. Primers that simultaneously detect AS isoforms of MAG mRNAs are depicted by half-arrows. (B) Immunoblot detects siRNA knockdown of hnRNP F/H (siF/H) as compared to negative control siRNA (siNC) in CG4 cells (top panel). Signal density of hnRNP F/H was normalized to β-actin and graphically displayed (bottom panel). (C and D) Representative image of semi-quantitative RT-PCR products (top panels) of alternatively spliced endogenous MAG pre-mRNA (C, S-MAG and L-MAG) and PLP pre-mRNA (D, PLP/DM20). The alternative exon is marked black and inclusion is depicted on the side for the corresponding band. The % inclusion of the alternative exon in each sample is calculated and results are statistically compared between siF/H and siNC-treated cells (bottom panels).

Figure 2-6


A
GGGTGGGGGTGGGG
TGGGGGTTGGTG
10
11
12
13
B
siNC
siF/H
hnRNP H
hnRNP F
β-actin
hnRNP F/H protein levels normalized to β-actin
C
S-MAG
L-MAG
Percent exon inclusion (%)
D
PLP
DM20

To directly test whether hnRNP F/H target the G-run elements to control inclusion of MAG Exon 12, we utilized a MAG minigene in which the genomic sequence for regulating AS of Exon 12 was fused in-frame downstream of the EGFP coding sequence (Figure 2-7A). Upon knockdown of hnRNP F/H, Exon 12 inclusion from the MAG minigene was significantly reduced in transfected CG4 cells (Figure 2-7B). Interestingly, hnRNP F/H knockdown in the neuronal cell line CAD that does not naturally express MAG also reduced Exon 12 inclusion from the minigene (Figure 2-7C). This result suggests that hnRNP F/H can regulate inclusion of MAG Exon 12 independent of OL-specific factors. Importantly, deletion of either G-run alone from the minigene, as illustrated in Figure 2-8A, reduced Exon 12 inclusion by 30–40% based on qRT-PCR (Figure 2-8B). Moreover, deletion of both G-runs led to an additive effect, resulting in greater than 60% reduction of Exon 12 inclusion (Figure 2-8B), which largely recapitulates the effects caused by hnRNP F/H knockdown (Figure 2-7B). Such effects were observed in CG4 and CAD cells (Figure 2-8C, D). These results identify both G-runs as equivalent functional targets of hnRNP F/H for regulating MAG Exon 12 inclusion. Furthermore, although hnRNP A1 also regulates AS of MAG (Zhao et al. 2010), the G-run deletion still causes comparable reduction of Exon 12 inclusion when hnRNP A1 is knocked down (Figure 2-9). This result suggests that hnRNP F/H can regulate inclusion of MAG Exon 12 through the G-runs independent of hnRNP A1.

Figure 2-7: hnRNP F/H regulates AS of a MAG minigene in OL and neuronal cell lines. (A) Schematic of the MAG minigene reporter construct in pEGFPC2 plasmid. Half-arrows indicate forward and reverse primers for simultaneous detection of AS isoform mRNAs from the MAG minigene. (B and C) Representative image of semi-quantitative RT-PCR products (top panels) upon treatment of the OL cell line CG4 (B) and the neuronal cell line CAD (C) with an siRNA to knockdown hnRNP F/H or a negative control siRNA (siNC). The % inclusion of the alternative exon in each sample is calculated and results are statistically compared between siF/H and siNC-treated cells (bottom panels).

Figure 2-7

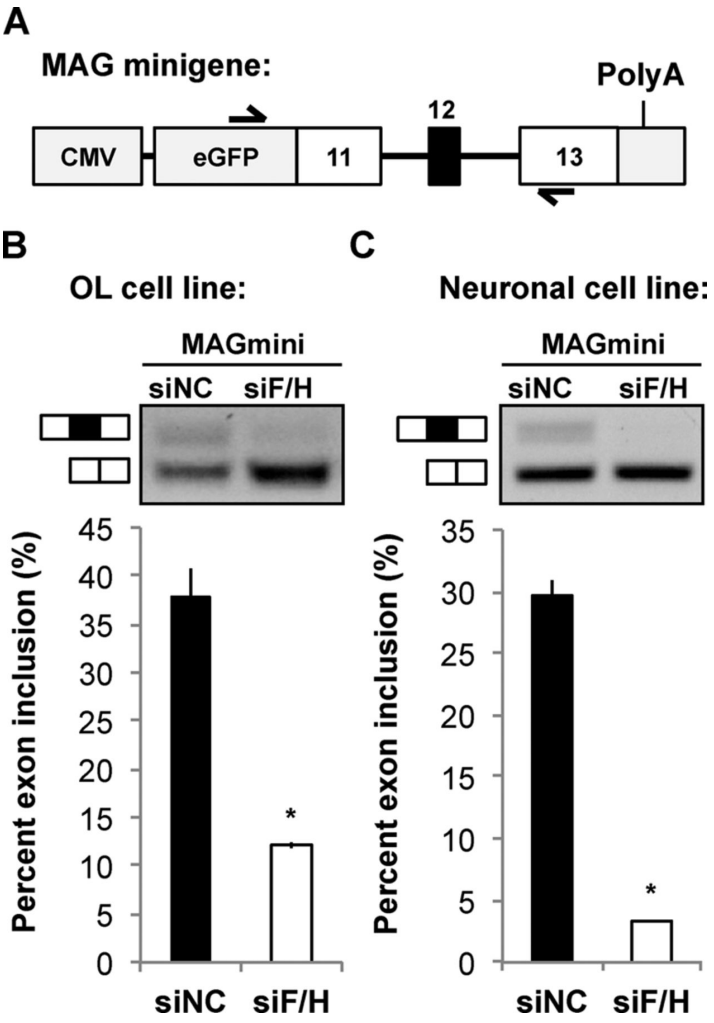

A
MAG minigene:
PolyA
12
CMV
eGFP
11
13
B
OL cell line:
C
Neuronal cell line:
MAGmini
siNC
siF/H
Percent exon inclusion (%)
45
40
35
30
25
20
15
10
5
0
*
siNC
siF/H
MAGmini
siNC
siF/H
Percent exon inclusion (%)
35
30
25
20
15
10
5
0
*
siNC
siF/H

Figure 2-8: Inclusion of MAG Exon 12 is reduced upon deletion of intronic G-runs. (A) Schematic of the MAG minigene reporter constructs illustrating deletion of individual or both intronic G-runs (mouse sequence). (B) qRT-PCR detection of mRNA isoforms derived from AS of the MAG minigene reporter that carries wild-type intronic G-runs (WT), deletion of the 5′-G-run (ΔSite#1), 3′-G-run (ΔSite#2) or both G-runs (ΔSite#1+2) expressed in the neuronal cell line CAD. Minigene-isoform-specific primers (Zhao et al. 2010) were used for qPCR, and the ΔΔCt of inclusion versus exclusion was calculated. The % change of Exon 12 inclusion in each mutant minigene was calculated and statistically compared with the WT minigene. (C and D) Semi-quantitative RT-PCR detects reduced Exon 12 inclusion of the MAG minigene when both G-runs are deleted (ΔSite#1+2) in the OL cell line CG4 (C) and the neuronal cell line CAD (D). The same primer set used in Figure 4 was employed for (C) and (D). The % inclusion of the alternative exon in each sample is calculated and results are statistically compared between the WT and mutant minigene.

Figure 2-8

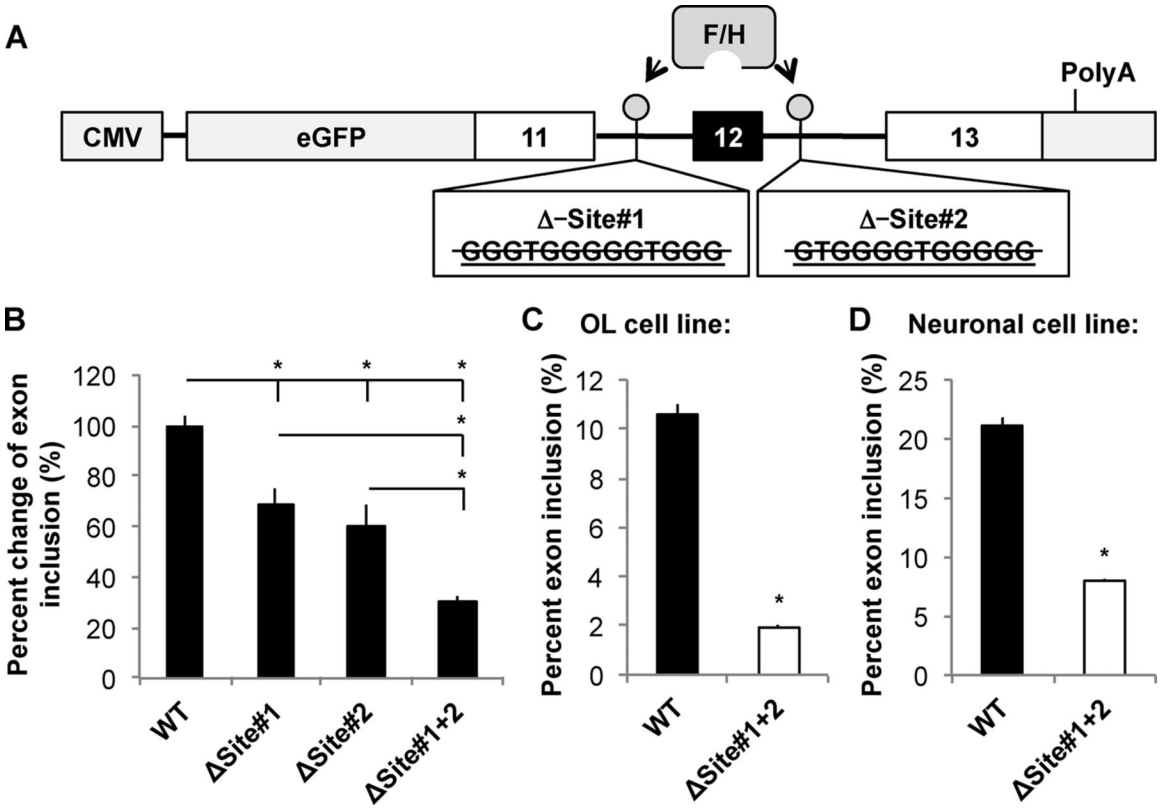
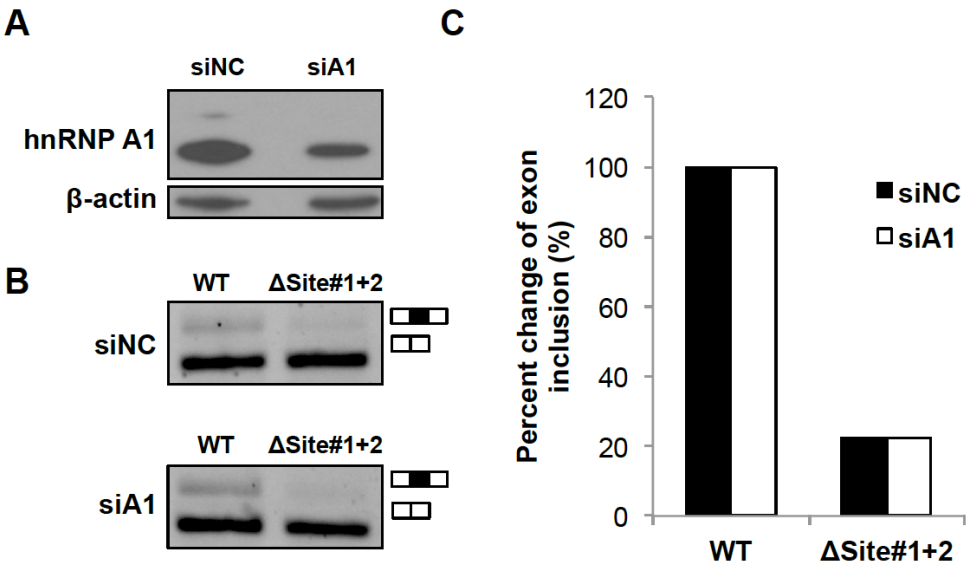

A
F/H
PolyA
CMV
eGFP
11
12
13
Δ-Site#1
GGGTGGGGGTGGG
Δ-Site#2
GTGGGGTGGGGG
B
Percent change of exon inclusion (%)
120
100
80
60
40
20
0
WT
ΔSite#1
ΔSite#2
ΔSite#1+2
C
OL cell line:
Percent exon inclusion (%)
12
10
8
6
4
2
0
WT
ΔSite#1+2
D
Neuronal cell line:
Percent exon inclusion (%)
25
20
15
10
5
0
WT
ΔSite#1+2

Figure 2-9: Deletion of G-runs in the MAG minigene results in comparable reduction of Exon 12 inclusion regardless the knockdown of hnRNP A1. (**A**) Immunoblot detects siRNA knockdown of hnRNP A1 (siA1) as compared to negative control siRNA (siNC) in CAD cells. β-actin was used as a loading control. (**B**) Representative image of semi-quantitative RT-PCR products of mRNA isoforms derived from AS of the MAG minigene reporter that carries wild type intronic G-runs (WT), or deletion of both G-runs (ΔSite#1+2), upon treatment with siNC or siA1. (**C**) qRT-PCR detection of mRNA isoforms derived from AS of the WT or mutant MAG minigene reporter in response to siNC or siA1. % change of inclusion of the alternative exon is calculated and graphically displayed (n=2).

Figure 2-9


A
siNC
siA1
hnRNP A1
β-actin
B
WT
ΔSite#1+2
siNC
WT
ΔSite#1+2
siA1
C
Percent change of exon inclusion (%)
120
100
80
60
40
20
0
siNC
siA1
WT
ΔSite#1+2

2.2.3 QKI deficiency affects the hnRNP F/H-dependent AS pathway in myelinating glia of the CNS and PNS

To test whether QKI regulates AS of additional pre-mRNAs targeted by hnRNP F/H, we identified and examined 26 putative functional targets of hnRNP F/H, including PLP and MAG. These pre-mRNAs either contain an alternative exon flanked by G-runs or display altered AS in response to changes of hnRNP F/H expression in various cell types, including a mouse OPC line Oli-neu (Lefave et al. 2011, Mauger, Lin and Garcia-Blanco 2008, Paul et al. 2011, Sun et al. 2012, Wang et al. 2012) (Table 2-1). A number of these pre-mRNAs are also known to bind human hnRNP H in HEK293T cells through G-run motifs, as revealed by CLIP-RNA sequencing (CLIP-seq) (Katz et al. 2010, Xiao et al. 2009). We detected mRNA expression from all the aforementioned genes in OLs or OPNs by RT-PCR. Among these transcripts, 19 generate alternative mRNA splice variants, and are potential hnRNP F/H targets in OLs. In addition, we observed dysregulated AS in eight of these hnRNP F/H targets in the qk^{v}/qk^{v} OPNs (Table 2-1). Quantification of exon inclusion/exclusion is shown in Figure 6. Among these dysregulated AS targets, the enhancer of yellow 2 transcription factor homolog (ENY2) was shown to be regulated by hnRNP F/H in OPCs in a recent report (Wang et al. 2012). Consistent with the finding that siRNA-mediated knockdown of hnRNP F/H in OPCs enhanced inclusion of the alternative exon in ENY2 (Wang et al. 2012), the abnormal over-expression of hnRNP F/H in the qk^{v}/qk^{v} OPNs repressed exon inclusion of ENY2 (Figure 2-10A). In addition, dysregulated AS in qk^{v}/qk^{v} OPNs was also observed in the pre-mRNAs encoding myelin protein zero-like 1 (MPZL1), UDP-acetylglucosamine pyrophosphorylase 1 (UAP1), ataxin-2 (ATXN2), and Z-band alternatively spliced PDZ-

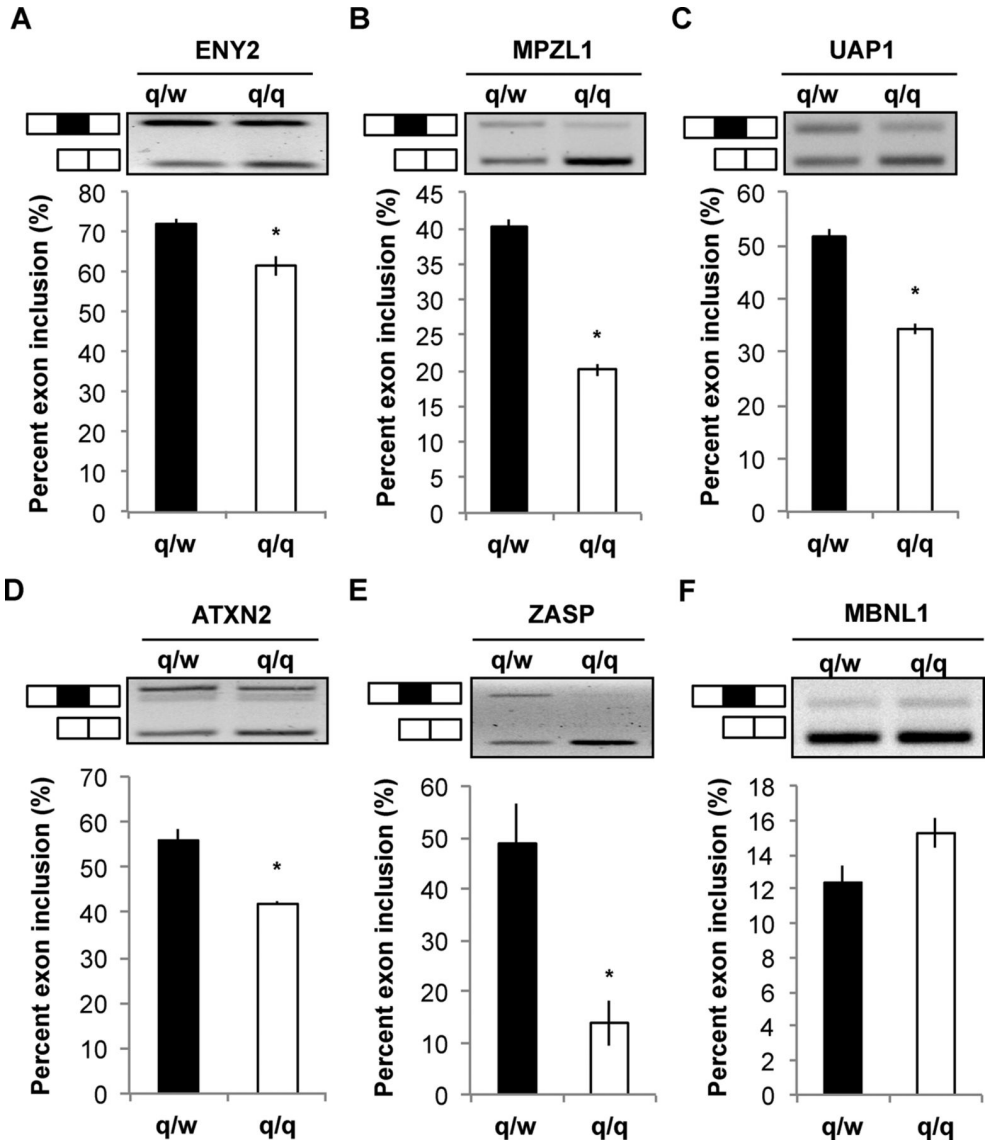
motif containing protein (ZASP) (Figure 2-10B-E). Hence, QKI regulates hnRNP F/H-dependent AS to control expression of genes that play important roles in CNS myelination, metabolism, synaptic function and neuronal degeneration (Liu-Yesucevitz et al. 2011, Mio et al. 1998, Wang, Dimova and Cambi 2007, Zhao et al. 2010).

Table 2-1:

Gene name	Gene Id (mouse)	mRNA in OLs	Splice variants in OLs	Abnormal AS in qk^v/qk^v	hnRNP H CLIP-tag in HEK293T	Species/Cell-types examined from literature	References
PLP	18823	Yes	Yes	Yes		Mouse, Oli-neu	(25)
MAG	17136	Yes	Yes	Yes		Mouse	This study
ENY2	223527	Yes	Yes	Yes		Mouse, Oli-neu	(28)
ZASP/LDB3	24131	Yes	Yes	Yes		Human, Skmc	(33)
UAP1	107652	Yes	Yes	Yes		Human, HeLa	(37)
MPZL1	68481	Yes	Yes	Yes		Human, HeLa	(37)
ATXN2	20239	Yes	Yes	Yes	✓	Human, HEK293T	(37)
NF2	18016	Yes	Yes	Yes		Human, HeLa	(38)
SIRT2	64383	Yes	Yes	No	✓	Human, HEK293T	(38)
MBNL1	56758	Yes	Yes	No		Human, Skmc	(33)
MBNL2	105559	Yes	Yes	No		Human, Skmc; mouse Oli-neu	(33) (37)
SMC2	14211	Yes	Yes	No		Mouse, Oli-neu	(28)
ELP3	74195	Yes	Yes	No		Mouse, Oli-neu	(28)
NFIB	18028	Yes	Yes	No		Mouse, Oli-neu	(28)
MADD	228355	Yes	Yes	No	✓	Human, HEK293T, HeLa, U373	(27) (38)
PTB	19205	Yes	Yes	No		Mouse	This study
nPTB	56195	Yes	Yes	No		Mouse	This study
FGFR2	14182	Yes	No	No		Human, HEK293T	(36)
TPM1	22003	Yes	No	No		Human, HeLa	(37)
BAT2 Domain	226562	Yes	Yes	N/A		Mouse, Oli-neu	(28)
DDEF1	13196	Yes	Yes	N/A		Mouse, Oli-neu	(28)
DSCR3 (CG4)	13185	Yes	No	N/A	✓	Human, HEK293T	(38)
HGS (CG4)	15239	Yes	No	N/A	✓	Human, HEK293T	(38)
TARBP2 (CG4)	21357	Yes	No	N/A	✓	Human, HEK293T	(38)
TRMU (CG4)	72026	Yes	No	N/A	✓	Human, HEK293T	(38)
MAST1 (CG4)	56527	Yes	No	N/A	✓	Human, HEK293T	(38)

Figure 2-10: Alternative splicing of hnRNP F/H target mRNAs are dysregulated in the qk^{v}/qk^{v} mouse. Semi-quantitative RT-PCR using primers that simultaneously detect AS isoforms of ENY2 (A), MPZL1 (B), UAP1 (C), ATXN2 (D), ZASP (E) and MBNL1 (F) in the OPNs of q/w and q/q mice. Representative images of the semi-quantitative RT-PCR products derived from the splice variants of the aforementioned genes are shown in the top panels. The alternative exons in the PCR products are indicated in black and inclusion is depicted on the side of the corresponding bands. The % inclusion of the alternative exon in each sample is calculated and results are statistically compared between the q/q mutant and the q/w control in the bottom panels.

Figure 2-10


A
ENY2
q/w
q/q
Percent exon inclusion (%)
80
70
60
50
40
30
20
10
0
*
q/w
q/q
B
MPZL1
q/w
q/q
Percent exon inclusion (%)
45
40
35
30
25
20
15
10
5
0
*
q/w
q/q
C
UAP1
q/w
q/q
Percent exon inclusion (%)
60
50
40
30
20
10
0
*
q/w
q/q
D
ATXN2
q/w
q/q
Percent exon inclusion (%)
70
60
50
40
30
20
10
0
*
q/w
q/q
E
ZASP
q/w
q/q
Percent exon inclusion (%)
60
50
40
30
20
10
0
*
q/w
q/q
F
MBNL1
q/w
q/q
Percent exon inclusion (%)
18
16
14
12
10
8
6
4
2
0
q/w
q/q

Not all the reported hnRNP F/H targets are dysregulated by QKI deficiency, represented by the muscle blind-like protein 1 (MBNL1) (Figure 2-10F). Consistent with the previously reported regulation of AS of MBNL1 by hnRNP F/H in skeletal muscle cells (Paul et al. 2011), siRNA knockdown of hnRNP F/H significantly altered AS of MBNL1 in the CAD neuronal cell line (Figure 2-11). However, AS of MBNL1 was not affected in the OL cell line CG4 upon knockdown of hnRNP F/H (Figure 2-11B). This result suggests that the functional influence of hnRNP F/H on AS of MBNL1 might be masked by OL-specific factors. In contrast, AS of ATXN2 was equivalently affected by hnRNP F/H knockdown in both the CAD and CG4 cell lines (Figure 2-11C and D), suggesting that hnRNP F/H regulates AS of ATXN2 in both neurons and OLs.

The qk^{v} mutation also causes QKI deficiency in SWCs that myelinate the PNS (Hardy et al. 1996). QKI-6 is preferentially reduced among QKI isoforms in the qk^{v}/qk^{v} SCN (Figure 2-12A), which is highly enriched of SWCs (Bourre et al. 1982). Both hnRNP F and hnRNP H proteins were abnormally up-regulated in the qk^{v}/qk^{v} SCNs, recapitulating what was found in qk^{v}/qk^{v} OPNs. Elevated expression of hnRNP A1, a known target of QKI-6 in OLs (Zhao et al. 2010), was also detected in the qk^{v}/qk^{v} SCNs (Figure 2-12A). Thus, the functional consequence of QKI-6 deficiency on splicing factor expression appears to be comparable in both the CNS and the PNS. Among our identified hnRNP F/H targets that display abnormal AS in the qk^{v}/qk^{v} OPNs, the MPZL1 pre-mRNA also displayed dysregulated AS in the qk^{v}/qk^{v} SCNs (Figure 2-12B). However, unlike the dysregulated AS of UAP1 and ATXN2 in qk^{v}/qk^{v} OPNs (Figure 2-10), transcripts from neither gene were affected in the qk^{v}/qk^{v} SCNs (Figure 2-12C, D). Thus, QKI deficiency differentially affects AS of hnRNP F/H targets in myelinating glia from

the CNS and PNS, presumably due to modulation of hnRNP F/H-dependent AS by undefined SWC-specific factors.

Figure 2-11: hnRNP F/H-dependent alternative splicing of MBNL1 and ATXN2 in neuronal and OL cell lines. (**A**) Semi-quantitative RT-PCR was performed using one primer set to simultaneously detect splice variants of MBNL1 in the neuronal cell line CAD upon siRNA knockdown of hnRNP F/H (siF/H) as compared to scrambled negative control siRNA (siNC). (**B**) Same experiment was performed in the OL cell line CG4. Representative images are shown in the top panels. The alternative exons in the PCR products are indicated in black and inclusion is depicted on the side of the corresponding bands. % inclusion of the alternative exon in each experiment is calculated and statistically compared between siF/H and siNC-treated cells in the bottom panels. (**C,D**) Representative image of semi-quantitative RT-PCR of ATXN2 upon knockdown of hnRNP F/H compared to scrambled negative control in the neuronal cell line CAD and the OL cell line CG4.

Figure 2-11

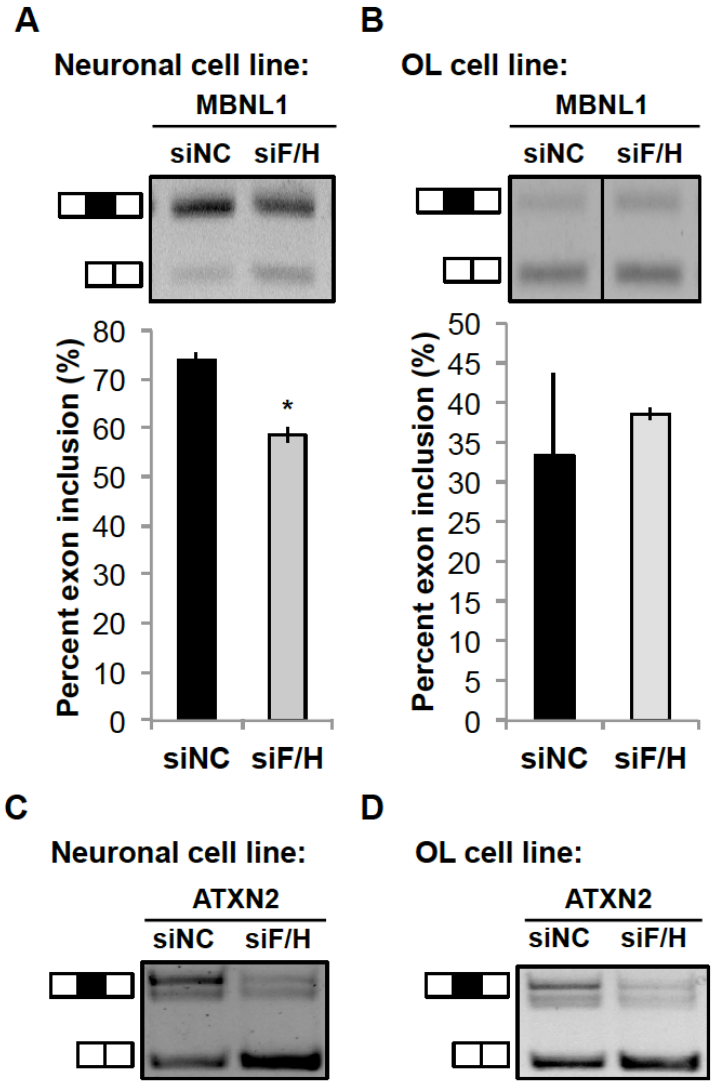
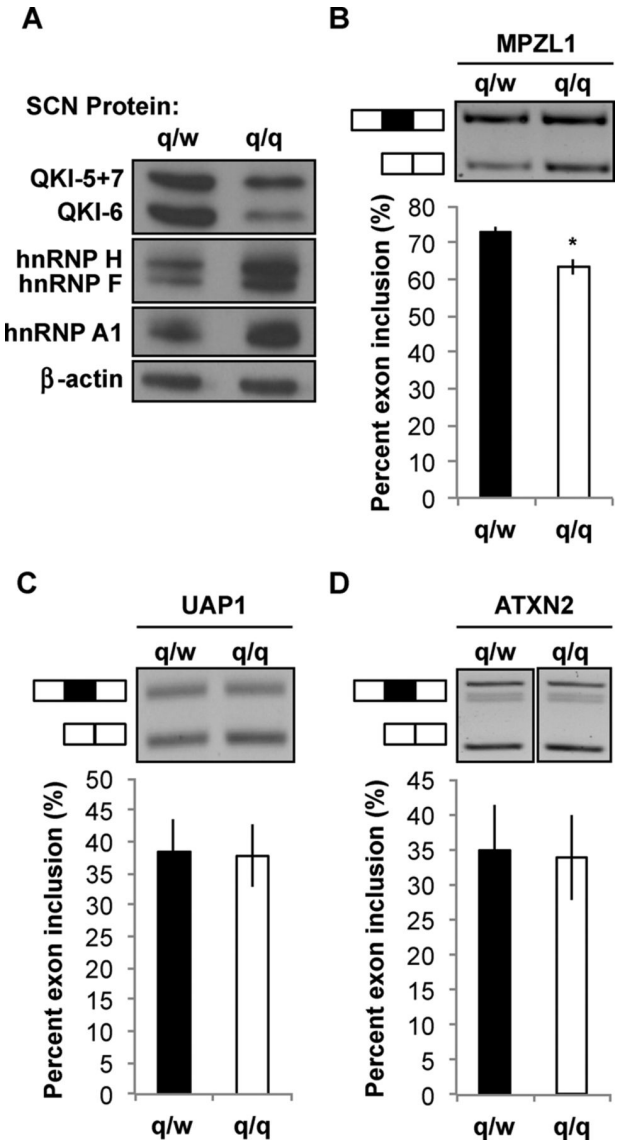

A
Neuronal cell line:
MBNL1
siNC
siF/H
Percent exon inclusion (%)
80
70
60
50
40
30
20
10
0
*
siNC
siF/H
B
OL cell line:
MBNL1
siNC
siF/H
Percent exon inclusion (%)
50
45
40
35
30
25
20
15
10
5
0
siNC
siF/H
C
Neuronal cell line:
ATXN2
siNC
siF/H
D
OL cell line:
ATXN2
siNC
siF/H

Figure 2-12: The hnRNP F/H targets are differentially affected in Schwann cells as compared to OLs by QKI deficiency. (A) Immunoblot detects QKI deficiency and aberrant over-expression of hnRNP F, H and A1 in the Schwann-cell-rich sciatic nerve (SCN) of the homozygous qk^{v} mice (q/q) as compared to the heterozygous non-phenotypic control (q/w). β-actin serves as the loading control. (B–D) Semi-quantitative RT-PCR of RNA isolated from q/w and q/q SCNs for quantification of MPZL1 (B), UAP1 (C) and ATXN2 (D) as described in the legend of Figure 6. Representative RT-PCR products of the splice variants are shown in the top panels. The % inclusion of the alternative exon in each sample is calculated and results are statistically compared between the q/q mutant and the q/w control in the bottom panels.

Figure 2-12


A
SCN Protein:
q/w
q/q
QKI-5+7
QKI-6
hnRNP H
hnRNP F
hnRNP A1
β-actin
B
MPZL1
q/w
q/q
Percent exon inclusion (%)
80
70
60
50
40
30
20
10
0
*
q/w
q/q
C
UAP1
q/w
q/q
Percent exon inclusion (%)
50
45
40
35
30
25
20
15
10
5
0
q/w
q/q
D
ATXN2
q/w
q/q
Percent exon inclusion (%)
45
40
35
30
25
20
15
10
5
0
q/w
q/q

2.3 Discussion

Our studies discovered that QKI acts upstream of two canonical splicing factors hnRNP F/H, which forms a novel post-transcriptional pathway to regulate AS in myelinating glia of the CNS and PNS. We found that in contrast to nuclear QKI-5 that directly regulates AS in muscle cells (Hall et al. 2013), cytoplasmic QKI-6 controls AS in myelinating glia by repressing expression of hnRNP F/H. To our knowledge, QKI-6 is the first factor identified that governs the cellular abundance of hnRNP F/H and selective exon inclusion of hnRNP F/H targets. Furthermore, we identified *in vivo* functional targets of the QKI-6-hnRNP F/H pathway, which play diverse roles in glia function and myelin development, some of which are also implicated in neurological diseases.

hnRNP F and hnRNP H are functional orthologues expressed in many cell types and control AS of numerous G-run containing pre-mRNAs (Caputi and Zahler 2001, Nilsen and Graveley 2010, Wang, Dimova and Cambi 2007). CLIP-Seq has identified hundreds of human pre-mRNAs bound by hnRNP H through G-run motifs in HEK293T cells (Katz et al. 2010, Xiao et al. 2009). In addition, AS of a number of pre-mRNAs are regulated upon manipulation of hnRNP F/H expression in other human cell types (Lefave et al. 2011, Mauger, Lin and Garcia-Blanco 2008, Paul et al. 2011, Sun et al. 2012). Furthermore, a recent report discovered 190 pre-mRNAs that display altered splicing in response to siRNA knockdown of hnRNP F/H in a mouse OPC line (Wang et al. 2012). However, there is minimal overlap between these published human and mouse hnRNP F/H targets. Nonetheless, all of the functional AS targets of hnRNP F/H we identified in OLs (Table 2-1) contain G-runs flanking the alternatively spliced exons in both the human and the mouse gene. The locations of these G-runs are within optimal distance for

regulating AS based on large number of CLIP-seq targets of hnRNP H (Katz et al. 2010, Wang et al. 2012, Xiao et al. 2009). In fact, a number of the transcripts are also CLIP-seq targets of hnRNP H, represented by ATXN2 (Xiao et al. 2009). The functional importance of conserved G-runs in MAG and PLP is validated by mutation studies (Figure 2-8) (Wang, Dimova and Cambi 2007). In addition, a conserved QRE is present in the human and the rodent hnRNP H 3′UTR. Thus, AS under control of the QKI-6-hnRNP F/H pathway appears to be conserved between mouse and human. One possible explanation for the diversity in previously reported mouse and human hnRNP F/H targets could be the existence of cell type-specific factors that modulate hnRNP F/H-dependent AS.

Regulation of hnRNP F/H expression by cytoplasmic QKI-6 provides one example for modulating hnRNP F/H-dependent AS in a cell type-specific manner. Expression of the qkI gene is detected in nearly all cell types except brain neurons (Lu et al. 2003). Nuclear QKI-5 is the predominant isoform in most cell types examined, which can directly regulate AS of its bound pre-mRNAs (Hall et al. 2013, Lu et al. 2003). In contrast, cytoplasmic QKI-6 is the most abundant QKI isoform in myelinating glia (Lu et al. 2003, Zhao et al. 2006). Moreover, the cytoplasmic isoforms QKI-6 and QKI-7 are vigorously up-regulated during CNS myelin development, whereas QKI-5 and hnRNP F/H are down-regulated (Bockbrader and Feng 2008, Hardy et al. 1996, Wang et al. 2012, Wang, Dimova and Cambi 2007). In the qk^{v}/qk^{v} OLs, although all QKI isoforms are reduced, deficiency of QKI-6 quantitatively exceeds the other isoforms (Zhao et al. 2006). The impairment of hnRNP F/H decline in the qk^{v}/qk^{v} OLs during myelin development, together with the correction of aberrant hnRNP F/H over-expression by the

OL-specific FLAG-QKI-6 transgene, clearly demonstrates the essential role of QKI-6 in regulating hnRNP F/H and their downstream AS targets in CNS myelination. Importantly, the FLAG-QKI-6 transgene neither rescued the deficiency of QKI-5 (Zhao et al. 2006) nor increased the nuclear abundance of QKI-5 (Zhao et al. 2010). Hence, although QKI-5 regulates AS in other cell types (Hafner et al. 2010, Hall et al. 2013, Wu et al. 2002), the deficiency of QKI-6 appears to be the major mechanism that affects hnRNP F/H-dependent splicing in myelinating glia.

Although QKI isoforms can form heterodimers and shuttle between the nucleus and the cytoplasm (Wu et al. 1999), both the endogenous QKI-6 and the FLAG-QKI-6 transgene product are predominantly localized to the cytoplasm of mature OLs *in vivo* (Hardy et al. 1996, Wu et al. 1999, Zhao et al. 2010, Zhao et al. 2006). Consistent with the known function of QKI-6 in repressing mRNA translation (Zhao et al. 2010), QKI deficiency in qk^{v}/qk^{v} OLs leads to over-expression of hnRNP F/H proteins without affecting the levels of their mRNAs. However, we were unable to detect association of QKI-6 with the hnRNP F mRNA in OLs even under non-denaturing conditions. This could possibly be caused by unknown RNA-binding proteins that mask the QRE in hnRNP F mRNA, which either renders the hnRNP F mRNA inaccessible by QKI-6 or permits only labile interactions with QKI-6 in OLs. Considering the emerging evidence that QKI regulates expression of selected microRNAs (Hafner et al. 2010, Wang et al. 2013), whether QKI-6-mediated miRNA expression also inhibits hnRNP F translation is an intriguing possibility to be explored by future studies.

All the AS targets of the QKI-6-hnRNP F/H pathway we identified in this study encode protein isoforms that play distinct functions, often reciprocally regulated during

myelin development. MAG and PLP are two examples of such regulation, which are also severely dysregulated in the qk^v/qk^v mutant (Wu et al. 2002, Zhao et al. 2010, Zhu et al. 2012). AS of MAG generates two transmembrane proteins that differ in their cytoplasmic domains, and hence are involved in distinct cellular signaling pathways that promote myelin formation (Quarles 2007, Zhao et al. 2010). AS of PLP also generates two protein isoforms that perform non-overlapping functions in myelin-axon integrity (Gudz et al. 2002, Zhu et al. 2012). It is important to note that an AS event could be regulated by the cooperation of multiple splicing factors. In particular, QKI-6 also represses translation of hnRNP A1 (Zearfoss et al. 2011, Zhao et al. 2010), which also affects AS of MAG pre-mRNA *in vivo* in the same direction as that by hnRNP F/H. However, besides coordination with hnRNP H (Fisette et al. 2010), hnRNPA1 was also shown to oppose hnRNP F in regulating AS of the insulin receptor pre-mRNA (Talukdar et al. 2011). The hnRNP F/H targets we analyzed could potentially be regulated by the coordination of multiple splicing factors downstream of QKI specifically in myelinating glia. Whether QKI-6 regulates additional splicing factors and how such regulation may cooperate to control AS in different cell types are challenging questions to be addressed by future studies.

Emerging evidence suggests dysregulation of AS in human disorders that involve deficiency of QKI, such as schizophrenia and glioma (Bockbrader and Feng 2008, Chen et al. 2012). Reduction of QKI isoforms and myelin-specific targets of QKI are observed in schizophrenic brains, including MAG and PLP (Aberg et al. 2006). However, whether hnRNP F/H and/or hnRNP A1 are dysregulated, and furthermore to what extent the downstream AS pathway is affected, which in turn contribute to the myelin impairments

in schizophrenia, still remain elusive. On the other hand, aberrant over-expression of hnRNP F/H and dysregulated AS of hnRNP F/H targets were found in human glioma specimens (Lefave et al. 2011). In particular, hnRNP H was found to control an oncogenic splicing switch in gliomas, and was reported to promote expression of proliferation-inducing genes (Lefave et al. 2011, Wang et al. 2012). Consistent with the repressive role of QKI in hnRNP F/H expression, the human qkI locus is frequently mutated in glioblastoma multiforme (GBM) derived from astrocytic origin identified by SNP-Chip analysis (Yin et al. 2009). Hence, besides its role in myelinating glia, QKI-6 may regulate hnRNP F/H-dependent AS to control normal growth and function of astrocytic glia. Whether the aberrant elevation of these hnRNPs in GBMs is caused by QKI-6 deficiency remains to be determined. Moreover, whether QKI-6 acts as a tumor suppressor to inhibit GBM tumorigenesis is an important question that warrants rigorous investigation.

Chapter 3: The regulation of splicing factor (SF) expression by QKI-6

3.1 Introduction

Tight regulation of alternative splicing (AS) is important for the normal development and function of oligodendroglia (OLs) (Wang et al. 2012, Wang, Dimova and Cambi 2007). The selective RNA-binding protein quaking I (QKI) plays crucial roles in governing AS during OL development, which regulates many genes that function in multiple cellular processes (Hall et al. 2013, Mandler, Ku and Feng 2014, Zearfoss et al. 2011, Zearfoss, Johnson and Ryder 2013, Zhao et al. 2010, Zhu et al. 2012).

There are three main QKI isoforms, the nuclear isoform QKI-5, and the cytoplasmic isoforms QKI-6 and QKI-7, which are derived from AS of the QKI pre-mRNA (Kondo et al. 1999). QKI-5 and QKI-6 were reported to control AS by distinct mechanisms in muscle and OLs (Hall et al. 2013, Zhao et al. 2010). In muscle cells, QKI-5 directly binds pre-mRNA of Capzb and enhances inclusion of exon 9 (Hall et al. 2013). In contrast, the cytoplasmic QKI-6 plays primary roles in controlling AS in OLs through undefined posttranscriptional cascades (Mandler, Ku and Feng 2014, Zhao et al. 2010). One unique feature of OL differentiation is the developmentally programmed downregulation of a large number of SFs, which is accompanied with a reciprocal increase of QKI-6 expression (Campagnoni and Macklin 1988, Hardy et al. 1996, Larocque et al. 2005, Scherer et al. 1994, Wang, Dimova and Cambi 2007). In fact, QKI-6 was reported to suppress expression of splicing factors (SFs) during OL development, represented by hnRNP A1 and hnRNP F/H (Mandler, Ku and Feng 2014, Zhao et al. 2010). Unlike controlling mRNA stability of myelin structural genes, QKI-6 does not alter mRNAs that encode the aforementioned SFs, suggesting a role for QKI-6 as a translation inhibitor (Larocque et al. 2005, Li et al. 2000, Mandler, Ku and Feng 2014,

Zhang et al. 2003, Zhao et al. 2010). However, the precise mechanism by which QKI-6 regulates translation of SFs in OLs still remains elusive.

The quakingviable (qk^v/qk^v) mutant mouse model is deficient in QKI specifically in myelinating glia, due to a deletion of the promoter region that contains enhancer elements likely targeted by a transcription factor that is strictly expressed in myelinating glia and results in reduced transcription of the *qkI* gene (Lu et al. 2003). Despite this, for yet unidentified reasons there is a preferential reduction of QKI-6 in this mutant mouse, which results in extensive defects in AS that are rescued by transgenic expression of QKI-6 specifically in OLs without increasing nuclear levels of QKI-5 (Lu et al. 2003, Mandler, Ku and Feng 2014, Zhao et al. 2006). The broad spectrum of abnormal AS events in the qk^v/qk^v mouse suggests that QKI deficiency might cause deregulation of additional SFs besides hnRNP A1 and hnRNP F/H reported in previous studies (Mandler, Ku and Feng 2014, Zhao et al.). Alternatively, deficiency of QKI may also affect AS due to impaired cooperation between QKI and specific SFs. In fact, QKI and the canonical SF PTBP1, also known as hnRNP I, were recently reported to have overlapping downstream targets in muscle (Hall et al. 2013). PTBP1 regulates AS by binding to pyrimidine-rich tracts, often composed of alternating cytosines and uracils (Xue et al. 2009). PTBP1 is expressed in all neural cell types with the exception of post-mitotic mature neurons (Boutz et al. 2007). In contrast, a paralog of PTBP1, called PTBP2 or nPTB is predominantly expression in neurons, and is thought to regulate AS of some overlapping and some distinct downstream targets (Boutz et al. 2007, Grabowski 2011, Zheng et al. 2012). In neural stem cells, PTBP1, PTBP2, and QKI are abundantly expressed and differentially regulated during neuronal and glial lineage development (Boutz et al. 2007,

Hardy 1998, Zheng et al. 2012). In OL progenitor cells (OPCs), PTBP1 and QKI are abundantly expressed in OLs, whereas PTBP2 is only detected at low levels (unpublished data from the Feng lab). Whether QKI and PTBP1 might coordinate to regulate AS in OLs is not known. In particular, whether QKI could act upstream of PTBP1 to regulate AS, similar to that of hnRNPA1 and F/H, is an intriguing possibility to be examined. Additionally, whether and how QKI deficiency may affect expression of PTBP2 is unknown.

Aberrant AS is reported in cancer and neurodegenerative diseases (NDDs) (Carpenter et al. 2006, Tazi, Bakkour and Stamm 2009). Elevated expression of several hnRNPs was predicted to promote tumorigenesis, including hnRNP A1, hnRNP H, and hnRNP M (Carpenter et al. 2006, Chen et al. 2014, Han, Li and Zhang 2013). QKI deficiency is also observed in several types of cancer, including glioma and colon cancer (Novikov et al. 2011, Yang et al. 2010). In NDDs, cytoplasmic aggregation of the core spliceosome factor U1-70K and its absence in the spliceosome was identified in Alzheimer's brain (Bai et al. 2013). In addition, in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), the SFs tar DNA-binding protein (TDP-43) and PTB-associated splicing factor (PSF), are implicated in the formation of cytoplasmic inclusions, potentially leading to splicing abnormalities (Alami et al. 2014, Seyfried et al. 2012). Particularly, PSF was abnormally sequestered to the cytoplasm in OLs of FTLD (Seyfried et al. 2012). Whether and how QKI contributes to dysregulation of SFs in cancer and/or NDDs is an intriguing possibility to be explored.

Here, I report that expression of multiple SFs, besides hnRNP A1 and F/H is aberrantly elevated as a result of QKI deficiency in the qk^{v}/qk^{v} mouse, including PTBP1,

PTBP2, and hnRNP M. Such abnormality is rescued by the OL-specific QKI-6 transgene, suggesting that QKI-6 may act as a major regulator of SFs in OLs. I further show that QKI-6 associates with mRNAs encoding PTBP1/2 yet does not affect the steady-state levels of these mRNAs. Thus, QKI-6 apparently regulates expression of SFs by targeting the mRNAs and inhibiting mRNA translation. However, not all SFs were affected by QKI deficiency, as TDP-43 is unaltered in qk^{v}/qk^{v} mutant. Finally, I found that QKI deficiency results in elevated expression and abnormal cleavage of PSF. Moreover, the C-terminal cleavage of PSF enhances cytoplasmic aggregate formation in OLs. Altogether, these preliminary data provide important clues elucidating a complex novel function of QKI-6 in controlling SF expression and AS.

3.2 Results

3.2.1 QKI-6 inhibits translation of hnRNP F/H in myelinating glia

I previously showed that QKI deficiency in the qk^{v}/qk^{v} mouse results in aberrantly elevated expression of hnRNP F/H without affecting their mRNA levels (Mandler, Ku and Feng 2014), suggesting that QKI-6 likely inhibits translation of hnRNP F/H mRNAs. To further assess whether translation of hnRNP H and F mRNA is affected by QKI deficiency, linear sucrose gradient fractionation was performed followed by qRT-PCR using the mouse brain stem derived from the qk^{v}/qk^{v} mutant (q/q) that harbor QKI deficiency specifically in OLs and the non-phenotypic qk^{v}/wt littermates (q/w). The brain stem was chosen because it contains more abundant OLs than other brain regions and can provide enough material for gradient fractionation. I observed a slight but detectable shift of hnRNP F and H mRNAs from mRNP fractions (fraction 1 and 2) into polyribosome

fractions (4-10) in the qk^v/qk^v mutant as compared to the qk^v/wt littermates (Figure 3-1), consistent with the idea of defects in QKI-dependent repression of translation initiation. However, such abnormality is likely underestimated due to the normal translation of hnRNP F/H mRNA in other cell types of the brain stem that lack the QKI deficiency.

QKI-6 has previously been shown to bind hnRNP H mRNA but not hnRNP F mRNA (Mandler, Ku and Feng 2014). As QKI-6 was recently shown to regulate expression of microRNAs (miRNA) in glia (Wang et al. 2013), I hypothesized QKI-6 may control expression of miRNAs which in turn target hnRNP F mRNA. I searched for putative miRNAs that may specifically target hnRNP F 3'UTR by using algorithms at microRNA.org and PITA (http://genie.weizmann.ac.il/pubs/mir07/index.html), and chose the overlapping hits with no mismatch in the seed region or G/U wobble (seed column) (Table 3-1). The ΔΔG value by PITA estimates the strength of binding between a miRNA and its putative target mRNA based on calculating free energy upon transition from unbound state to binding of the target sequence. A ΔΔG of -10 or below suggests a likely target site by a miRNA, but values above -10 could still predict putative targets if the expression level of the miRNA is very high. Considering the high expression level of some miRNAs that may potentiate miRNA binding to the hnRNP F 3'UTR, I included miRNA species that have a ΔΔG of -9 or below in Table 3-1. The majority of the twelve miRNAs identified were detected in OL progenitor cell lines (Wang and Cambi 2012). Importantly, none of these miRNAs are predicted to target the hnRNP H 3'UTR. In fact, using the above criteria, I only identified miR-154 with a ΔΔG of -9.33 that could potentially target hnRNP H 3'UTR. The bioinformatic analysis suggests miRNAs that could target hnRNP F 3' UTR to inhibit mRNA translation. Whether QKI-6 may govern

expression of some of these miRNAs to repress hnRNP F translation is an intriguing possibility to be explored in the future.

To determine whether the abnormal upregulation of hnRNP F/H due to QKI-6 deficiency indeed leads to increased nuclear levels of hnRNP F/H, nuclear fractionation was performed from brain stem of qk^{v}/qk^{v} mouse (q/q) in parallel with the non-phenotypic heterozygote control littermates (q/w). Immunoblot of nuclear extracts indicated elevated hnRNP F/H levels in the q/q nucleus (Figure 3-2). In contrast, the abundance of nuclear marker Parp was similar in q/w and q/q nuclear extracts on the same blot. I also show that hnRNP F/H levels are not upregulated in heart tissue of the qk^{v}/qk^{v} mouse at the age of 1.5 months and 5 months (Figure 3-3), which is consistent with the previous report that QKI deficiency in the qk^{v}/qk^{v} mouse is specific to myelinating glia (Lu et al. 2003).

Figure 3-1: Polyribosome association profiles of hnRNP F/H mRNAs in the brain stems of qk^{v}/qk^{v} mutant and the qk^{v}/wt littermate. (A, B) qRT-PCR was performed using cytoplasmic fractions on linear sucrose gradient derived from the brain stem of the qk^{v}/qk^{v} mutant (q/q) and the non-phenotypic qk^{v}/wt littermate control (q/w). Primers specific to hnRNP H (A, left panel) and hnRNP F (B, left panel) were used. Percent distribution of hnRNP F/H mRNA was calculated. On the right panels, the percentages of hnRNP F/H mRNAs in the mRNP fractions (fractions 1 and 2), monoribosomes (fraction 3), and polyribosomes (fraction 4 to 10) were graphically displayed (n=1).

Figure 3-1

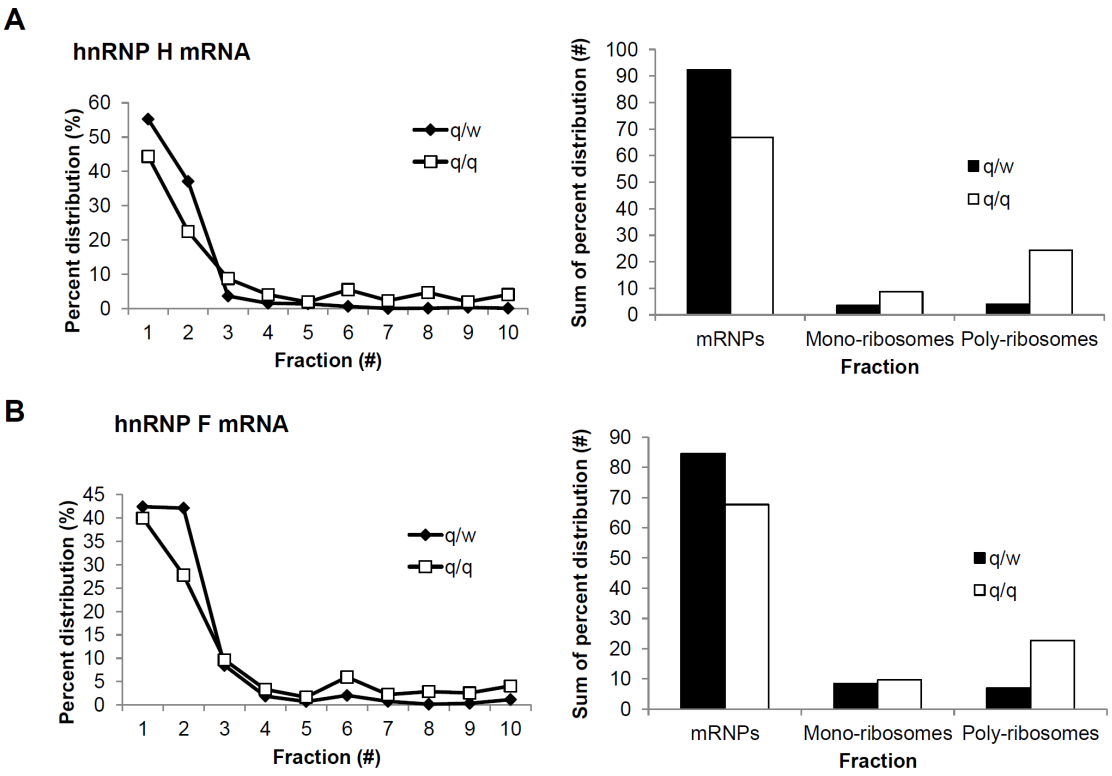

A
hnRNP H mRNA
Percent distribution (%)
Fraction (#)
q/w
q/q
Sum of percent distribution (#)
mRNPs
Mono-ribosomes
Poly-ribosomes
Fraction
B
hnRNP F mRNA
Percent distribution (%)
Fraction (#)
q/w
q/q
Sum of percent distribution (#)
mRNPs
Mono-ribosomes
Poly-ribosomes
Fraction

Table 3-1: Putative microRNAs targeting the 3'UTR of hnRNP F mRNA in mouse.

The table summarizes two bioinformatic searches performed to predict putative microRNAs (miRNA) that may specifically target hnRNP F 3'UTR using the algorithms at microRNA.org and PITA (http://genie.weizmann.ac.il/pubs/mir07/index.html). Shown in the table are overlapping putative miRNAs from both independent searches. PITA provides the position of the miRNA in the 3'UTR, an assessment of the seed region with the number of matches:mismatches:G/U wobble sites (seed column), and the ΔΔG, which estimates the strength of binding between a miRNA and its putative target by calculating free energy upon transition from unbound state to binding of the target. The last column indicates reported expression of these miRNAs in the OL progenitor cell line oli-neu (Wang and Cambi 2012).

Table 3-1

Gene (mouse)	microRNA	Position	Seed	ddG	Expressed in OLs
hnRNP F	mmu-miR-27a	522	7:0:0	-12.64	✔
hnRNP F	mmu-miR-202-3p	139	6:0:0	-11.66	
hnRNP F	mmu-miR-27b	522	7:0:0	-11.11	✔
hnRNP F	mmu-let-7g	139	6:0:0	-10.86	✔
hnRNP F	mmu-miR-139-5p	480	7:0:0	-10.76	
hnRNP F	mmu-let-7c	139	6:0:0	-10.76	✔
hnRNP F	mmu-let-7d	139	6:0:0	-10.75	✔
hnRNP F	mmu-miR-101b	520	7:0:0	-10.5	
hnRNP F	mmu-let-7b	139	6:0:0	-9.76	✔
hnRNP F	mmu-miR-128	522	6:0:0	-9.74	✔
hnRNP F	mmu-miR-218	298	7:0:0	-9.22	
hnRNP F	mmu-let-7f	139	6:0:0	-9.06	✔

Figure 3-2: Abnormal increase in hnRNP F/H detected in qk^v/qk^v nuclear extracts. Nuclear fractionation of brain stem was performed from qk^v/qk^v mouse (q/q) and non-phenotypic heterozygote control (q/w) (Li et al. 2000). Nuclear lysates were subjected to immunoblot, and probed with antibodies against hnRNP F/H, as well as the nuclear protein Parp.

Figure 3-2

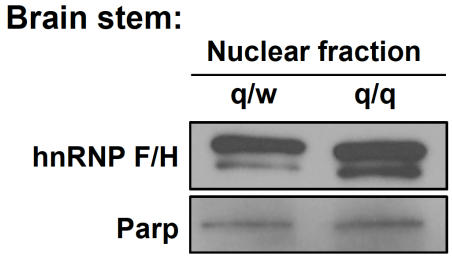
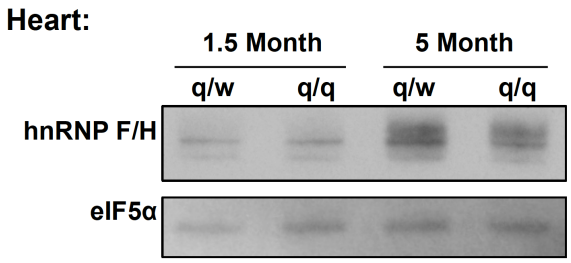


Figure 3-3: hnRNP F/H levels are not elevated in the heart of qk^{v}/qk^{v} mouse. Immunoblot performed on heart tissue from the qk^{v}/qk^{v} mouse (q/q) and the non-phenotypic heterozygote control in 1.5 and 5 months of age, using antibodies against hnRNP F/H and eIF5α, which serves as a loading control.

Figure 3-3



3.2.2 QKI deficiency in OLs leads to dysregulation of PTBP1 and PTBP2 levels

QKI and PTBP1 were recently shown to play overlapping roles in regulating AS in muscle (Hall et al. 2013). Here I asked whether or not QKI could regulate expression of PTBP1 and/or the neuronal paralog PTBP2 in the brain. I first examined whether QKI may bind the mRNAs that encode PTBP1 and PTBP2. Although no classical QRE of ACUAAY was identified, multiple single UAAY sites were found in PTBP1 and PTBP2 3' UTRs, as well as a few bipartite UAAY…UAAY motifs identified in PTBP2, which could serve as putative binding sites for QKI (Figure 3-4A) (Galarneau and Richard 2005, Hafner et al. 2010, Teplova et al. 2013). To test this hypothesis, UV cross-linking immunoprecipitation (CLIP) was performed on brain stem of a transgenic mouse that expresses FLAG-tagged QKI-6 specifically in OLs (Mandler, Ku and Feng 2014). FLAG-QKI-6 coimmunoprecipitates with both PTBP1 and PTBP2 mRNAs as compared to inputs, above the background binding to the negative control GAPDH mRNA (Figure 3-4B), suggesting QKI-6 may bind PTBP1 and PTBP2 mRNAs in OLs.

I next assessed whether or not QKI deficiency in the qk^{v}/qk^{v} mouse affected expression of PTBP1 and PTBP2. In the brain stem, which contains a mixture of glia and neurons but is rich in OLs, I found that both PTBP1 and PTBP2 protein levels were significantly elevated in the qk^{v}/qk^{v} mouse (q/q) as compared to the control (q/w), and both were rescued by OL-specific transgenic expression of QKI-6 (q/qtg, Figure 3-5A, B). However, steady-state PTBP1 and PTBP2 mRNA levels were unaffected (Figure 3-5C, D). These results suggest that QKI-6 controls expression of PTBP1 and PTBP2 in OLs, likely by suppressing mRNA translation, consistent with the mechanism by which QKI-6 regulates hnRNP A1 and hnRNP F/H (Mandler, Ku and Feng 2014, Zhao et al.

2010). Since PTBP2 is normally more abundant in neurons, the QKI deficiency that causes abnormalities in OL differentiation and myelin development might also contribute to the elevated PTBP2 levels through potential neuron-glia interactions.

Figure 3-4: QKI-6 can be UV cross-linking immunoprecipitated (CLIPed) with PTBP1 and PTBP2 mRNAs in brain OLs. (A) On the left, is a diagram of the mouse PTBP1 and PTBP2 mRNAs with UAAY sites that could potentially be targeted by QKI identified in the PTBP1 and PTBP2 3'UTRs, marked by blue balls. On the right, are representative UAAY sequences from the 3'UTRs highlighted by blue bold font. (B) RT-PCR of RNA isolated from cross-linking immunoprecipitation (IP) of a mouse expressing OL-specific FLAG-tagged QKI-6 transgene and inputs was performed using primers for PTBP1 and PTBP2, as well as the negative control GAPDH mRNA.

Figure 3-4

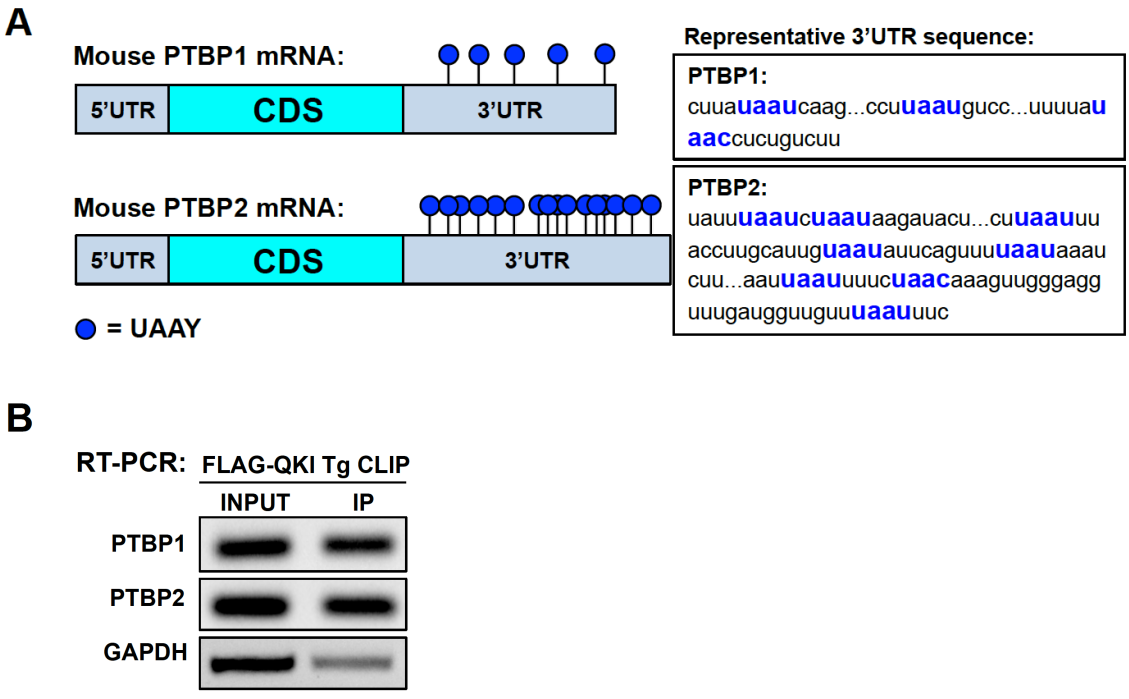
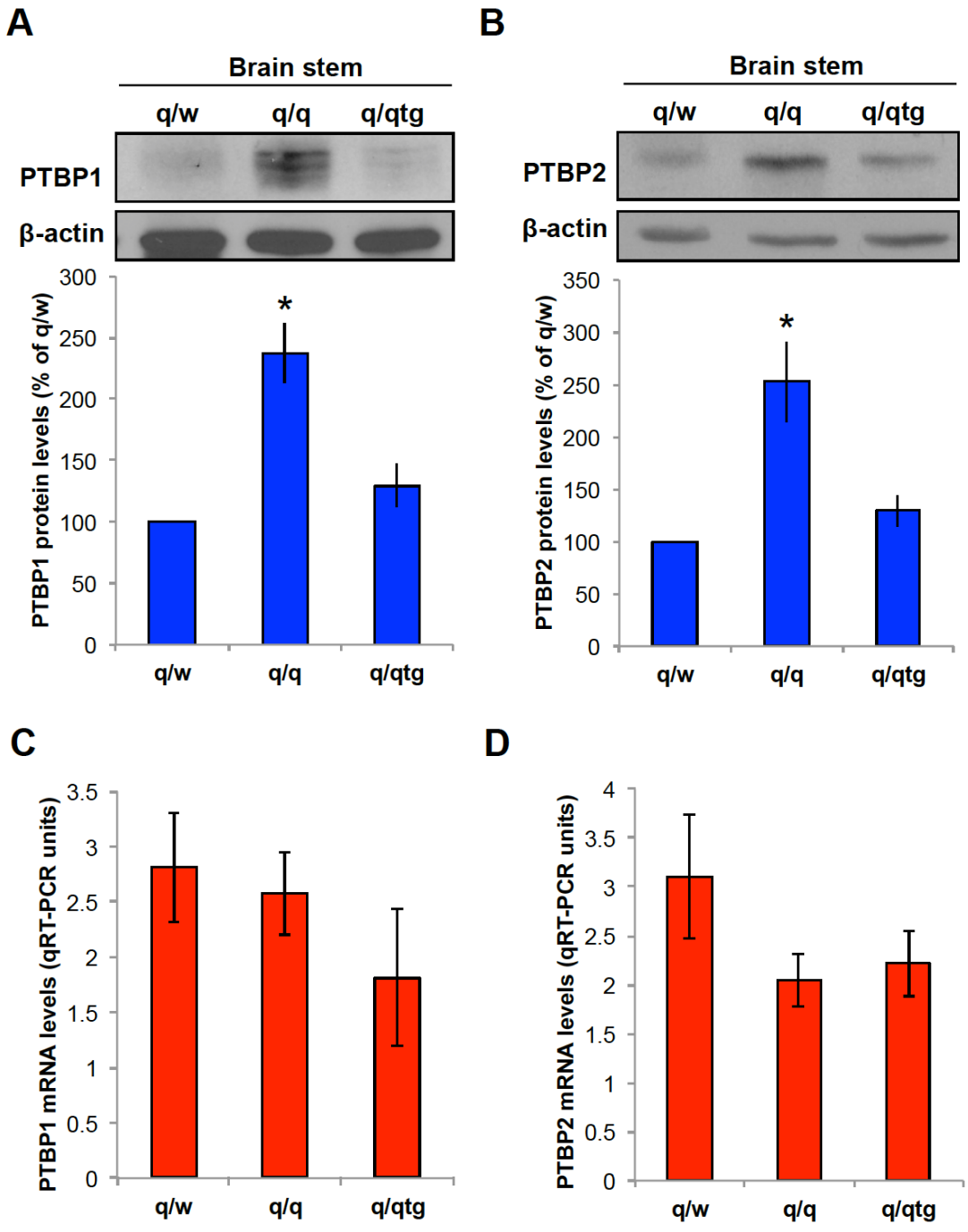

A
Mouse PTBP1 mRNA:
5'UTR
CDS
3'UTR
Mouse PTBP2 mRNA:
5'UTR
CDS
3'UTR
= UAAY
Representative 3'UTR sequence:
PTBP1:
cuuauaaucaag...ccuuaaugucc...uuuuauaaccucugucuu
PTBP2:
uauuuaaucuaauaagauacu...cuuaauuuaccuugcauuguaauauucaguuuuaauaaaucuu...aauuaauuuucuaacaaaguugggagguuugaugguuguuuaauuuc
B
RT-PCR:
FLAG-QKI Tg CLIP
INPUT
IP
PTBP1
PTBP2
GAPDH

Figure 3-5: QKI deficiency leads to an abnormal increase in PTBP1 and PTBP2 protein, without affecting steady-state mRNA levels. (A, B) Immunoblot detects aberrantly increased PTBP1 and PTBP2 protein in the brain stem of the qk^{v}/qk^{v} mouse (q/q) as compared to the non-phenotypic heterozygote control (q/w), which is rescued by OL-specific expression of the QKI-6 transgene (q/qtg). Quantification of PTBP1 and PTBP2 protein levels against β-actin is graphically displayed below, and represented as % of q/w control (n=3, * indicates p-value < 0.05). (C, D) qRT-PCR was performed on RNA isolated from brain stems of q/w, q/q and q/qtg mice, using primers against PTBP1, PTBP2 and β-actin. The results were graphically displayed as qRT-PCR units by calculating the ΔΔCt of PTBP1 or PTBP2 to β-actin control (n=3).

Figure 3-5


A
Brain stem
q/w
q/q
q/qtg
PTBP1
β-actin
PTBP1 protein levels (% of q/w)
300
250
200
150
100
50
0
*
q/w
q/q
q/qtg
B
Brain stem
q/w
q/q
q/qtg
PTBP2
β-actin
PTBP2 protein levels (% of q/w)
350
300
250
200
150
100
50
0
*
q/w
q/q
q/qtg
C
PTBP1 mRNA levels (qRT-PCR units)
3.5
3
2.5
2
1.5
1
0.5
0
q/w
q/q
q/qtg
D
PTBP2 mRNA levels (qRT-PCR units)
4
3.5
3
2.5
2
1.5
1
0.5
0
q/w
q/q
q/qtg

3.2.3 QKI deficiency affects expression of additional SFs that are implicated in human diseases

QKI is dysregulated in gliomas and is deficient in several different types of cancer (Chenard and Richard 2008, Novikov et al. 2011, Yang et al. 2010). Many hnRNPs are abnormally expressed in different types of cancer, including the QKI targets hnRNP H and hnRNP A1 in glioma (Babic et al. 2013, Lefave et al. 2011). In addition, hnRNP M was recently shown to promote epithelial to mesenchymal transition (EMT) and breast cancer metastasis (Han, Li and Zhang 2013, Xu et al. 2014). Interestingly, I found that hnRNP M protein levels were abnormally increased in the brain stem of the qk^v/qk^v mouse, and rescued by the QKI-6 transgenic expression (Figure 3-6A), suggesting QKI deficiency could allow increased expression of hnRNP M, which could in turn promote tumorigenesis.

Defects in myelin are thought to contribute to the pathogenesis of NDDs, which are also marked by dysregulation of multiple SFs (Bankston, Mandler and Feng 2013, Tazi, Bakkour and Stamm 2009). TDP-43 and PSF are RNA-binding proteins that are dysregulated in ALS and FTLD (Alami et al. 2014, Seyfried et al. 2012). These two structurally and functionally similar nuclear proteins were sequestered in the detergent-insoluble fractions of FTLD (Seyfried et al. 2012). I found that TDP-43 levels were unaffected in the brain stem of qk^v/qk^v mouse (Figure 3-6B). In contrast, when using a C-terminal antibody to PSF demarcated in Figure 3-7A, I found that the full length PSF protein (100 kDa) and a cleaved PSF product (70 kDa) were abnormally increased in the brain stem of 1 month and 4 month old qk^v/qk^v mouse (Figure 3-7), which was rescued by OL-specific QKI-6 transgene expression. Conversely, a smaller cleavage product of

20 kDa was reduced in the brain stem of 1 month and 4 month old qk^v/qk^v mouse (Figure 3-7), and partially rescued by OL-specific QKI-6 transgene expression. Thus, QKI deficiency may not only lead to abnormal expression of PSF but also abnormal cleavage of PSF by an undefined mechanism. Currently, the cleavage sites in PSF have not been completely identified.

Both the full length and the C-terminal PSF fragment were enriched in the detergent insoluble fraction specifically in FTLD postmortem brains (Seyfried et al. 2012). Moreover, PSF was shown to be sequestered into cytoplasmic aggregates in FTLD OLs. Interestingly, the 20 kDa C-terminal product deregulated in the qk^v/qk^v mutant is similar in size to the cleavage product found to be enriched in FTLD cytoplasmic aggregates (Seyfried et al. 2012). Importantly, PSF contains a nuclear localization signal (NLS) that determines its normal nuclear localization, which is found at the C-terminal end of PSF protein (Seyfried et al. 2012). The proteolytically cleaved PSF protein fragments are thought to have lost the NLS, potentially explaining the cytoplasmic localization that can result in protein aggregate formation. In fact, in HEK293T cells, a PSF construct lacking the NLS was shown to be mislocalized in the cytoplasm, whereas full length PSF localized to the nucleus (Seyfried et al. 2012). To test whether loss of the NLS indeed causes cytoplasmic aggregation of PSF in OLs, a human OL cell line (HOG) was transfected with a plasmid encoding either an N-terminal-GFP-tagged full length PSF (fPSF) or a truncated PSF lacking the C-terminal NLS (ΔNLS) (Dye and Patton 2001). At 24 or 70 hours after transfection, cells were treated with Na Arsenite to induce cellular stress, which can promote aggregate formation (Zhang et al. 2011). Live cells were then observed under a fluorescent microscope and live-images were captured

(Figure 3-8). As expected, fPSF localizes to the nucleus whereas ΔNLS is predominantly cytoplasmic (Figure 3-8A). Interestingly, even at time zero cytoplasmic aggregates formed in the ΔNLS, which became larger over time after Na Arsenite treatment (Figure 3-8B). In contrast, the fPSF presented minimal aggregate formation even after 70 hours of drug treatment (Figure 3-8B). Despite potential cleavage of the GFP-tagged proteins, both constructs expressed their respective full length forms (Figure 3-8C). The results suggest the lack of an NLS results not only in predominantly cytoplasmic localization of PSF, but also increased potential for cytoplasmic aggregate formation, which is detrimental to the cell and could contribute to white matter degeneration in FTLD.

Figure 3-6: QKI deficiency leads to an abnormal increase in hnRNP M protein levels but not TDP-43. (A, B) Immunoblot was performed on lysates of brain stems obtained from the qk^{v}/qk^{v} mouse (q/q) and the non-phenotypic heterozygote control (q/w), and a q/q mutant mouse expressing OL-specific QKI-6 transgene (q/qtg), using antibodies against hnRNP M and TDP-43. β-actin and eIF5α antibodies were used to re-probe the blots and served to indicate similar loading.

Figure 3-6

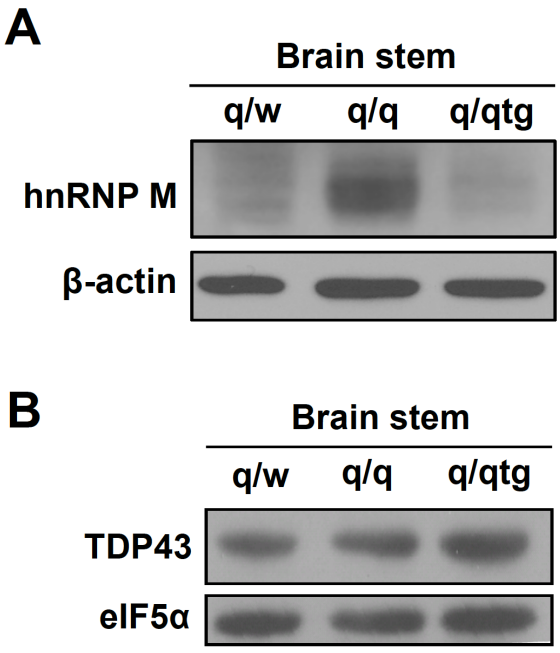

A
Brain stem
q/w
q/q
q/qtg
hnRNP M
β-actin
B
Brain stem
q/w
q/q
q/qtg
TDP43
eIF5α

Figure 3-7: Abnormal PSF expression detected in brain stems of qk^v/qk^v mice. (A) A schematic of full-length PSF and predicted cleavage (lightening bolts) products denoted by size. The immunogen to create the PSF C-terminal antibody (Abcam) is demarcated by a line. (B) Immunoblot was performed using lysates derived from 1 and 4 months old brain stems of qk^v/qk^v mice (q/q), non-phenotypic heterozygote control (q/w), and mice expressing OL-specific QKI-6 transgene (q/qtg) using the PSF C-terminal antibody. The full length and cleavage products that are detected using the C-terminal antibody are indicated by the respective labels. β-actin served as a loading control.

Figure 3-7

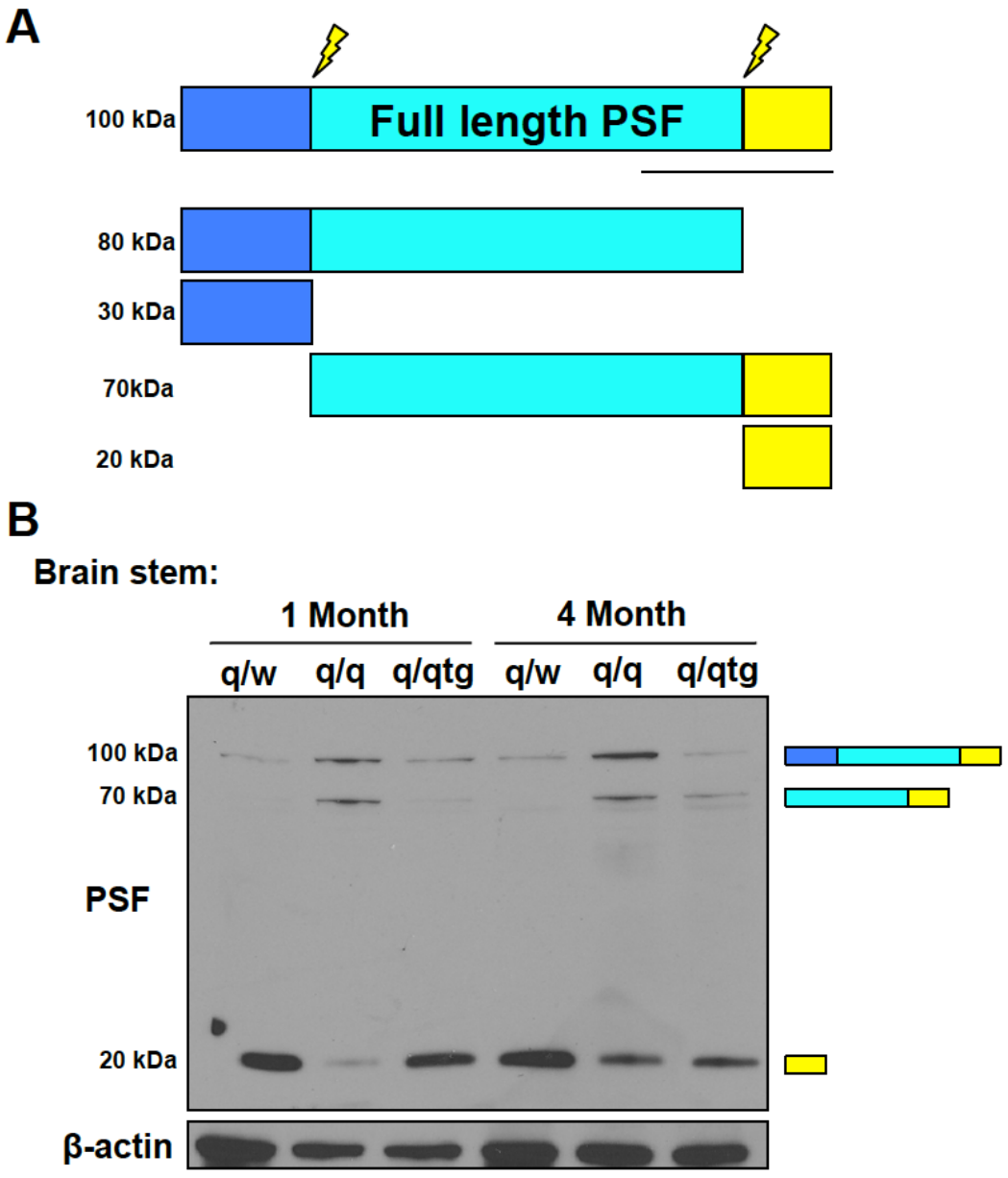
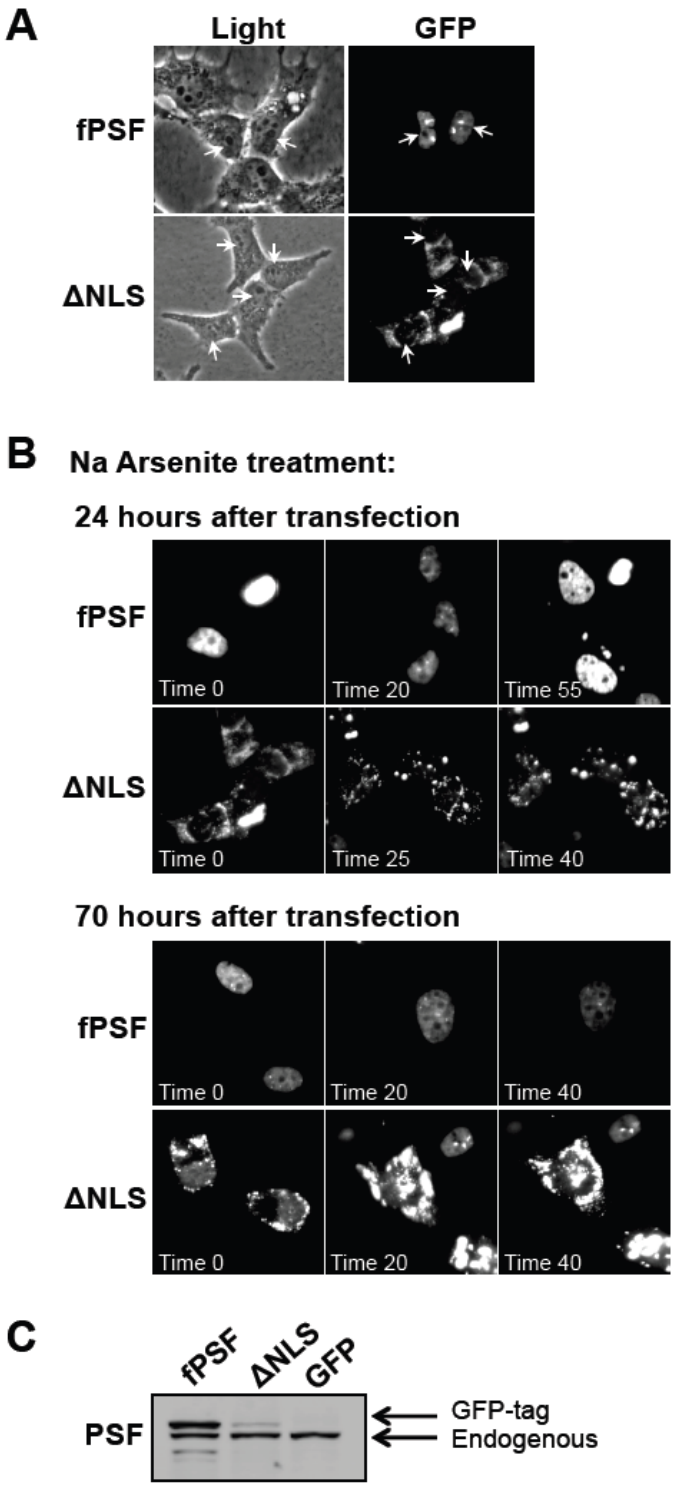

A
100 kDa
Full length PSF
80 kDa
30 kDa
70kDa
20 kDa
B
Brain stem:
1 Month
4 Month
q/w
q/q
q/qtg
q/w
q/q
q/qtg
100 kDa
70 kDa
PSF
20 kDa
β-actin

Figure 3-8: Lack of NLS from PSF results in increased formation of cytoplasmic aggregates in OLs. (A, B) OL cell line HOG was transfected with full-length GFP-tagged PSF (fPSF), or the plasmid lacking the NLS (ΔNLS). (A) Light field and fluorescent images were taken of cells expressing GFP-tagged PSF constructs. Arrows indicate the nuclei. (B) Live-cell images showing GFP-tagged PSF proteins, captured at 24 hours and 70 hours after treatment with Na Arsenite. (C) Immunoblot of protein lysates from transfected HOG cells using C-terminal PSF antibody that indicates expression of respective full length proteins from the GFP-tagged constructs.

Figure 3-8


A
Light
GFP
fPSF
ΔNLS
B
Na Arsenite treatment:
24 hours after transfection
fPSF
Time 0
Time 20
Time 55
ΔNLS
Time 0
Time 25
Time 40
70 hours after transfection
fPSF
Time 0
Time 20
Time 40
ΔNLS
Time 0
Time 20
Time 40
C
fPSF
ΔNLS
GFP
PSF
GFP-tag
Endogenous

3.3 Discussion

Here I report on the role of QKI-6 in regulating AS in OLs. QKI-6 seems to regulate expression of multiple SFs by suppressing translation, either by directly targeting the mRNA or possibly through the involvement of microRNAs (miRNAs). In addition to previously identified hnRNP targets of QKI-6 hnRNP A1 and hnRNP F/H, I provide evidence that PTBP1, PTBP2, hnRNP M and PSF are dysregulated by QKI deficiency in OLs of the qk^{v}/qk^{v} mouse, and are rescued by OL-specific expression of QKI-6. The identified SFs downstream of QKI-6 play crucial roles in brain development and function (Boutz et al. 2007, Licatalosi et al. 2012, Wang et al. 2012). Dysregulated expression or function of the identified SFs is implicated in multiple types of NDDs and cancer.

I previously showed that QKI-6 deficiency leads to abnormally high levels of hnRNP F/H protein expression, without affecting mRNA steady-state levels (Mandler, Ku and Feng 2014). Here I showed by linear sucrose fractionation that QKI-6 controls hnRNP F/H expression potentially by repressing mRNA translation. The limitation of this experiment was that QKI deficiency and abnormal over-expression of hnRNP F/H are restricted to OLs whereas hnRNP F/H are broadly expressed in the heterogeneous mixture of brain cell types (Lu et al. 2003, Nilsen and Graveley 2010). The optic nerve is most enriched of OLs but unfortunately does not provide sufficient material for linear-sucrose gradient fractionation. The brain stem is the next OL-rich tissue in which OLs may account for no more than 30% of cells. Despite the potentially underestimated effect due to use of the brain stem for analysis, a shift in mRNAs towards the polyribosomal fraction was observed (Figure 3-1).

In my previous study, hnRNP F mRNA did not coimmunoprecipitate with QKI-6, suggesting an indirect mechanism controls its expression (Mandler, Ku and Feng 2014). Interestingly, although the coding sequences for hnRNP F and H are quite similar, the 3' UTRs are fairly different, which could allow distinct forms of regulation. QKI-6 was previously shown to regulate expression of miRNAs (Wang et al. 2013), which could target hnRNP F mRNA to repress translation. The bioinformatic analysis predicted multiple miRNAs that might be regulated by QKI-6 to target hnRNP F mRNA (Table 3-1). Future studies will elucidate the potential for miRNA-mediated regulation of hnRNP F expression in myelinating glia.

Similar to mechanisms by which QKI-6 controls expression of hnRNP F/H and hnRNP A1, I showed that two other SFs, PTBP1 and PTBP2, displayed abnormally high protein levels due to QKI-6 deficiency without affecting mRNA steady-state levels (Figure 3-5). In addition, PTPB1 and PTBP2 mRNAs coimmunoprecipitated with QKI-6 protein (Figure 3-4), suggesting QKI-6 might target the mRNAs and prevent translation. Altogether, the data suggest that the regulation of mRNA translation is a common mechanism by which QKI-6 regulates expression of SFs. Whether or not QKI-6 regulates expression of hnRNP M and PSF by the same mechanism will be addressed in future studies.

QKI and PTBP1 have overlapping targets in muscle (Hall et al. 2013). Recombinant QKI-5 binds to wild type *in vitro* transcribed RNA in a QRE-dependent manner and regulates AS in muscle cells (Hall et al. 2013). However, the siRNA used to deplete QKI in this study could not distinguish between the three QKI isoforms, which are all expressed in myoblasts (Hall et al. 2013). In particular, QKI-6 expression is

upregulated during myoblast differentiation, which is accompanied by a decrease in PTBP1 expression (Hall et al. 2013). Here I showed QKI-6 is upstream of PTBP1 in the brain, but whether QKI-6 could mediate PTBP1-dependent AS in muscle will be interesting to address in future studies.

As PTBP2 is predominantly expressed in neurons, whereas QKI deficiency is restricted to myelinating glia, the marked increase of PTBP2 in the brain stem of qk^{v}/qk^{v} mutant suggests a potential role for QKI in controlling neuronal AS through neuron-glia interactions. Both PTBP1 and PTBP2 are developmentally regulated in the early stages of neural stem cell (NSC) differentiation (Grabowski 2011, Zheng et al. 2012). QKI expression is also tightly regulated during the differentiation of NSCs to neurons or glia (Hardy 1998). Potentially, QKI could play a role in the regulation of PTBP1 and PTBP2 expression during neuron or glia specification in the differentiation of NSCs. Future studies will aim to determine the cell types that elicit abnormal PTBP1 and PTBP2 protein levels by OL-specific QKI deficiency, and further delineate the mechanism by which QKI might control expression of both PTBP1 and PTBP2 in the brain.

I showed that QKI deficiency results in abnormally high expression of hnRNP M in brain stem, which was rescued by QKI-6 expression (Figure 3-6). Similar to hnRNP F/H, hnRNP M can target G-runs, and could also potentially control AS of the G-run containing genes previously thought to be dysregulated in the qk^{v}/qk^{v} mouse (Datar, Dreyfuss and Swanson 1993, Mandler, Ku and Feng 2014). Additionally, hnRNP M is among the group of hnRNPs that has been largely implicated in cancer progression by promoting epithelial to mesenchymal transition, and is implicated in dysregulation of AS in breast cancer (Dery et al. 2011, Xu et al. 2014). QKI is reduced or abnormally

expressed in several types of cancer, including breast cancer (Novikov et al. 2011). Perhaps a reduction in QKI contributes to increased expression of hnRNP M, which in turn can promote tumorigenesis.

Not all SFs were dysregulated by QKI deficiency in the qk^{v}/qk^{v} mutant, represented by TDP-43, which forms cytoplasmic inclusions causing neurodegeneration in ALS and FTLD (Alami et al. 2014, Seyfried et al. 2012). However, QKI deficiency caused abnormal expression of the SF PSF, which is also enriched in the detergent-insoluble fractions and particularly in the cytoplasmic aggregates of OLs from several FTLD cases (Seyfried et al. 2012). Interestingly, along with increased levels of full length PSF in QKI deficient mice, aberrant levels of PSF cleavage products were detected, and rescued by the OL-specific QKI-6 transgene (Figure 3-7). Additionally, in an OL cell line, PSF that lacks an NLS forms cytoplasmic aggregates that grow larger over time and under cell stress, whereas full length PSF localizes normally to the nucleus and remains innocuous (Figure 3-8). Cleavage of PSF could cause removal of the NLS, which would result in cytoplasmic localization and increased aggregate formation similar to what is observed in OLs of FTLD (Seyfried et al. 2012). Determining the composition of the cleavage products identified in the QKI deficient mouse will reveal which product lacks the NLS, and the role for QKI in contributing to formation of cytoplasmic aggregates.

In summary, QKI-6 clearly plays a crucial role in regulating expression of multiple SFs in OLs that act together to regulate AS of downstream targets. Some of the identified SFs were shown to have distinct and overlapping downstream targets, such as those regulated by PTBP1 and PTBP2 (Boutz et al. 2007). The known function for both hnRNP F/H and hnRNP M to target G-runs suggests a potentially coordinated or

antagonistic effort to regulate splice site selection (Datar, Dreyfuss and Swanson 1993, Mandler, Ku and Feng 2014). PSF and hnRNP M interact and colocalize to nuclear paraspeckles to regulate mRNA processing in the nucleus (Marko et al. 2010). In fact, both hnRNP M and PSF were recently shown to regulate AS of SMN2 to promote inclusion of exon 7 (Cho et al. 2014). The QKI deficiency in myelinating glia results in abnormal expression of selective SFs in the brain, which could explain the large number of AS defects in the qk^{v}/qk^{v} mutant mouse. Additionally, as discussed, many of these SFs were previously shown to be dysregulated in NDD and cancer. In this regard, dysregulation of QKI expression could contribute to neurodegeneration in the brain or in cancer progression, which will be important and challenging questions to be addressed by future studies.

Chapter 4: Exploring mechanisms that regulate QKI isoform expression

4.1 Introduction

The selective RNA-binding protein quaking I (QKI) is critical for the normal function and differentiation of oligodendroglia (OLs), which are the myelinating glia of the central nervous system (CNS). There are three main QKI isoforms, named QKI-5 QKI-6 and QKI-7, which are derived from alternative splicing (AS) of the 3' terminal coding exons of the QKI pre-mRNA (Kondo et al. 1999). QKI isoform expression is tightly regulated during cell fate specification of neural progenitor cells, OL differentiation, and myelin formation (Hardy 1998, Hardy et al. 1996). Dysregulation of QKI isoform expression has been implicated in several diseases, including glioma and myelin-associated disorders such as schizophrenia (Chenard and Richard 2008). Delineating what regulates QKI isoform expression would be crucial to understand how QKI dysregulation may contribute to various human diseases.

The three QKI isoforms share common N-terminal amino acids, which contain the RNA binding and heterodimerization domains but differ in their C-terminal amino acids (Kondo et al. 1999). The QKI-5 isoform contains a nuclear localization signal (NLS) and is highly concentrated in the nucleus (Wu et al. 1999). In contrast, QKI-6 and QKI-7 lack an NLS and are predominantly cytoplasmic at steady state (Zhao et al. 2010), despite the reported formation of heterodimers between QKI isoforms and the ability to shuttle between the nucleus and cytoplasm (Pilotte, Larocque and Richard 2001, Wu et al. 1999). Such distinct nuclear *versus* cytoplasmic distribution enables differential function of QKI isoforms to regulate RNA metabolism at different posttranscriptional steps (Bockbrader and Feng 2008). The steady-state localization of QKI-5 to the nucleus can explain the observed functional role for binding pre-mRNA and regulating splicing, which has been

shown in different cell types (Hafner et al. 2010, Hall et al. 2013, Wu et al. 2002), whereas QKI-6 and QKI-7 were shown to control mRNA translation and stability in the cytoplasm (Larocque et al. 2005, Li et al. 2000, Saccomanno et al. 1999, Zhao et al. 2006).

QKI expression is developmentally regulated during neuron-glia lineage specification. All three QKI isoforms are expressed in neural progenitor cells, but are diminished when differentiated to become mature neurons (Hardy 1998). In contrast, all QKI isoforms remain abundantly expressed in glia lineages. The abundant expression of cytoplasmic QKI-6 and QKI-7 is a unique feature of glia cells, because QKI-5 is the predominant isoform in other non-neural cells examined (Lu et al. 2003). However, the mechanism(s) that control QKI isoform expression are currently unknown.

All three QKI isoforms can promote OL differentiation in OL progenitor cell culture (Chen et al. 2007). However, expression of nuclear and cytoplasmic QKI isoforms is reciprocally regulated during brain development (Hardy et al. 1996). QKI-5 is detected in high abundance in OL progenitor cells (OPCs), and is expressed during the proliferative state of OPCs (Hardy et al. 1996, Kondo et al. 1999). Whereas, QKI-6 and QKI-7 expression is upregulated concomitant with a decline of QKI-5 during myelin formation in mouse brain development (Hardy et al. 1996, Kondo et al. 1999, Larocque et al. 2009, Larocque et al. 2005). Expression of QKI-6 and QKI-7 leads to cell cycle arrest and promotes OL differentiation by stabilizing the cyclin dependent kinase inhibitor $p27^{kip}$ (Larocque et al. 2005). Moreover, QKI-6 is necessary for myelin gene expression and advancing myelination, whereas QKI-5 overexpression prevents myelination by sequestration of myelin gene mRNAs in the nucleus (Larocque et al. 2005, Larocque et

al. 2002, Li et al. 2000, Zhao et al. 2006). Thus, regulation of QKI isoform expression is critical for properly controlling the balance between OL proliferation and differentiation as well as myelin formation. Besides AS of the 3' terminal coding exons, alternative poly-adenylation (APA) site usage also results in distinct 3' untranslated regions (3'UTRs) in the QKI isoform mRNAs (Kondo et al. 1999). However, it is not understood whether QKI isoform expression is regulated by AS or APA, or by 3' UTR-mediated mRNA translation and mRNA stability, during OL differentiation and neuron-glia cell fate decisions.

In this study, I investigated the potential molecular mechanisms that control differential QKI isoform expression in developing neural cells. I determined that regulation of QKI isoform expression in differentiation of early OL progenitors (OPCs) is likely controlled by differential translation of QKI mRNA isoforms. In contrast, I observed distinct QKI mRNA isoform profiles at steady-state levels in mouse-derived neuronal and OL cell lines, suggesting differential processing of the QKI primary transcripts in neurons and glia. I also provide evidence that the splicing factor (SF) RBFOX2 (FOX2) controls splice site selection to promote QKI-7 expression. Finally, I constructed a QKI minigene that contains just the sequence critical for alternative splice site selection of the coding exons, which will allow specific assessment as to whether AS alone might dictate QKI isoform expression, apart from potential influence of APA in QKI 3' UTRs. The preliminary data obtained suggest that multiple mechanisms coordinate to determine QKI isoform expression, which may depend on the stage of differentiation or the type of cells.

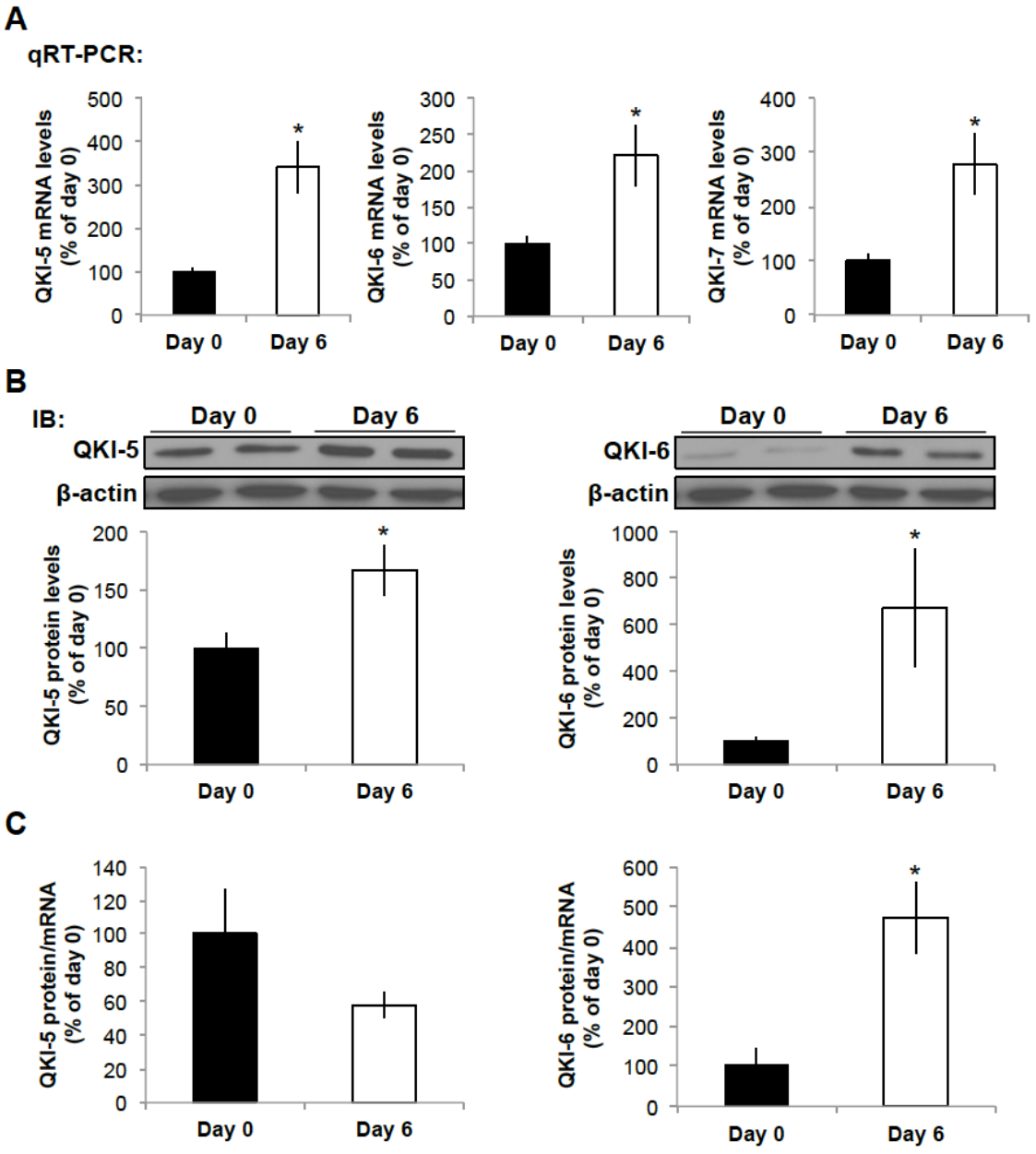
4.2 Results

4.2.1 mRNA translation underlies QKI isoform expression in OPC differentiation

The biogenesis of QKI mRNA isoforms depends on alternative processing of the QKI pre-mRNA, but whether this mechanism underlies the QKI isoform protein switch during OL differentiation remains elusive. To assess whether the mRNA levels determine protein expression, I compared mRNA and protein levels of the QKI isoforms during differentiation of the rat OPC cell line CG4. CG4 cells were harvested on day 0 and day 6 after induced differentiation, and processed for quantitative analysis of QKI mRNA and protein isoforms. The endogenous steady-state QKI mRNA isoforms were detected by qRT-PCR using rat mRNA isoform-specific primer sets, and normalized to GAPDH mRNA as a loading control. QKI-5, QKI-6 and QKI-7 mRNAs are all upregulated with similar fold after 6 days of OPC differentiation (Figure 4-1A). In addition, protein samples from day 0 and day 6 after OPC differentiation were analyzed by immunoblot using isoform-specific QKI antibodies and normalized to β-actin (Figure 4-1B). QKI-7 levels are not expressed at detectable levels in CG4 cells. Interestingly, although QKI mRNA isoforms were increased comparably, the increase of QKI-5 and QKI-6 protein levels differed. QKI-5 protein levels increased less than 2 fold by day 6 of OPC differentiation, whereas QKI-6 protein levels increased close to 6 fold (Figure 4-1B). The protein to RNA ratio for QKI-5 and QKI-6, which can estimate translation efficiency of the corresponding mRNA, was markedly different (Figure 4-1C). No significant change was observed for QKI-5 whereas a five-fold increase was detected for QKI-6 (Figure 4-1C). The analysis suggests differential translation may be the major mechanism regulating the switch in relative levels of QKI isoforms during early OL differentiation.

Figure 4-1: Increase in QKI mRNA levels does not recapitulate protein levels during early OL progenitor cell (OPC) differentiation. (A) qRT-PCR was performed on total RNA isolated from the OPC cell line CG4 on day 0 or day 6 after induced differentiation, using QKI isoform specific primers and β-actin. The results were calculated by ΔΔCt of the QKI mRNA isoforms to β-actin mRNA, and graphically displayed as % of day 0 (n=6, * indicates p-value < 0.05). (B) Immunoblots were performed on protein lysates isolated from OPCs on day 0 or day 6 after induced differentiation using specific antibodies against QKI-5 on the left and QKI-6 on the right, and β-actin serving as a loading control. The upper panel shows representative immunoblots. Densitometry readings of QKI isoforms from two independently collected sets of samples were quantified by ImageJ, normalized to β-actin reading and graphically displayed in the bottom panel below as % of day 0 (day 0, n=6; day 6, n=5, * indicates p-value < 0.05). (C) Translation indexes were calculated by taking the ratio of protein to RNA on day 0 and day 6 for QKI-5 on the left panel and QKI-6 on the right panel. The ratios were graphically displayed as % of day 0 (n=3, * indicates p-value < 0.05).

Figure 4-1

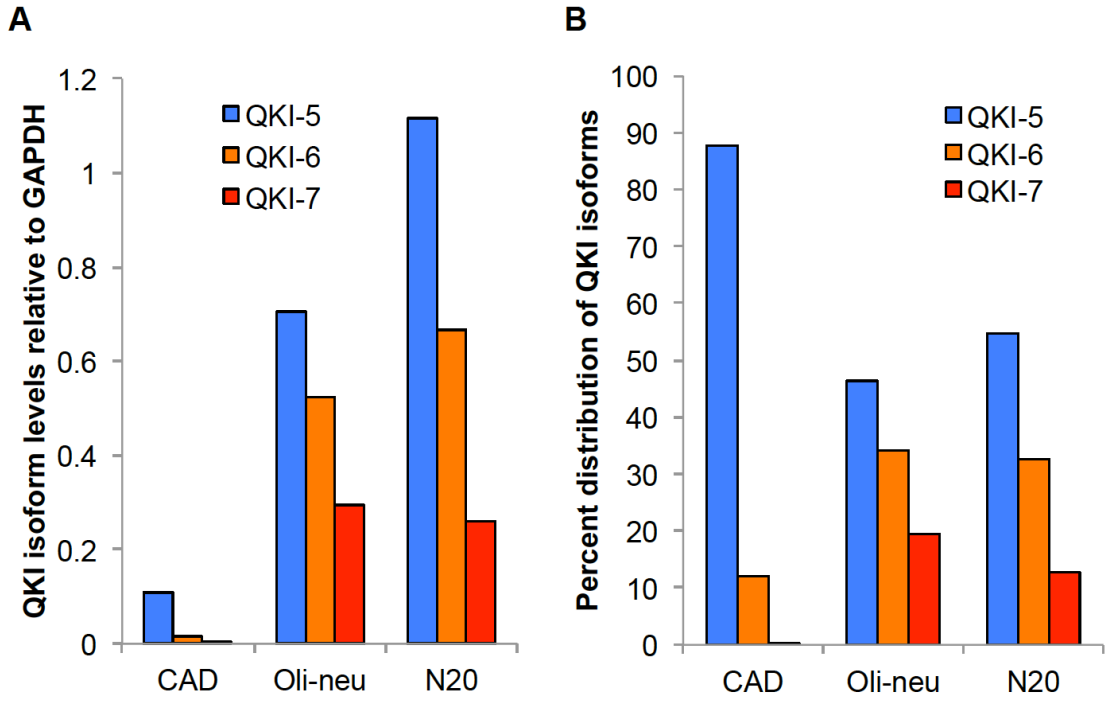

A
qRT-PCR:
QKI-5 mRNA levels (% of day 0)
QKI-6 mRNA levels (% of day 0)
QKI-7 mRNA levels (% of day 0)
Day 0
Day 6
B
IB:
QKI-5
QKI-6
β-actin
QKI-5 protein levels (% of day 0)
QKI-6 protein levels (% of day 0)
C
QKI-5 protein/mRNA (% of day 0)
QKI-6 protein/mRNA (% of day 0)

4.2.2 Steady-state levels of QKI mRNAs display differential patterns in mouse neuronal and OL cell lines

Neural progenitors display distinct regulation of QKI isoform expression upon cell fate specification (Hardy 1998). However, the underlying regulatory mechanisms are unknown. To begin to understand the molecular mechanisms dictating differential QKI isoform expression, I examined mRNA levels in the mouse neuronal progenitor cell line CAD, and two mouse OL progenitor cell lines oli-neu and N20. qRT-PCR using QKI isoform-specific primers was performed to determine endogenous QKI mRNA levels. The QKI mRNA isoform levels relative to internal loading control are shown in Figure 4-2A. Neuronal progenitor cells express significantly less QKI mRNA overall as compared to OLs (Hardy 1998). Consistent with the literature, I detected lower levels of QKI mRNAs in CAD cells than in the OL cell lines (Figure 4-2A). The relative percentages of QKI isoforms in each cell line were calculated and graphically displayed in Figure 4-2B. The pattern of QKI isoform expression is consistent between the two mouse OL cell lines. Interestingly, the neuronal cell line displays a different QKI isoform expression pattern as compared to the OL cell lines. Although QKI-5 mRNA is the most abundant isoform in all cell lines examined, the relative amount of QKI-5 mRNA versus QKI-6 and QKI-7 is much higher in CAD cells as compared to the OL cell lines (Hardy 1998). These results suggest that regulation of QKI mRNA levels is an important factor that determines QKI isoform expression during neuronal-glial cell fate specification.

Figure 4-2: Steady-state mRNA levels of QKI isoforms in neuronal and OL cell lines. (A) qRT-PCR was performed on RNA isolated from neuronal cell line CAD, and OL cell lines Oli-neu and N20, using specific primers for QKI mRNA isoforms and the GAPDH mRNA, which serves as an internal control. The results were calculated by ΔΔCt of the QKI mRNA isoforms to GAPDH control, and graphically displayed (n=1). (B) The percent of each QKI mRNA was calculated based on dividing the isoform level by total QKI mRNA levels measured to determine the percentage of QKI mRNA isoform expression, which is graphically displayed.

Figure 4-2


A
QKI isoform levels relative to GAPDH
1.2
1
0.8
0.6
0.4
0.2
0
QKI-5
QKI-6
QKI-7
CAD
Oli-neu
N20
B
Percent distribution of QKI isoforms
100
90
80
70
60
50
40
30
20
10
0
QKI-5
QKI-6
QKI-7
CAD
Oli-neu
N20

4.2.3 Knockdown of splicing factor FOX2 specifically reduces QKI-7 mRNA levels

Factors that might regulate AS of QKI pre-mRNA in OLs remain unidentified. Cross-linking immunoprecipitation (CLIP) followed by high-throughput RNA sequencing identified QKI pre-mRNA as a target for the SF FOX2, also known as RBM9, in human embryonic stem cells (hESCs) (Yeo et al. 2009). Previous studies identified the consensus-binding site for FOX2, GCAUG, which was functionally validated by Affymetrix exon-tiling array and CLIP-seq combined with Z-score statistics (Ponthier et al. 2006, Yeo et al. 2009, Yeo et al. 2007). CLIP-seq studies in combination with shRNA knockdown of FOX2 allowed the authors to establish a splicing code in hESCs. They determined that FOX2 binding within 200 bps upstream of an alternative exon correlates with exon exclusion, whereas binding within 200 bps downstream of an alternative exon correlates with exon inclusion (Yeo et al. 2009).

I mapped the sites in the QKI pre-mRNA and confirmed the presence of putative binding sites between exons 7a and 7b (Figure 4-3A). In collaboration with the Yeo lab, I obtained FOX2 shRNA to assess whether FOX2 regulates QKI isoform expression in a human OL cell line. In fact, knockdown of FOX2 (Figure 4-3B) significantly repressed QKI-7 mRNA levels (Figure 4-3C), without affecting QKI-6 or QKI-5 mRNA levels (Figure 4-3D). The results suggest that FOX2 may promote exon 7a splice site selection, which is consistent with the mode of regulation by FOX2 (Yeo et al. 2009). I also examined FOX2 protein levels in the brain stem of QKI deficient mouse, and found FOX2 expression is unaffected by QKI deficiency in 1 month and 4 month old mice, suggesting FOX2 only acts upstream of QKI to regulate AS of QKI pre-mRNA (Figure 4-4).

AS is critical for the biogenesis of the QKI isoforms. However, in addition to alternative splice site selection, the nuclear and cytoplasmic QKI isoforms utilize distinct poly-A sites, thus QKI isoform expression may also depend on alternative poly-A site selection (APA). To definitively dissect the potential role for regulation of AS alone in determining QKI isoform expression, I constructed a human QKI minigene construct that contains just the sequence critical for splice site selection of the alternatively spliced 3' terminal coding exons from QKI pre-mRNA (Figure 4-5). The sequence includes the 3' half of common exon 6, downstream introns and exons 7a, 7b and the majority of exon 7c, fused in-frame downstream of GFP in the mammalian expression reporter plasmid pEGFPC2. Expression of each QKI minigene-derived transcript utilizes the poly-A site from the vector (Figure 4-5A). Splicing to exon 7a and 7b results in use of a natural stop codon at the 3' end of each exon to form GFP-QKI-7-mini and GFP-QKI-6-mini fusion proteins, respectively (Figure 4-5A, C). However, splicing to exon 7c results in use of the vector stop codon, which is artificial for GFP-QKI-5-mini expression (Figure 4-5A, C). The minigene sequence was confirmed by restriction mapping (Figure 4-5B) and sequencing. The mRNA transcripts produced from the minigene can be detected using QKI isoform minigene-specific primers, as indicated by the half arrows in Figure 4-5C. The minigene will allow direct testing of AS regulation by FOX2. Moreover, it will address whether AS of QKI minigene could mimic endogenous QKI mRNA isoform expression in various cell types that harbor distinct abundance of QKI mRNA isoforms, in a manner that is independent of APA.

Figure 4-3: FOX2 knockdown reduces QKI-7 mRNA expression. (A) Diagram of QKI pre-mRNA illustrating the putative FOX2 binding sites. FOX2 is proposed to promote inclusion of an upstream exon (green ball and stem), and/or inhibit inclusion of a downstream exon (red ball and stem). (B) Immunoblot of protein lysate from human OL cells (HOG) treated with either negative control shRNA virus (shNegC) or shRNA virus against Fox2 (shFox2), performed with antibodies against FOX2 and β-actin, which serves as loading control. (C, D) qRT-PCR was performed on RNA isolated from shRNA treated cells using QKI mRNA isoform specific primers. The results were calculated by ΔΔCt of the QKI mRNAs to β-actin control. Results of shFox2 were taken as a % of shNegC and graphically displayed for QKI-7 mRNA (C), QKI-5 and QKI-6 mRNAs (D) (n=6, * indicates p-value < 0.05).

Figure 4-3

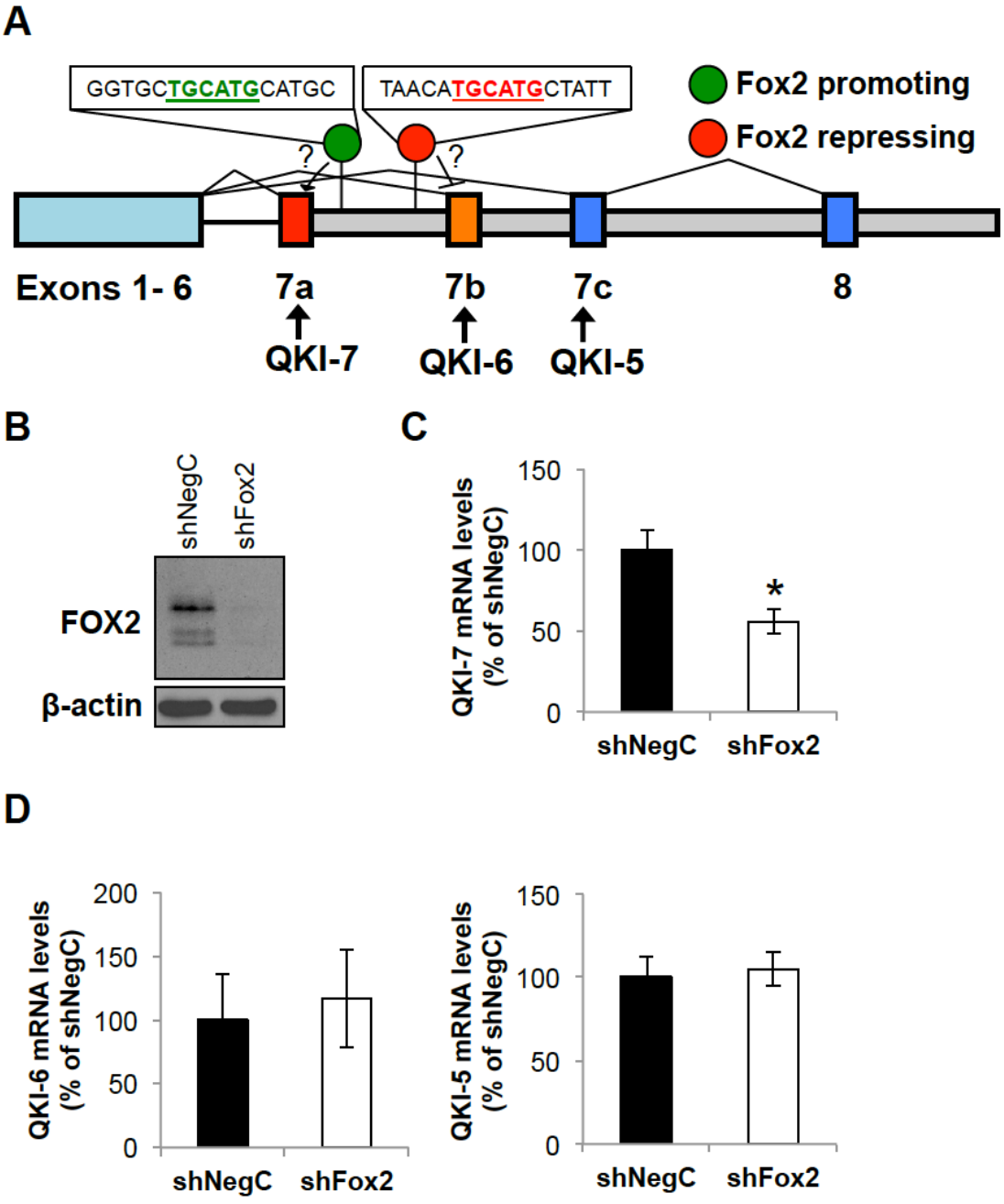

A
GGTGCTGCATGCATGC
TAACATGCATGCTATT
Fox2 promoting
Fox2 repressing
?
?
Exons 1- 6
7a
7b
7c
8
QKI-7
QKI-6
QKI-5
B
shNegC
shFox2
FOX2
β-actin
C
QKI-7 mRNA levels (% of shNegC)
150
100
50
0
*
shNegC
shFox2
D
QKI-6 mRNA levels (% of shNegC)
200
150
100
50
0
shNegC
shFox2
QKI-5 mRNA levels (% of shNegC)
150
100
50
0
shNegC
shFox2

Figure 4-4: FOX2 protein levels are unaffected by QKI deficiency in brain stem of qk^{v}/qk^{v} mutant mice. Upper panel shows immunoblot of protein lysates from 1 and 4 months old brain stems of qk^{v}/qk^{v} mice (q/q) as compared to non-phenotypic heterozygote control (q/w), and mice expressing OL-specific QKI-6 transgene (q/qtg) using antibodies against FOX2 and β-actin, which serves as loading control. The bands were quantified and normalized to β-actin. The percent of either q/q or q/qtg to q/w were calculated and graphically displayed in the lower panel.

Figure 4-4

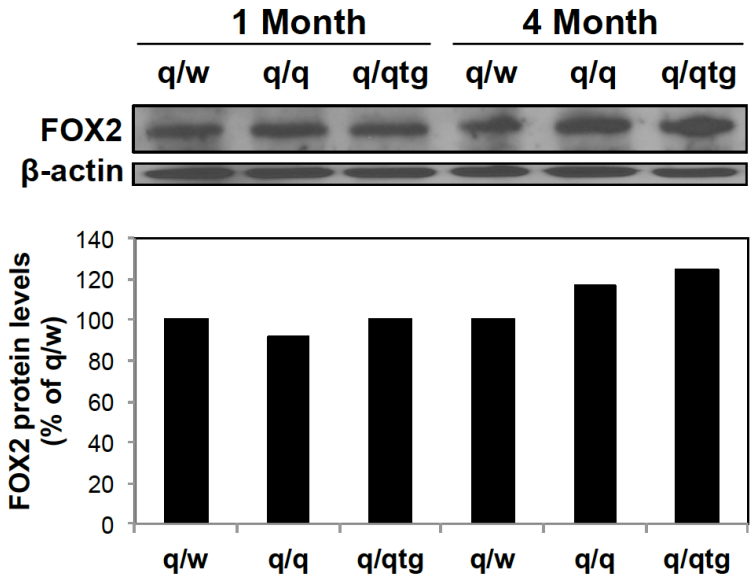
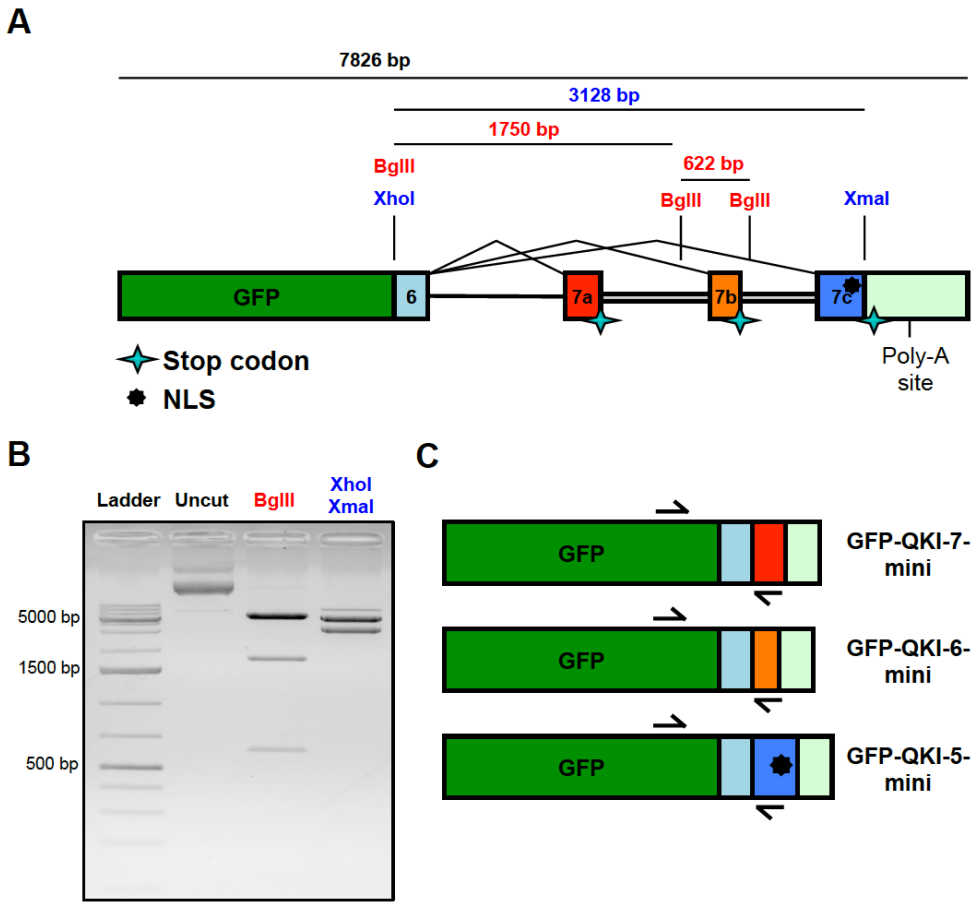

1 Month
4 Month
q/w
q/q
q/qtg
q/w
q/q
q/qtg
FOX2
β-actin
FOX2 protein levels (% of q/w)
140
120
100
80
60
40
20
0
q/w
q/q
q/qtg
q/w
q/q
q/qtg

Figure 4-5: A QKI minigene construct is established, which can be used to determine the regulation of AS of QKI terminal coding exons. (A) Diagram of QKI minigene construct showing QKI pre-mRNA containing a human genomic fragment covering 71 bps of exon 6 to exon 7c fused in frame downstream of GFP in the reporter plasmid pEGFPC2. Each exon is labeled, and shown in boxes that are joined by the intervening lines or the thicker grey line representing introns and/or 3'UTRs. The stop codons are indicated by the blue diamonds and the NLS by a black star. The adjoining lines represent alternative splice site selection. The sizes of the entire plasmid and the fragments formed by using the color-coded restriction enzymes are indicated. (B) The plasmid was mapped by restriction enzymes BglII and XhoI+XmaI, versus an uncut control, and run on a 1% agarose gel alongside a 1 Kb DNA ladder. Band ladder sizes are indicated on the left of the gel image. (C) The minigene-derived QKI mRNA transcripts are diagrammed and respectively labeled. The primers used to determine minigene-specific QKI mRNA isoform expression are shown by half arrows.

Figure 4-5


A
7826 bp
3128 bp
1750 bp
BglII
XhoI
622 bp
BglII
BglII
XmaI
GFP
6
7a
7b
7c
Stop codon
NLS
Poly-A site
B
Ladder
Uncut
BglII
XhoI
XmaI
5000 bp
1500 bp
500 bp
C
GFP
GFP-QKI-7-mini
GFP
GFP-QKI-6-mini
GFP
GFP-QKI-5-mini

4.3 Discussion

The QKI isoforms are derived from AS, but what regulates the splice site selection is not known. Additionally, whether or not AS alone is sufficient to control QKI isoform expression between different neural cell types or during OL differentiation has never been explicitly shown. Here I investigated potential mechanisms that might control QKI isoform expression, from biogenesis of the mRNA isoforms to mRNA translation. I determined that mRNA translation likely controls QKI isoform expression during differentiation of OL progenitors. However, the distinct expression pattern of QKI mRNAs in neuron and glia progenitor cell lines suggests regulation of mRNA levels during cell fate specification. Finally, I identified that the SF FOX2 regulates expression of QKI-7 in an OL cell line. The data suggests that regulation of QKI isoform expression is complex, and may require distinct regulation in different types of cells or the stage of differentiation.

Regulation of mRNA translation is likely a main contributing factor controlling QKI isoform expression in early OL differentiation. In fact, signaling controlled by the mammalian target of rapamycin (mTOR), which regulates translation initiation (Martin and Blenis 2002), was found to be critical for regulating expression of genes important for differentiation of OPCs, including QKI (Tyler et al. 2011). Consistently, I found that all three QKI mRNAs were similarly upregulated during OPC differentiation, which was not recapitulated by the fold increase in protein levels. Additionally, consistent with a role for QKI-6 in OL differentiation (Larocque et al. 2005), I observed a sharp increase in QKI-6 protein levels suggesting enhanced translation of QKI-6 mRNA. Whether regulation of QKI isoform expression by mRNA translation is sufficient during later

stages of OL differentiation or is accompanied by other factors regulating gene expression will be an interesting question to investigate in future studies.

Unlike OL differentiation, I observed a difference in the relative amount of QKI mRNA isoforms at steady-state between neuronal and OL cell types, which reveals regulation at the mRNA level. The neuronal cell line CAD expressed much lower amounts of total QKI mRNA, as compared to the OL cell lines (Figure 4-2A), reminiscent of the QKI decline upon commitment to the neuronal lineage (Hardy 1998), with QKI-5 as the predominant mRNA isoform (Figure 4-2B). Whereas, cells that are committed to become OLs expressed much higher levels of QKI-6 and QKI-7 compared to that in CAD cells (Figure 4-2B). The steady-state expression of mRNA levels in these neuronal and OL progenitor cell lines is consistent with the level of QKI protein expression during neural cell fate specification (Hardy 1998). Thus, the regulation of mRNA levels potentially underlies QKI isoform expression during neuron-glia cell fate decisions.

Although AS is unlikely regulating the QKI isoform switch during early stages of OPC differentiation (Figure 4-1), I found that the SF FOX2 controls QKI isoform mRNA expression in OLs. Previous studies identified QKI pre-mRNA as a CLIP-seq target of FOX2 in hESCs (Yeo et al. 2009). Here I show that knockdown of FOX2 specifically reduced endogenous QKI-7 mRNA levels in OLs, suggesting FOX2 promotes QKI-7 expression in OLs (Figure 4-3). The location of the putative FOX2 binding sites downstream of exon 7a is consistent with the mode of regulation to promote inclusion of exon 7a (Yeo et al. 2009). Additionally, although QKI was shown to regulate expression of several different SFs, initial analysis of FOX2 levels in the QKI deficient mouse show

no change (Figure 4-4), suggesting FOX2 may act upstream of QKI to regulate AS of QKI pre-mRNA *in vivo*. QKI-7 is implicated in late phase of myelination, and may be important for maintenance of myelin (Pilotte, Larocque and Richard 2001, Wu et al. 2001, Zhang et al. 2003). Interestingly, studies have shown that QKI-7 is specifically reduced in schizophrenia, and of the three isoforms QKI-7 responds better to anti-psychotic drugs than the other QKI isoforms (Aberg et al. 2006, Jiang et al. 2009). However, very little is known about what regulates expression of QKI-7. Whether or not FOX2 plays a role in the abnormal expression of QKI-7 in schizophrenia, or potentially other psychiatric illnesses will be an interesting question to address in future studies. The QKI minigene I constructed (Figure 4-5) will be a useful tool to further address whether and how AS determines QKI isoform expression in different cell types.

The biogenesis of the QKI-6 and QKI-7 cytoplasmic isoforms relies on the usage of a common downstream poly-A site upstream of exon 8 (Figure 1-5). However, biogenesis of nuclear QKI-5 occurs by splicing from exon 6 to exon 7c, followed by an additional constitutive splicing event downstream to exon 8 resulting in the removal of the sequence that contains the proximal poly-A site common for QKI-6 and QKI-7 and usage of the distal poly-A site (Kondo et al. 1999). Differential use of the poly-A sites by APA could determine the biogenesis of the different isoforms, which could be dependent or independent of splicing (Elkon, Ugalde and Agami 2013). The constructed QKI minigene will help to tease apart the regulation of QKI isoform expression by AS from the potential influence of APA (Figure 4-5). Future studies will investigate the influence of APA to regulate QKI isoform expression in various cells types. Additionally, differences in mRNA stability, perhaps mediated by the distinct 3' UTRs, could also

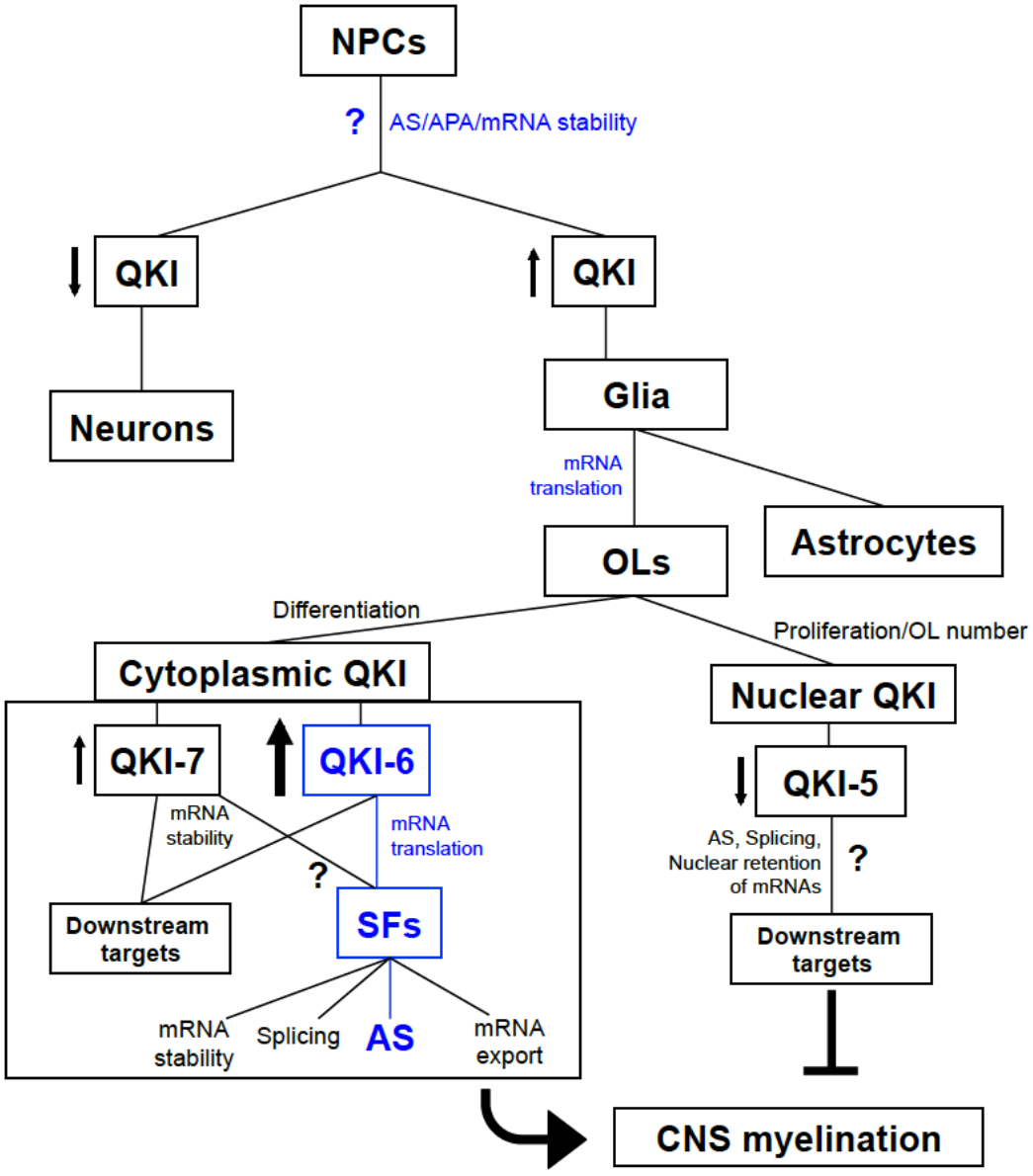
contribute to the regulation of QKI isoform expression. The cytoplasmic isoforms share mostly overlapping 3' UTRs, which are completely distinct from the 3' UTR for QKI-5 (Kondo et al. 1999). Quite possibly, microRNAs or other RNA-binding proteins could target QKI-6 and QKI-7 in a manner distinct from QKI-5 that could differentially regulate mRNA stability and/or translation. In conclusion, the potential coordination between mechanisms that could regulate AS, APA, mRNA stability, as well as mRNA translation to control QKI isoform expression are challenging questions to be addressed by future studies.

Chapter 5: Conclusions and Future Directions

In this dissertation, I identified novel pathways controlling alternative splicing (AS) in oligodendroglia (OLs), the myelinating glia of the central nervous system (CNS) (summarized in Figure 5-1). I provided strong evidence that the selective RNA-binding protein, quaking I (QKI), plays crucial roles in regulating AS in myelinating glia *in vivo*. While the nuclear isoform QKI-5 was previously shown to control AS in other cell types (Hall et al. 2013, Wu et al. 2002), here I unveiled a role for cytoplasmic QKI-6 as a major regulator of AS in OLs, through controlling expression of splicing factors (SFs) (summarized in Figure 5-2). The defects in AS in a mouse deficient in QKI specifically in myelinating glia are largely due to abnormally upregulated expression of SFs including hnRNP F/H, hnRNP M, PTBP1, PTBP2 and PSF. These changes in expression are rescued with OL-specific expression of cytoplasmic QKI-6. The mechanism by which QKI-6 regulates expression of these SFs is to target the mRNAs either directly or indirectly potentially in a microRNA-mediated manner, and consequently inhibit mRNA translation. Finally, I explored the mechanisms that control the expression of QKI isoforms. I present data suggesting that mRNA translation may control QKI isoform expression during differentiation of an OL progenitor cell line. However, QKI mRNA isoform levels display distinct patterns between neuronal and OL progenitor cell lines, suggesting regulation of QKI mRNA steady-state levels during neuron-glia cell fate specification. I identified a role for SF FOX2 in preferentially enhancing expression of the QKI-7 mRNA, likely by regulating AS. Finally, I constructed a minigene for future studies to test the underlying mechanisms by which AS regulates QKI isoform expression in different cell types and during OL differentiation.

Figure 5-1: Diagram of the QKI pathway in neural cells. I propose that mRNA levels, regulated by AS/APA/mRNA stability controls QKI expression upon neuron-glia cell fate specification. QKI expression is increased in glia, and decreased in neurons. During OL differentiation, mRNA translation likely dictates QKI isoform expression. Nuclear isoform QKI-5 is expressed in proliferating OLs, which maintain OL cell number. QKI-5 may also control AS/splicing in the nucleus and can retain mRNAs in the nucleus, but expression goes down during myelin formation. Cytoplasmic QKI expression increases during OL differentiation and myelination. QKI-6 is most abundant in OLs, and was previously shown to regulate mRNA stability and translation. I found QKI-6 regulates expression of SFs, likely by inhibiting mRNA translation, which in turn control alternative splicing, and other mRNA processing events. QKI-7 targets mRNAs to regulate mRNA stability in the cytoplasm, and may also inhibit translation of SFs. A proper balance between the levels of the QKI isoforms is crucial to control OL differentiation and myelin formation. My contributions to the QKI pathway are colored in blue.

Figure 5-1


NPCs
?
AS/APA/mRNA stability
QKI
QKI
Neurons
Glia
mRNA translation
Astrocytes
OLs
Differentiation
Proliferation/OL number
Cytoplasmic QKI
Nuclear QKI
QKI-7
QKI-6
QKI-5
mRNA stability
mRNA translation
AS, Splicing, Nuclear retention of mRNAs
?
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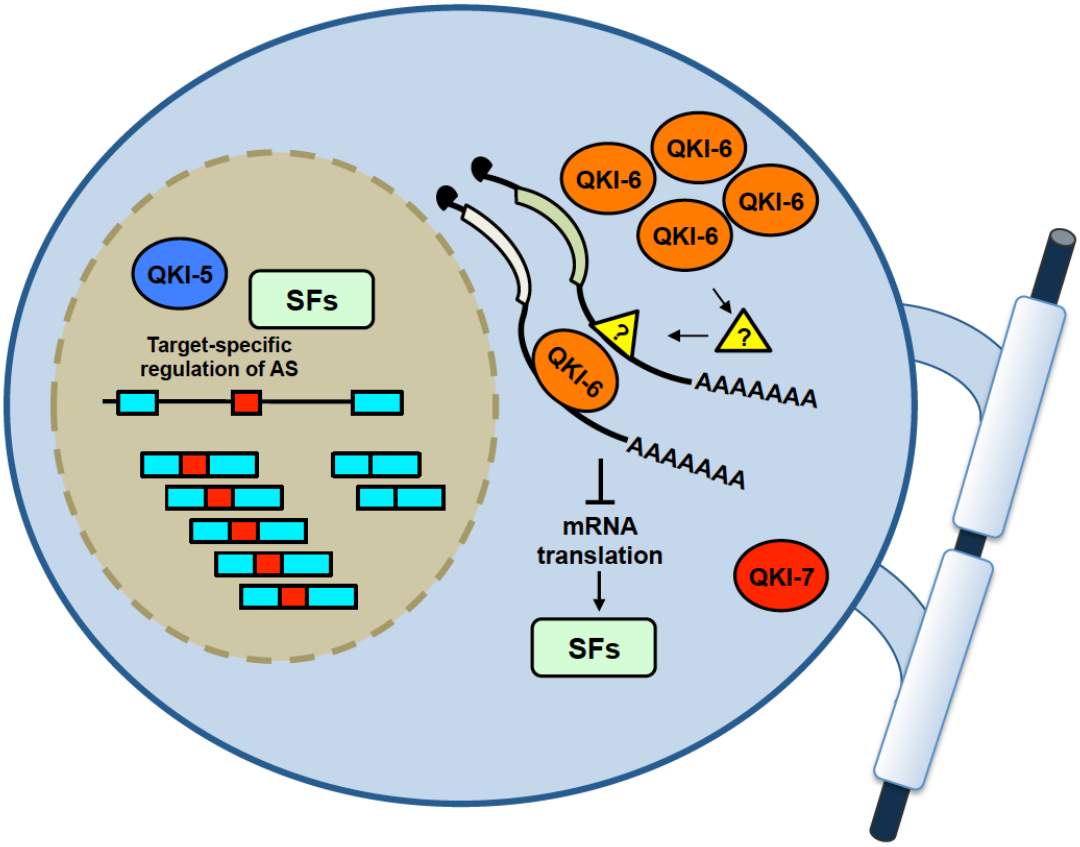
Downstream targets
SFs
Downstream targets
mRNA stability
Splicing
AS
mRNA export
CNS myelination

Figure 5-2: Cytoplasmic QKI-6 controls AS in myelinating glia by suppressing expression of SFs, likely by inhibiting mRNA translation. QKI-6 is abundant in myelinating glia and regulates expression of splicing factors (SF), likely due to inhibition of mRNA translation in the cytoplasm. QKI-6 can directly bind the mRNAs by targeting the 3' UTRs, or indirectly target the mRNAs through a yet unidentified mechanism (indicated by the yellow triangle), potentially by regulating microRNAs. QKI-6 regulation of SF expression allows for normal target-specific regulation of AS in the nucleus (tan circle) in myelinating glia. QKI-5 is predominantly nuclear, and QKI-7 is localized to the cytoplasm. The cell represents an oligodendroglia (OL) myelinating a neuronal axon, separated by a node of Ranvier, which is illustrated by the gap in myelin.

Figure 5-2


QKI-6
QKI-6
QKI-6
QKI-6
QKI-5
SFs
Target-specific
regulation of AS
?
?
QKI-6
AAAAAAA
AAAAAAA
mRNA
translation
QKI-7
SFs

5.1 A novel AS pathway controlled by QKI-6 in myelinating glia

A mouse deficient for all three QKI isoforms specifically in myelinating glia (quakingviable or qk^v/qk^v mutant) displays severe defects in AS of the pre-mRNAs expressed from myelin-specific genes. These defects could be rescued by reintroducing the cytoplasmic QKI-6 transgene expressed specifically in OLs (Zhao et al. 2010, Zhu et al. 2012). Two well-studied examples of myelin-specific genes that display dysregulated AS in the qk^v/qk^v mutant and are rescued by QKI-6 are myelin-associated glycoprotein (MAG) and proteolipid protein (PLP) (Zhao et al. 2010, Zhao et al. 2006, Zhu et al. 2012). An important clue to understand how QKI-6 could regulate AS comes from the fact that SFs hnRNP F/H were previously shown to regulate AS of PLP pre-mRNA in OLs (Mandler, Ku and Feng 2014, Wang, Dimova and Cambi 2007). Here I identified a novel pathway by which QKI-6 regulates expression of SFs hnRNP F/H in myelinating glia, which in turn regulates AS of multiple downstream targets along with PLP (Mandler, Ku and Feng 2014). QKI deficiency resulted in abnormally high levels of hnRNP F/H protein in OLs, which was rescued by OL-specific QKI-6 expression, although mRNA levels were unaffected. Polysome profiling suggested that QKI-6 represses translation of hnRNP F/H mRNAs, which may explain the developmental downregulation of hnRNP F/H expression that occurs concomitantly with QKI-6 upregulation during myelin formation (Hardy et al. 1996, Larocque et al. 2005, Mandler, Ku and Feng 2014, Wang, Dimova and Cambi 2007). The data also suggested that the QKI-6-hnRNP F/H axis is restricted to myelinating glia, since levels of hnRNP F/H were not abnormally elevated in the heart of qk^v/qk^v mutant mice. Whether or not QKI-6

expression in other unidentified cell types could confer a similar regulation of hnRNP F/H will be interesting to investigate in the future.

I provide evidence that QKI-6 binds to hnRNP H mRNA in a QRE-dependent manner. However, QKI-6 did not coimmunoprecipitate with hnRNP F mRNA *in vivo* or in transfected cells, suggesting regulation of hnRNP F expression is not by direct binding. This finding led to the possibility of an independent mechanism by which QKI-6 could regulate hnRNP F expression. QKI was recently shown to regulate miRNAs in glia (Chen et al. 2012, Wang et al. 2013). QKI-6 could control hnRNP F expression by regulating expression of miRNAs that could target the mRNA and inhibit translation. Additionally, QKI-6 might control expression of other RNA-binding proteins that could target hnRNP F mRNA 3' UTR to prevent translation. As a result, the potential QKI-6 regulation could be further expanded to control translation of multiple downstream mRNAs by indirect mechanisms. Future studies will delineate the underlying mechanism by which QKI-6 controls hnRNP F expression in myelinating glia that could potentially be applied to other downstream targets.

We previously showed that QKI-6 regulates expression of the SF hnRNP A1 (Zhao et al. 2010), which could contribute to AS of myelin-associated glycoprotein (MAG). Here, I identified a more prominent mechanism for QKI-6 to control AS of MAG pre-mRNA (Mandler, Ku and Feng 2014). I provide evidence that the SFs hnRNP F/H control AS of MAG pre-mRNA by targeting functionally important G-runs in the introns flanking alternative exon 12. Previous studies have reported on hnRNP F/H coordinating with or antagonizing hnRNP A1 to control AS of downstream targets (Fisette et al. 2010, Talukdar et al. 2011). Thus, I assessed whether or not the SFs could

influence each other to regulate MAG. Deletion of either G-run alone from the MAG minigene was sufficient to reduce exon inclusion, which was further enhanced by deleting both G-runs. However, the manipulation of hnRNP A1 levels in cell culture did not influence G-run mediated regulation of exon inclusion in the MAG pre-mRNA (Mandler, Ku and Feng 2014). Additionally, the G-runs seem to have a stronger effect on MAG exon inclusion, as compared to the hnRNP A1 binding site previously identified (unpublished data from the Feng lab) (Zhao et al. 2010). However, I was unable to assess the strength of a different hnRNP A1 binding site identified by another group, which is overlapping with a splice site (Zearfoss, Johnson and Ryder 2013). Potentially, hnRNP A1 and hnRNP F/H and other unidentified SFs could still coordinate to regulate AS of MAG *in vivo*. Rescuing expression of hnRNP A1 and/or hnRNP F/H to normal levels specifically in OLs of the qk^{v}/qk^{v} mutant mice could determine the sufficiency for either SF to control AS of MAG pre-mRNA or other alternatively spliced targets *in vivo*.

Delineating cell-type specific AS pathways using mouse models is complicated by the difficulties in manipulating gene expression. Yet, the qk^{v}/qk^{v} mutant mouse became a powerful tool that enabled me to understand mechanisms regulating AS *in vivo*, which could be investigated in further detail by using *in vitro* models. However, in this case the use of *in vitro* models proved to be difficult due to the inability to maintain sustained abundant QKI-6 expression in a manner that is representative of QKI-6 levels *in vivo*. Overexpression of QKI-6 in culture did not affect endogenous hnRNP F/H expression to a detectable level, potentially due to the stability and abundance of hnRNP F/H proteins in cell culture. Despite this obstacle, I was still able to detect an effect of QKI-6 overexpression in an OL cell line resulting in reduced luciferase activity from a

reporter containing the hnRNP H 3' UTR. Moreover, I showed an attenuated effect upon deletion of the QRE from the reporter; further suggesting QKI-6 could in fact directly control hnRNP H expression in cell culture, although potentially underestimating what occurs *in vivo*. Other factors upstream of QKI-6 might regulate or cooperate with QKI-6 function during myelin development *in vivo*, which could explain difficulties in the ability to recapitulate QKI-6 function *in vitro*. Determining the factors that modulate QKI-6 function and/or abundance to regulate expression of SFs *in vivo* will be addressed in future investigations.

High-throughput studies using CLIP-seq and RNA-seq previously identified hundreds of alternatively spliced targets of hnRNP F/H, highlighting the nuclear function for these SFs (Katz et al. 2010, Wang et al. 2012, Xiao et al. 2009). Indeed, I detected the abnormal increase in hnRNP F/H protein levels in the nuclear fraction of the qk^{v}/qk^{v} mutant, suggesting the QKI deficiency influences hnRNP F/H function in the nucleus, including regulation of AS. I was able to identify a broader spectrum of AS genes that play crucial roles in the brain, which undergo hnRNP F/H-dependent AS events in OLs and were dysregulated with the QKI deficiency. Some of these targets are implicated in neurodegenerative diseases (NDDs). One example is ATXN2, which is dysregulated in spinocerebellar ataxia and linked to several other NDDs (Ross et al. 2011). Other targets identified are critical for cell function and development, such as UAP1 and MPZL1 (Mio et al. 1998, Zhao and Zhao 2003). AS of MPZL1 pre-mRNA, also known as protein zero-related (PZR), forms the two transmembrane protein isoforms PZR and PZR1b that differentially regulate tyrosine phosphatase SHP-2, which plays important roles in cell differentiation/development (Zhao and Zhao 2003). The dysregulated splice variant

expression of the MPZL1 isoforms may contribute to the defects in the development of myelin observed in the qk^{v}/qk^{v} mutant.

The qk^{v}/qk^{v} mutant allowed the analysis of differences in AS between the myelinating glia of the CNS and PNS. Schwann cells (SWCs) also harbor QKI deficiency and abnormally high hnRNP F/H levels (Mandler, Ku and Feng 2014). Of the analyzed hnRNP F/H targets that were dysregulated in OLs, only MPZL1 was also dysregulated in SWCs. Other targets were unaffected, suggesting QKI-6 regulates hnRNP F/H-dependent AS in a cell-type-specific manner, even between two functionally similar cell types. Potentially, QKI-6 could regulate distinct SFs in OLs and SWCs that could contribute to differences in AS regulation of downstream targets. Additionally, MAG expression is distinct between OLs and SWCs, since L-MAG is expressed in OLs but is almost undetectable in SWCs (Quarles 2007). The molecular differences between OLs and SWCs that determine cell-type specific decisions in regulating expression of splice variants will be an interesting question to investigate in the future.

5.2 QKI-6 as a major regulator of splicing factor expression

We have shown that QKI-6 can regulate expression of splicing factors (SFs) hnRNP A1 and hnRNP F/H in myelinating glia to control AS of myelin-specific genes (Mandler, Ku and Feng 2014, Zhao et al. 2010). However, due to the large number of AS defects observed in the qk^{v}/qk^{v} mutant, I suspected QKI-6 might regulate expression of additional SFs. In Chapter 3, I investigated other potential SFs that could be regulated by QKI-6. The data revealed that several SFs were abnormally expressed in brain stem of the qk^{v}/qk^{v} mutant that were rescued with OL-specific QKI-6 expression.

Two of the SFs, PTBP1 and PTBP2, were regulated in a similar manner to hnRNP A1 and hnRNP H in that steady-state mRNA levels were unaffected, and both mRNAs coimmunoprecipitated with QKI-6 in a UV cross-linking immunoprecipitation assay. Taken together, these results suggest QKI-6 may control expression of SFs through a common mechanism to inhibit mRNA translation. Future studies will delineate how QKI-6 targets PTBP1 and PTBP2 mRNAs to reduce expression, whether directly through the indicated UAAYs identified in the 3' UTRs or via indirect mechanisms. Additionally, several miRNAs target PTBP1 and PTBP2 mRNAs and regulate expression in brain and muscle (Boutz et al. 2007, Makeyev et al. 2007). These miRNAs could be affected by QKI-6. The effect of QKI deficiency and OL-specific QKI-6 rescue was strong enough to allow detection of abnormalities in PTBP1 and PTBP2 protein expression even in the brain stem, which contains a mixture of neural cell types. Interestingly, PTBP2 is a neuronal-specific paralog of PTBP1 (Keppetipola et al. 2012). Whether or not abnormal expression of PTBPs is restricted to OLs, or occurs in other cell types potentially affected by neuron-glia interactions, will be addressed in future investigations. Furthermore, the possibility for QKI-6 to regulate expression of PTBPs in neuronal or OL progenitor cells should be explored, due to the downregulation of PTBP1 and PTBP2 expression that is accompanied by an increase in QKI-6 expression during mouse brain development (Hardy et al. 1996, Larocque et al. 2005, Zheng et al. 2012). Additionally, PTBP1 represses PTBP2 expression (Boutz et al. 2007). Thus, the abnormal increase in expression of both might be explained by independent mechanisms in distinct neural cell types. Finally, expression of both PTBP1 and PTBP2 is tightly regulated during neurogenesis and neuronal differentiation (Keppetipola et al. 2012, Zheng et al. 2012).

QKI deficiency that causes abnormal expression of these SFs could also impact neurogenesis.

QKI and PTB have overlapping downstream targets in muscle (Hall et al. 2013). Interestingly, evidence from this study suggested that QKI-6 expression was upregulated during differentiation of myoblasts, concomitant with a decrease in PTBP1 expression (Hall et al. 2013). Possibly, QKI-6 could be upstream of PTBP1 in muscle (Hall et al. 2013). Another potential mechanism for QKI-6 to regulate AS in muscle is supported by an AS target of hnRNP F/H. MPZL1 was one of the targets in the splicing microarray that had slightly increased exon inclusion during differentiation of myoblasts into myotubes (Hall et al. 2013). Whether and how hnRNP F/H expression is regulated during myoblast differentiation is unknown, which could be addressed in future studies to open doors for potential QKI-6 function in muscle.

I also found that the QKI deficiency upregulates expression of SFs hnRNP M and PTB-associated splicing factor (PSF) but not tar-DNA binding protein (TDP-43) in brain stem. Changes in the steady-state levels of hnRNP M and PSF are rescued by OL-specific QKI-6 expression. Along with hnRNP A1, hnRNP M and PSF were also both recently shown to regulate AS of the transcripts that encode the motor neuron proteins SMN1 and SMN2 (Cho et al. 2014, Cho et al. 2014, Kashima, Rao and Manley 2007). Patients with the motor neuron disease spinal muscular atrophy (SMA) have a deletion or mutation in the *SMN1* gene. Due to splicing of exon 7 from SMN2 pre-mRNA, SMN2 fails to rescue the deficiency of SMN1 in these patients (Cho et al. 2014, Cho et al. 2014). The overlapping roles of hnRNP A1, hnRNP M and PSF begs the question of which other downstream targets are affected that could be dysregulated in disease. Whether or not

QKI-6 controls the overlapping hnRNP A1, PSF and/or hnRNP M-dependent AS events should be addressed in the future.

PSF is dysregulated in the neurodegenerative disease (NDD) frontotemporal lobar degeneration (FTLD) (Seyfried et al. 2012). FTLD is characterized by insoluble cytoplasmic protein aggregates, which are enriched in full length and cleaved forms of PSF (Seyfried et al. 2012). I observed elevated expression of both full length and a cleaved form of PSF, which was inversely proportional to the amount of a smaller cleavage product, using a C-terminal antibody. Such abnormalities were rescued by OL-specific expression of QKI-6. The full length PSF has an NLS that determines its nuclear localization, which is predicted to be lost in the cleavage product (Seyfried et al. 2012). I showed that in a human OL cell line deletion of the NLS led to cytoplasmic localization of PSF and markedly enhanced formation of cytoplasmic aggregates. The abnormal expression of PSF in the QKI deficient mouse suggests a potential role for QKI in regulating cleavage of PSF in addition to controlling PSF expression. The underlying mechanism by which QKI regulates expression and cleavage of PSF will be analyzed in future studies.

Despite evidence from some of the SF targets suggesting the role for QKI-6 in targeting the mRNA directly or indirectly and inhibiting translation, the underlying mechanism by which QKI-6 is regulating expression of these SFs still remains elusive. A potential explanation as to how QKI-6 could regulate expression of SFs without affecting steady-state mRNA levels could be by the sequestration of the mRNAs directly or indirectly from translation machinery to prevent translation initiation. This hypothesis is supported by evidence that in primary rat OLs cytoplasmic QKI-6 and QKI-7 colocalize

with stress granules, which can store translationally dormant mRNAs (Wang et al. 2010). Another explanation could be a potential novel role for QKI-6 in affecting protein stability. Whether or not the mechanism by which QKI-6 controls expression of SFs is common to all of its downstream targets, and how QKI-6 performs such a function are crucial questions to be addressed in future studies to precisely define QKI-6 function.

An intriguing future experiment is to integrate high-throughput proteomic analysis as well as RNA-seq analysis to reveal a genome wide map for dysregulation of SFs and AS events in the QKI deficient mouse, as compared to non-phenotypic heterozygote control littermates, that are rescued by QKI-6. Additionally, whether and how the targets are regulated, by direct or indirect mechanisms, can be determined by high-throughput CLIP-seq assays using the mice that express OL-specific FLAG-tagged QKI-6 transgene. The potential for QKI-6 to control expression of miRNAs can also be analyzed in the future using miRNA-seq, which can be further validated *in vitro* to define the mechanism by which QKI-6 controls expression of certain SFs, such as hnRNP F. Additionally, determining whether QKI-6 mediates a multitude of RNA processing events beyond AS that might be regulated by the affected SFs would further increase the global significance of RNA biochemistry dictated by QKI-6 in myelinating glia. For example, hnRNP A1 regulates transcription, splicing, export and stability of mRNAs (Jean-Philippe, Paz and Caputi 2013). Additionally, hnRNP A1 regulates processing and expression of miRNAs (Michlewski and Caceres 2010). Perhaps, QKI could regulate miRNA expression directly and indirectly in an hnRNP A1-dependent manner (Wang et al. 2013). Finally, how QKI-mediated pathways differ to distinctly regulate AS between myelinating glia and other cell types will be an intriguing question to address in future

investigations. These studies may reveal overlapping or distinct targets downstream of the SFs, which could open doors to determine the interplay between SFs that might coordinate or antagonize different splicing events.

5.3 Potential mechanisms controlling QKI isoform expression

The regulation of QKI isoform expression is crucial for the normal function and development of myelin (Larocque et al. 2005, Zhao et al. 2006). Additionally, the QKI isoforms are differentially expressed in different neural cell types (Hardy 1998, Lu et al. 2003). However, the underlying molecular mechanisms that regulate QKI isoform expression are unknown. In this dissertation, I discovered that although the biogenesis of QKI mRNAs is a crucial initial step for QKI protein expression that the mRNA levels do not recapitulate the protein levels during differentiation of OL progenitor cells in culture. The data revealed that regulation of translation likely dictates QKI isoform expression in early stages of OL differentiation. These results are consistent with recent findings that mTOR signaling differentially regulates QKI expression in early OLs (Tyler et al. 2011). Whether or not mRNA translation is a major mechanism that regulates QKI isoform expression during later stages of OL differentiation and myelin formation, or if other additional regulatory mechanisms are at play, will be a challenge for future studies.

I showed that in a neuronal progenitor cell line compared to two OL progenitor cell lines, QKI mRNA expression patterns were distinct, suggesting differential regulation to determine the steady-state levels of QKI mRNAs during neuron-glia cell fate specification. Additionally, although mRNA translation might determine QKI isoform expression in early OPCs, the mRNAs first must be produced, which is likely

regulated even during OL differentiation. Thus, I investigated potential mechanisms controlling the biogenesis of the mRNAs, which depends on AS and alternative poly-adenylation (APA) site selection, due to the usage of two poly-A sites, that generates QKI isoform mRNAs. I found that the SF FOX2 specifically regulates expression of QKI-7 in an OL cell line. Whether FOX2 directly binds to QKI pre-mRNA in OLs, and whether it promotes inclusion of exon 7a or blocks splicing to exon 7b to promote QKI-7 expression, remains unclear. The underlying mechanism by which FOX2 regulates QKI isoform expression will be investigated with future experiments.

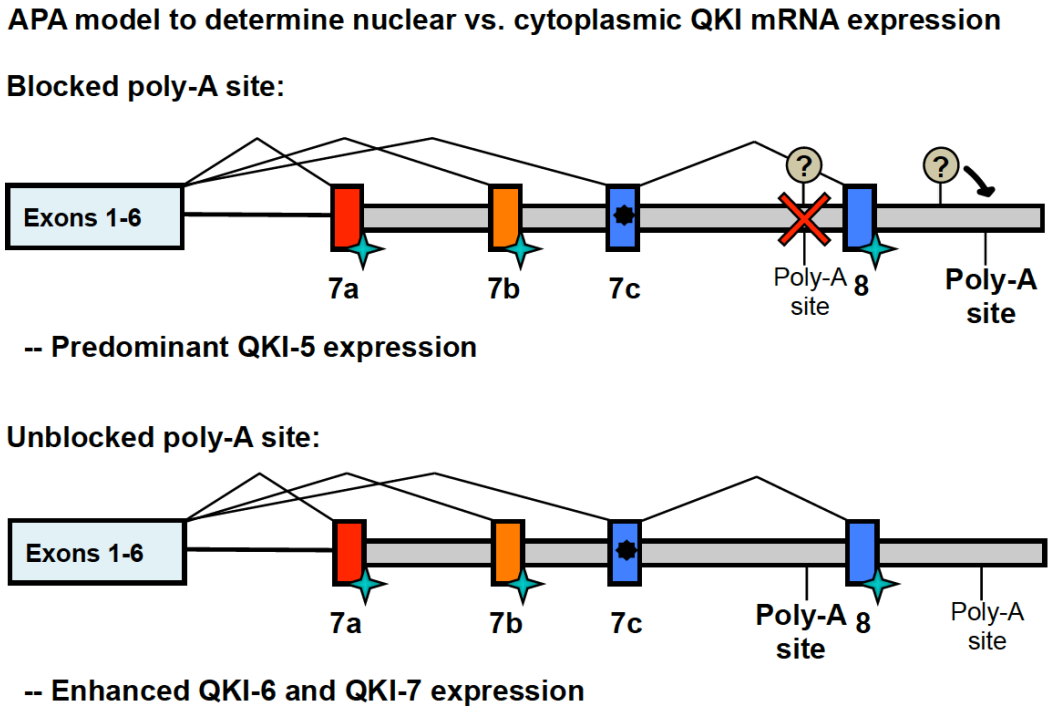
APA could play a role in determining the biogenesis of the different isoforms during OL differentiation, which could be dependent or independent of splicing (Elkon, Ugalde and Agami 2013). Interestingly, QKI-6 and QKI-7 mRNAs use the first poly-A site, which is found in an intron that is spliced out when QKI-5 is formed. A component of the spliceosome, U1 snRNP has recently been shown to control poly-A site selection by blocking usage of pre-mature cryptic poly-A sites (Berg et al. 2012, Elkon, Ugalde and Agami 2013, Kaida et al. 2010). Interestingly, the rapid increase in transcription upon neuronal activation is not matched by increasing U1 levels, thus resulting in a shortage of U1 (Berg et al. 2012). Potentially a similar U1 shortage might occur during OL differentiation that could allow usage of the early poly-A sites, such as in QKI, and in turn could enhance QKI-6/7 expression. Other potential RNA-binding proteins or non-coding RNAs could target either of these sites to promote or block poly-A site selection and regulate QKI isoform expression. For QKI-5 mRNA biogenesis, the allowed usage of the first poly-A site would result in a premature cleavage event that would affect the necessary splicing event between exon 7c and 8 and likely block QKI-5 production,

potentially contributing to the reciprocal reduction in QKI-5 mRNA with increased QKI-6/7 mRNA observed in mouse brain development (Hardy et al. 1996). A model by which APA could regulate QKI isoform expression is illustrated in Figure 5-3.

AS and/or APA could be at play to control QKI mRNA expression, which might depend on the specific factors expressed in different cell types or during stages of OL differentiation. Minigenes can be used to tease apart the two mechanisms. The QKI minigene presented in Chapter 4 will be used to determine AS site selection, and whether or not expression of the alternatively spliced minigene-derived isoforms mimics expression of endogenous isoforms. The minigene can also be used to assess regulation of AS by different SFs, such as FOX2. In the future, inclusion of the two poly-A sites downstream of the alternative exons in the minigene could be constructed to determine the interplay between AS and APA to in turn control QKI isoform expression. Additionally, a construct just containing the sequences critical for APA could be made to specifically assess the regulation of APA independent of splicing. By comparing these QKI 3' end minigene constructs one could address whether APA and/or AS determine QKI isoform expression, which can then further be used to identify underlying regulatory factors involved.

Figure 5-3: Model for APA to determine QKI isoform expression. The pre-mRNA diagram is illustrated to show common exons 1-6 (light grey), the connecting intron and downstream exons 7a (red), 7b (orange), 7c and 8 (blue), to form QKI-7, QKI-6 and QKI-5, respectively. The grey rectangle refers to the sequence that either becomes 3' UTR upon splicing to the upstream respective exon, or intron that is spliced out. The blue diamonds denote stop codons and a nuclear localization signal is represented by the black star. The two poly-A sites are demarcated; the first poly-A site is utilized by QKI-6 and QKI-7, whereas the poly-A site downstream of exon 8 is utilized by QKI-5. The model illustrates how APA could regulate biogenesis of QKI mRNAs independent of alternative splicing. Trans-acting factors could block selection of the first poly-A site, and/or promote selection of the downstream poly-A site (bold) resulting in predominant levels QKI-5 mRNA. If the first poly-A site were unblocked there would likely be a relative enhancement of QKI-6 and QKI-7 mRNAs.

Figure 5-3


APA model to determine nuclear vs. cytoplasmic QKI mRNA expression
Blocked poly-A site:
Exons 1-6
7a
7b
7c
?
Poly-A site
8
?
Poly-A site
-- Predominant QKI-5 expression
Unblocked poly-A site:
Exons 1-6
7a
7b
7c
Poly-A site
8
Poly-A site
-- Enhanced QKI-6 and QKI-7 expression

Despite a unique portion in the QKI-7 3' UTR upstream of exon 7b, QKI-6 and QKI-7 mRNAs share an overlapping 3' UTR, which is completely distinct from the 3' UTR of QKI-5 mRNA. The 3' UTRs, which are often decorated with RNA-binding proteins and/or regulatory RNAs, could potentially determine nuclear vs. cytoplasmic isoform expression by differentially controlling mRNA stability and/or translation. Whether or not distinct regulatory factors that target the 3' UTRs could also contribute to determining the expression of the QKI isoforms between different cell types or during OL differentiation is unknown.

How AS and/or APA underlie the expression of the QKI isoforms and/or the influence of mRNA stability and translation are intriguing questions to be answered. Future studies will tease apart the different underlying mechanisms. Additionally, whether and how a combination of multiple potential regulatory steps determines QKI isoform expression will be addressed by future investigations. Finally, whether or not different cell types employ distinct mechanisms to regulate QKI isoform expression, and/or if different stages of OL differentiation rely on a complex array of regulatory factors, will be the challenges of upcoming studies.

5.4 Emerging studies on the mechanisms regulating AS of pre-mRNA

Accumulating evidence within the last decade has highlighted the influence of transcription on the regulation of AS, identifying mechanisms by which AS can be regulated in a co-transcriptional manner (Braunschweig et al. 2013). Early studies investigating the pre-mRNA processing from one of the largest human genes, dystrophin, which has 79 exons, led to strong initial evidence that alternative splicing occurs co-

transcriptionally (Tennyson, Klamut and Worton 1995). The C-terminal domain (CTD) of RNA polymerase II (RNAPII) can carry SFs involved in splicing regulation (Hsin and Manley 2012). Additionally, hyperphosphorylation of the CTD can affect RNAPII rate (Braunschweig et al. 2013, Munoz et al. 2009). A kinetic model was proposed to explain how the rate of transcription by RNAPII could co-transcriptionally determine the positioning of the spliceosome on a nascent pre-mRNA (Dujardin et al. 2013). A slow RNAPII rate was thought to allow inclusion of exons that have weak splice sites that are missed when transcription is fast. In contrast, a recent paper identified that slow RNAPII rate can allow recruitment of SFs to inhibit exon inclusion, such as ETR-3 suppression of alternative exon 9 inclusion in CFTR pre-mRNA (Dujardin et al. 2014). In fact, weakening of the downstream 5' splice site led to reduced exon inclusion and increased binding by the ETR-3, but a stronger splice site promoted exon inclusion (Dujardin et al. 2014). If downregulation of splice site usage requires inhibition by a SF, it also likely needs time for interaction between a SF and its target site (Dujardin et al. 2014). When RNAPII transcription rate is fast, the ability for the SF to inhibit splicing is attenuated, and the exon is included (Dujardin et al. 2014). Thus, even the kinetics of RNAPII regulating AS seems to be largely target-specific due to differences in splice site strength and the availability/interaction with different SFs, potentially ones regulated by QKI-6.

Recent studies have found increasing evidence of a role for epigenetics in regulation of AS, which could affect RNAPII pausing or rate of transcription (Luco et al. 2011). Chromatin modifications can influence inclusion or exclusion of an exon from an emerging pre-mRNA (Zhou et al. 2014). Additionally, many SFs have previously been shown to be downregulated during cell differentiation and development (Wang, Dimova

and Cambi 2007, Zheng et al. 2012). How cells maintain their regulatory pathways to ensure correct temporal protein expression is unclear. There seems to be an intragenic chromatin signature, such as hypermethylated regions near intron/exon boundaries that are thought to have been evolutionarily evolved (Shukla and Oberdoerffer 2012). Binding of the chromatin modifier CTCF to cause pausing of RNAPII and promote exon inclusion is blocked by DNA methylation (Shukla et al. 2011). Additionally, recent studies identified interactions between chromatin remodeling proteins and SFs, such as histone deacetylase 1 and 2 and SRSF1 (Khan et al. 2014). Interestingly, although AS events can change with cell differentiation, many splicing events are maintained, even in parallel with reduced expression of a regulatory factor. Whether or not SFs can deposit a signature in early stages of cell differentiation by interacting with methyltransferases or chromatin remodelers to methylate DNA at exons, which can in turn maintain regulation of AS events in a differentiated cell, is an intriguing possibility to be addressed in future studies. How widespread these phenomena are, and whether or not the epigenome plays a critical role in regulating AS in a differentiation-dependent or cell-type specific manner will be the focus of many future investigations. Additionally, how these newly identified mechanisms that regulate AS might be employed in brain cell types, such as OLs, should also be explored.

The role for epigenetics and transcription affecting expression of QKI isoforms is largely unknown. During OL differentiation and myelin formation there is an overall increase in QKI mRNA levels (Hardy et al. 1996), potentially due to an increase in transcription (unpublished data from the Feng lab). However, QKI-6 becomes the most abundant isoform (Lu et al. 2003, Zhao et al. 2006). Additionally, in the qk^{v}/qk^{v} mutant

the mutation is a deletion of the promoter region, which should theoretically affect transcription of all isoforms similarly (Lu et al. 2003). However, there is actually a preferential reduction of QKI-6 in the qk^{v}/qk^{v} mutant (Zhao et al. 2006). Potentially, transcription of the *qkI* gene is affected by local epigenetic marks at the alternative exons, such as methylation, which could cause RNAPII pausing or regulate transcription rate to control splice site selection and result in differential QKI isoform expression. Furthermore, QKI-6 might regulate its own expression by a feedback loop mechanism. For example, certain SFs may access the QKI pre-mRNA during slow RNAPII transcription that could reduce splicing to exon 7b, and thus result in low QKI-6 expression. During OL differentiation, an increased transcription rate along with the suppression of these inhibitory SFs potentially by QKI-6 could further allow enhanced QKI-6 expression. The role for epigenetics and/or the influence of SFs on RNAPII transcription of the *qkI* gene to in turn affect QKI isoform expression will be addressed in future studies.

5.5 Future directions and implications for human health and disease

Splicing is crucial for proper gene expression and protein production in eukaryotes (Braunschweig et al. 2013). AS is critical for the diverse development and function of cells in higher organisms (Kelemen et al. 2013). QKI-6 is upstream of the SFs that play important roles in regulating AS during early neuronal development, PTBP1 and PTBP2 (Keppetipola et al. 2012, Zheng et al. 2012). Dysregulation of AS is observed in multiple human diseases discussed in this dissertation, including neurodegenerative diseases (NDDs), schizophrenia (SCZ), and cancer (Han, Li and Zhang 2013, Mills and

Janitz 2012, Morikawa and Manabe 2010, Qian and Liu 2014, Schirer et al. 2014). In particular, OLs that produce myelin in the CNS are extensively regulated by AS (Mandler, Ku and Feng 2014, Wang et al. 2012, Wu et al. 2002, Zhao et al. 2010). Myelin deficiency and dysfunction is strongly implicated in neurodegeneration and psychiatric disorders (Bankston, Mandler and Feng 2013). I identified a potential role for QKI in the pathogenesis of NDDs. In addition to PSF dysfunction in FTLD (Seyfried et al. 2012), QKI-6 regulates expression of other SFs that are linked to NDDs, such as hnRNP H that binds to hexanucleotide repeats and hnRNP F that can bind G-quadruplexes observed in ALS/FTD (Haeusler et al. 2014, Lee et al. 2013).

Dysregulation of QKI expression is implicated in psychiatric diseases, including SCZ, bipolar disease and major depression (Aberg et al. 2006, Haroutunian et al. 2006, Klempan et al. 2009, Lauriat et al. 2008). Expression of QKI isoforms differentially responded to anti-psychotic treatments, in which QKI-7 was most abundantly upregulated (Jiang et al. 2009). Thus, defining the different mechanisms that dictate QKI isoform expression will be crucial to understanding how anti-psychotic treatment could be more effectively utilized. Additionally, many of the AS events that are controlled by QKI-6, which ensure proper myelin formation and maintenance during mouse brain development, are implicated in psychiatric illness (Sokolov 2007). Novel treatments could be identified to target specific pathways that could control QKI isoform expression, and AS, and prevent a potential dysfunction in myelin observed in these psychiatric disorders.

QKI is deficient in several types of cancer, which are marked by increased levels of SFs (Han, Li and Zhang 2013, Zhao et al. 2014, Zong et al. 2014). Dysfunction of QKI

leading to aberrant AS in cancer has already been proposed (Zong et al. 2014). Thus, a specific role for QKI as a tumor suppressor is coming under serious consideration (Zhao et al. 2014). However, the underlying mechanisms are still being investigated. Most of the SFs regulated by QKI-6 identified in this dissertation such as hnRNP F/H, hnRNP M and PSF are implicated in cancer (Chen et al. 2014, Mandler, Ku and Feng 2014, Tsukahara, Haniu and Matsuda 2013, Wang et al. 2012). In fact, hnRNP H is upregulated in glioma and regulates AS of MADD, which is dysregulated in glioblastoma multiforme (GBM) (Lefave et al. 2011). Furthermore, QKI has recently been implicated in regulating miRNAs in GBM (Chen et al. 2012, Wang et al. 2013). The inability to detect binding of QKI-6 to hnRNP F mRNA opened up the potential idea for indirect regulation of hnRNP F expression in a miRNA-mediated manner. However, whether or not hnRNP F is upregulated in glioma remains unknown. The dysregulation of the QKI-6 to hnRNP F/H pathway could contribute to tumorigenesis in glioma. Futures studies will likely investigate the therapeutic potential of QKI in cancer due to its function as a tumor suppressor (Chen et al. 2012).

As discussed above, QKI deficiency and/or dysregulation contribute to and potentially dictate abnormal posttranscriptional cascades linked to multiple diseases. High-throughput analysis *in vivo* along with molecular validation will reveal the extent of QKI-mediated pathways to unveil the underlying mechanisms controlling mRNA processing in normal brain function and development, which will ultimately help to understand pathogenesis of various human diseases.

Chapter 6: Material and Methods

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6.1 Chapter 2

Animal colonies and treatment All animals were treated under the rules and regulations held by the National Institutes of Health and Emory University Institutional Animal Care and Use Committee. The qk^v mouse colony was originally purchased from Jackson Laboratory and maintained on campus. The wt/qk^v, qk^v/qk^v and PLP-FLAG-QKI-6 transgenic mice were generated and identified by PCR-genotyping (Zhao et al. 2006). After euthanization, brain stems, optic nerves (OPNs) and sciatic nerves (SCNs) were dissected, and processed for protein or RNA extraction.

Plasmid constructs The FLAG-tagged QKI-6 cDNA expression construct was previously described (Chen et al. 2007). hnRNP H 3′ untranslated region (3′UTR) reporter was made by PCR amplification of mouse genomic DNA using primers with engineered XbaI and XhoI restriction sites for cloning downstream of the firefly luciferase coding sequence. Mutagenesis to delete the entire bipartite sequence of the putative QKI-response element (QRE) in the hnRNP H reporter, and the G-runs in the MAG minigene (Zhao et al. 2010) were performed using the QuikChange lightening mutagenesis kit (Agilent Technologies). All primers used for cloning and mutagenesis are listed in the table below.

Primer	Sequence
Actin	F:TGTTACCAACTGGGACGACA
	R:GGGGTGTTGAAGGTCTCAAA
ATXN2	F:GTTCAGCACCATCAGCACCAG
	R:GGAGGGGATTTGGCCTTTCG
DSCR3	F:CGTGCTGAAGCCAGGAAAGATT
	R:AGCTCTCTCTTTGACGTTCTGC
FGFR2	F:CCTCCACGGTGGTCGGAG
	R:GGGGGATGCGCTTGGTCAG
HGS	F:GTCAGGGGGACACACAAGCA
	R:GGGCTCTTTCAGCAGCGAAC

hnRNP F 3'UTR M/R	**F:GAGTTAAGTAGTTTAAGTATGTTGAGTG**
	R:GTCAAGGCCTCACCAATTCCTAC
hnRNP F qPCR M/R	**F:GACCACAGATATGGAGACAGCG**
	R:GTAGCAAACTCAACATCAGCTTCTC
hnRNP H 3'UTR/qPCR M/R	**F:ATTGCATAGGTAGCCAAGGAGCA**
	R:GGCATAAAACCACAAGGCTGGAT
hnRNP F 3'UTR H	**F:TTGAGAATTGAGACACAATACTAATAC**
	R:AGTAATCTGTGCCAGTCATTGTG
hnRNP H 3'UTR H	**F:TCATCTAGGATGTAACAGTGAAGC**
	R:TGTTCAATTTAACGTGGCAAAGGG
hnRNP H 3'UTR Cloning primers	**F:GGATCTCTTTACAGGTAGCCAAG**
	R:ACATCAAGTTCACAGGACACTAA
hnRNP H 3'UTR nested cloning primers	**F:CGCGCTCGAGGTAATGGAAGCCGAGCATCT**
	R:CGCGTCTAGACAAGGTTCACTTAGCGCAATAC
hnRNP H putative QRE mutagenesis primers	**F:gagcaaagctgagtgaactgtcctagcttttctctagtta**
	R:taactagagaaaagctaggacagttcactcagctttgctc
hnRNP H reporter	**F:AAGAGATCGTGGATTACGTCGCCA**
	R:GGCATAAAACCACAAGGCTGGAT
MADD	**F:TTGCTAGCAGCCTGTATCGGAA**
	R:GGTTCTCGCTAGAGTTGCTCG
MAG endogenous specific	**F:GTGCTGTGGTCGCCTTTGCC**
	R:CCCTCACCCCTACTACTCTC
MAGmini specific	**F:GGATCACTCTCGGCATGGAC**
	R:GTCTGAGTGGGAATAACTGAGG
MAGmini G-run site #1 mutagenesis primers	**F:aggatggcaaggttgctaaacaaaagggaggcc**
	R:ggcctccctttgtttagcaaccttgccatcct
MAGmini G-run site #2 mutagenesis primers	**F:ccctattgtgtgtccaaatgctatcaggagtagagagc**
	R:gctctctactcctgatagcatttggacacacaataggg
MAST1	**F:CCACTTAGGTAGCAGTCCTTTG**
	R:CCTTTGGGAACCGCTCCTTG
MBNL2	**F:AGGACTGATAAACTGGAGGTATG**
	R:TCTTTGGTAAGGGATGAAGAGCA
MPZL1	**F:CTCCACGTGGTGGAAATAGACAA**
	R:GAGTCCTCAAGGCCACATGC
NF2	**F:GAGGAGCCCATGAACCCAATTC**
	R:GGAGCAGCAGATGTGGCAGG
UAP1	**F:GCAAAGAAGTTTGTGGTGTATG**
	R:ATAACCTTCAAGGCCCTCTC
SIRT2	**F:CGGGACAGAGCAGTCGGTGA**
	R:GGCAGATGGTTGGCTTGAACTG
TARBP2	**F:GCGTTTGCCAGAGTACATGGT**
	R:CAGTTCCTCAATATCCAGATAGC
TRMU	**F:AGGGTGACGTGCTTGTGGCT**
	R:CTTACCCTTCTGGAGTGTGTAG
TPM1	**F:GGCGGAAGACCGGAGCA**
	R:CATGCCTCTCTCACTCTCATCT
Zasp/LBD3	**F:ATGACCGGGACAGAATACATGC**
	R:GCAGGCACTGGCTGGTAGA

Cell cultures and transfections The HOG, CG4 and CAD cell lines were propagated as previously described (Post and Dawson 1992, Qi et al. 1997, Zhao et al. 2006). Transfections were performed using Lipofectamine 2000 (Invitrogen) with DNA constructs and/or siRNAs following manufacturer's instructions. The FLAG-QKI-6 construct or pcDNA control plasmid (2 μg) was transfected into HOG cells on two sequential days, with 0.5 μg of reporter plasmids co-transfected on the first day. For the luciferase assay, all conditions were co-transfected with the Renilla plasmid. Firefly and Renilla luciferase activities were measured with a luminometer using dual luciferase assay reagents (Promega). Knockdown of hnRNP F/H was achieved by transfection with 100 pmol of a published siRNA (Wang, Dimova and Cambi 2007)or a Silencer Negative Control #1 (Invitrogen) on two consecutive days. Same transfection conditions were performed for knockdown of hnRNP A1 using 50 pmol hnRNP A1 siRNA (Santa Cruz) (Lopez-Manriquez et al. 2013). MAG minigene (1 μg) was co-transfected with siRNA on the second round of treatment. Cells were harvested 48 h after the first transfection for RNA or protein analysis.

Protein/RNA preparation and quantification assays Whole cell lysates were prepared by sonication in 1× Laemlli buffer (Li et al. 2000) for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot analysis with the following antibodies: anti-hnRNP F/H (1:4000; Abcam), anti-hnRNP A1 (1:5000; Sigma), anti-β-actin (1:10 000; Sigma), anti-Flag (1:2000; Sigma), anti-eIF5α (1:10 000; Santa Cruz). The pixel density of protein bands on X-ray films was quantified using Image J software. RNA was isolated by Trizol extraction (Invitrogen). Samples were DNase treated with

the Quantitect kit (Qiagen) prior to reverse transcription (RT) using random hexamer primers (Promega) and SSII reverse transcriptase (Invitrogen), or using the Quantitect kit. Semi-quantitative RT-PCR was performed using primers that detect the alternative splice variants simultaneously. After electrophoresis, the RT-PCR products of AS isoforms were quantified with ImageJ. The intensity of ethidium bromide staining of the PCR products that contain either exon included or skipped was quantified. The % inclusion of the alternatively spliced exon in each sample was calculated. Results in qk^{v}/qk^{v} mutant or siRNA knockdown samples were statistically compared with the corresponding control in each experiment and graphically displayed. Real-time RT-PCR (qRT-PCR) was performed using DyNaMo Sybr Green qPCR kit (Thermo-Scientific), and quantified by ΔΔCt. Primers are listed in Supplementary Table S2, or were previously published (Boutz et al. 2007, Paul et al. 2011, Wang et al. 2012, Zhao et al. 2010).

UV-crosslinking immunoprecipitation Brain stems derived from mice expressing the FLAG-QKI-6 transgene specifically in OLs were dissected, minced and immediately UV cross-linked at 400 mJ in a Stratalinker 1800 (Agilent Technologies). In addition, HOG cells transfected with FLAG-QKI-6 or pcDNA control, together with reporter constructs, were also subjected to UV cross-link under the same conditions. Cells or homogenized tissues were lysed on ice in a buffer containing 20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.5% Triton X, a cocktail of protease inhibitors including pepstatin, leupeptin, aprotinin, phenylmethanesulfonyl fluoride and RNase inhibitor. Lysates were centrifuged at 800 × g for 5 min at 4°C to remove nuclei. SDS was added to supernatants to a final concentration of 0.01% or 0.5% as indicated in the corresponding

figure legends and centrifuged at 16 000 × g for 15 min at 4°C. Four percent of total supernatant was kept for detecting RNA and protein inputs, respectively. Samples were pre-cleared for 30 min at 4°C with sepharose 4B beads (Sigma) that were pre-balanced in the IP buffer. Twenty microliters of pre-balanced anti-Flag-M2 beads (Sigma) were added to the supernatants and incubated overnight at 4°C. Centrifugation was performed at 100 × g at 4°C to isolate the immunoprecipitated complexes on the beads, which were washed three times in a buffer containing 20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl2, protease inhibitors and 1% Triton X. The beads were then resuspended in a buffer containing 5 mM Tris pH 7.5, 0.32 M sucrose and 2% SDS. Seventy-five percent of the immunoprecipitates on beads were taken out and treated with proteinase K for 20 min. After centrifugation at 8000 × g, the RNA was isolated from the supernatant by phenol/chloroform extraction, and subjected to RT-PCR to detect FLAG-QKI-6 associated RNA. For experiments using reporter plasmids, samples were DNase treated before RT, with an independent replicate that was not reverse transcribed, to assess complete removal of plasmid DNA contamination. The remaining 25% of the immunoprecipitates were incubated at 65°C for 3 min in 1× Laemlli buffer, centrifuged, and the supernatant was subjected to SDS-PAGE and immunoblot.

Statistical analysis Statistical analyses were performed using Student's t-tests to compare two sample sets, one-way ANOVA and Tukey's post-test when comparing three or more sample sets, or two-way ANOVA and Bonferroni's post-test when comparing three or more sets of paired conditions. Standard errors are depicted in all bar graphs. P-value < 0.05 was considered statistically significant and is indicated by *.

6.2 Chapter 3

Animal colonies and treatment All animals were treated under the rules and regulations held by the National Institutes of Health and Emory University Institutional Animal Care and Use Committee. The qk^v mouse colony was originally purchased from Jackson Laboratory and maintained on campus. The wt/qk^v, qk^v/qk^v and PLP-FLAG-QKI-6 transgenic mice were generated and identified by PCR-genotyping (Zhao et al. 2006). After euthanization, brain stems, optic nerves (OPNs) and sciatic nerves (SCNs) were dissected, and processed for protein or RNA extraction.

Fractionations Linear-sucrose gradient fractionation was performed from brain stem homogenate as previously described (Lu et al. 2004, Zhao et al. 2010). RNA was isolated by phenol chloroform extraction, and subjected to qRT-PCR using previously published primer for hnRNP F and hnRNP H mouse mRNAs (Mandler, Ku and Feng 2014). The percent distribution of mRNA levels from each fraction was calculated using the formula $2^{(0-n)}$, where n = Ct-value. The values for each fraction were added together, and the value from each fraction was divided by total to obtain the percent of a particular mRNA in that fraction from the total fractionation experiment. Nuclear fractionation was performed on brain stem as previously described (Li et al. 2000). Fractionated lysates were then subject to immunblot with the indicated antibodies.

Protein/mRNA preparation and quantification assays Whole cell lysates were prepared by sonication of cell pellets or tissues in 1xLaemlli buffer (Li et al. 2000), separated on a 12.5% (wt/vol) SDS-PAGE, transferred to PVDF membrane, and

immunoblotted with primary antibodies against specific proteins, followed by corresponding HRP-conjugated mouse or rabbit secondary antibodies. The immunoblots were exposed to film using enhanced chemiluminescence (ECL) reagent. The protein bands on the films were quantified using ImageJ software to measure pixel density. Anti-hnRNP F/H (1:4000; Abcam), anti-PTBP1 (1:2000; Invitrogen), anti-PTBP2 (1:5000; Novus Biologicals), anti-PSF (Seyfried et al. 2012) (1:2000; Abcam), anti-Parp (1:1000; BD Transduction laboratories), anti-hnRNP M (1:1000; Santa Cruz), anti-TDP-43 (Seyfried et al. 2012) (1:3000; ProteinTech), anti-β-actin (1:10 000; Sigma) and anti-eIF5α (1:10 000; Santa Cruz).

Total RNA was isolated by Trizol extraction following the manufacturer's protocol (Invitrogen). RT of samples was performed using random primers (Promega), SSII reverse transcriptase (Invitrogen), RNase inhibitor (Invitrogen), DTT, and dNTPs. Regular and semi-quantitative PCRs were performed using Taq polymerase and buffer (Invitrogen), dNTPs, MgCl2, and primers. Real-time PCR (qRT-PCR) was performed using DyNaMo Sybr Green qPCR kit (Thermo-Scientific), and quantified by ΔΔCt. The sequences of all primers used are listed in the table below.

Primer	Sequence
Mouse PTBP1 qPCR	**F:TCTACCCAGTGACCCTGGAC**
	R:GAGCTTGGAGAAGTCGATGC
Mouse PTBP2 qPCR	**F:ACCAGGCATTTTTGGAACTG**
	R:TGTGGTGCCACTAAGAGGTG
Mouse Gapdh qPCR	**F: GGTGAAGGTCGGTGTGAAC**
	R: CCTTGACTGTGCCGTTGAA
Human β-actin qPCR	**F:GGACTTCGAGCAAGAGATGG**
	R:AGCACTGTGTTGGCGTACAG

Cell Culture, Transfections The human OL cell line HOG was propagated as previously described (Post and Dawson 1992). HOG cells were cultured in DMEM

containing 4.5 g/L glucose, L-glutamine and sodium pyruvate (Cellgro) plus with FBS and antibiotics (Penicillin/Streptomycin). Transfections were performed with Lipofectamine 2000 (Invitrogen), using the Lipofectamine 2000 protocol, despite slight variations on lipofectamine amounts depending on cell-type for optimum transfection efficiencies. Transfections were performed for 5 hours in serum-free media, which was then replaced with serum-containing media. N-terminal-GFP-tagged full length and ΔNLS PSF constructs were previously published and were gifts from James Patton to our collaborator Nick Seyfried (Dye and Patton 2001, Seyfried et al. 2012). HOG cells were transfected with GFP alone, or the two GFP-tagged constructs. 24 and 70 hours after transfection the cells were treated with Na Arsenite (Zhang et al. 2011). Live-cell images were taken at the indicated time points after treatment using the Olympus IX-51 inverted fluorescent microscope.

Statistical analysis Statistical analyses were performed using Student's t-tests to compare two sample sets, one-way ANOVA and Tukey's post-test when comparing three or more sample sets. Standard errors are depicted in all bar graphs. P-value < 0.05 was considered statistically significant and is indicated by *****.

6.3 Chapter 4

Plasmid Constructs The QKI minigene was constructed by cloning 71 bps of the 3' end of exon 6 to the majority of exon 7c (56 bps from the 5' end) from the *qkI* gene of human U87 genomic DNA in frame downstream of GFP in the pEGFPC2 reporter plasmid. PCR amplification of the QKI minigene was performed by nested PCR using high fidelity polymerase. The first round of nested PCR was on genomic DNA from U87 using the forward primer 5'-CTATGAGTACCCCTACACATTG-3' and the reverse primer 5'-GAACTAAACTAACCTCGGTCTG-3'. The second round of nested PCR with the internal forward primer 5'-CGCTCTCGAGTGAGTACCCCTACACATTGGCACC-3' and the reverse primer 5'-CGCTCCCGGGCCTTTGGTAAGGATGGACACG-3', which contained the restriction enzyme sites for XhoI and XmaI, was performed on the product from the first PCR reaction. The resulting PCR product was cloned into pDrive and then subcloned into pEGFPC2 at the XhoI and XmaI sites in the multiple cloning sequence. Sequencing and restriction mapping was performed with XhoI, XmaI and BglII to confirm the final plasmid construct, run on a 1% agarose gel alongside 1 Kb DNA ladder (Promega). Restriction digests from New England Biolabs were performed following the recommended New England Biolabs protocols for each enzyme.

Cell Culture, Transfection, Infection The rat OPC cell line (CG4), mouse OL cell lines Oli-neu and N20, human OL cell line HOG and neuronal immortalized mouse brain catecholaminergic cell line (CAD) were propagated as previously described (Post and Dawson 1992, Qi et al. 1997). HOG cells were cultured in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate (Cellgro) plus with FBS and antibiotics

(Penicillin/Streptomycin). CG4 and oli-neu cells were cultured on poly-lysine coated plates, and grown in 30% B104 media (Zhao et al. 2006). CAD cells were cultured using DMEM F-12 50/50 (Cellgro) plus FBS. Transfections were performed with Lipofectamine 2000 (Invitrogen), using the Lipofectamine 2000 protocol, despite slight variations on lipofectamine amounts depending on cell-type for optimum transfection efficiencies. Transfections were performed for 5 hours in serum-free media, which was then replaced with serum-containing media. CAD cell transfections were performed in the presence of serum to prevent differentiation and cell death, although lipofectamine complex formation was performed in serum-free media. For the QKI minigene transfection, 1 ug of DNA was transfected into triplicate wells of HOG cells and were harvested 48 hours after transfection. For FOX2 knockdown in HOG cells, infections were performed by using 2 ul of high titer shRNA lentivirus against FOX2 (pshfox2-mRFP) or against GFP serving as a negative control (pshgfp-mRFP) (compliments of Gene Yeo) on two consecutive days. Virus was added to 1 ml cell suspensions on the first day. Cells were plated in 60 mm dishes and incubated at 37 C for 1 hr. 2 ml of fresh media were then added to the plates for overnight incubation. On day two, media was changed depending on cell death, and 2 ul of virus were added again to 3 ml of media. Cells were harvested 48 hours after the last infection, which were then processed for RNA and protein.

Protein/mRNA preparation and quantification assays Whole cell lysates were prepared by sonication of cell pellets or tissues in 1xLaemlli buffer (Li et al. 2000), separated on a 12.5% (wt/vol) SDS-PAGE, transferred to PVDF membrane, and

immunoblotted with primary antibodies against specific proteins, followed by corresponding HRP-conjugated mouse or rabbit secondary antibodies. The immunoblots were exposed to film using electrochemiluminescence (ECL) reagent. The protein bands on the films were quantified using ImageJ software to measure pixel density. Anti-QKI-5 antibody (1:3000; Bethyl laboratories), anti-QKI-6 antibody (1:2000; NeuroMab), anti-β-actin antibody (1:10,000; Sigma), anti-FOX2 (1:2000; Bethyl laboratories), and anti-eIF5α (1:10,000; Santa Cruz).

Total RNA was isolated by Trizol extraction following manufacturer's protocol (Invitrogen). RT of samples was performed using random primers (Promega), SSII reverse transcriptase (Invitrogen), RNase inhibitor (Invitrogen), DTT, and dNTPs. Regular and semi-quantitative PCRs were performed using Taq polymerase and buffer (Invitrogen), dNTPs, MgCl2, and primers. Real-time PCR (qRT-PCR) was performed using DyNaMo Sybr Green qPCR kit (Thermo-Scientific), and quantified by ΔΔCt. The sequences of all primers used are listed in the table below.

Primer	Sequence
Rat QKI-5 qPCR	**F: CTAACCACCCAGCCGCCACC**
	R: TGGTAAGACGAACGGACAAGTGCC
Rat QKI-6 qPCR	**F: CTACACACCCTATGAGTACCCCT**
	R: TTCTTGAATTCTTAGCCTTTCGTTGG
Rat QKI-7 qPCR	**F: CTACACACCCTATGAGTACCCCT**
	R: TCTAGTCCTTCATCCAGCAAGTC
Mouse QKI-5 qPCR	**F: CTACACACCCTATGAATACCCCT**
	R: AGAAGGTCATAGGTTAGTTGCCG
Mouse QKI-6 qPCR	**F: CTACACACCCTATGAATACCCCT**
	R: TTCTTGAATTCTTAGCCTTTCGTTGG
Mouse QKI-7 qPCR	**F: CTACACACCCTATGAATACCCCT**
	R: TCTAGTCCTTCATCCAGCAAGTC
Human QKI-5 qPCR	**F:CCCCTACACATTGGCACCAGCTAC**
	R:CTCGGTCTGCGGTCACAATCC
Human QKI-6 qPCR	**F:CCCCTACACATTGGCACCAGCTAC**
	R:CTTGAATTCTTAGCCTTTCGTTGGGA
Human QKI-7 qPCR	**F:GCAATAGTTCCTCCAGGGCCCG**
	R:AGTCCTTCATCCAGCAAGTCAATG

QKI-5-mini qPCR	**F: CCGCCCTGAGCAAAGACCCCAAC**
	R: CCTTCGAACTTTAGTAGCCACCG
QKI-6-mini qPCR	**F: CCGCCCTGAGCAAAGACCCCAAC**
	R: TAGCCTTTCGTTGGGAAAGCCATA
QKI-7-mini qPCR	**F: CCGCCCTGAGCAAAGACCCCAAC**
	R: AAATATCAGGCATGACTGGCATTTCA
Mouse Gapdh qPCR	**F: GGTGAAGGTCGGTGTGAAC**
	R: CCTTGACTGTGCCGTTGAA
Human β-actin qPCR	**F:GGACTTCGAGCAAGAGATGG**
	R:AGCACTGTGTTGGCGTACAG

Statistical analysis Statistical analyses were performed using Student's t-tests to compare two sample sets, one-way ANOVA and Tukey's post-test when comparing three or more sample sets. Standard errors are depicted in all bar graphs. P-value < 0.05 was considered statistically significant and is indicated by *****.

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