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Regulation and function of the Cdk5/QKI pathway in oligodendroglial development

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Abstract

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By Andrew N. Bankston

Proper development of oligodendroglia (OL), the cells of the central nervous system that provide myelin and neurotrophic and metabolic support to axons, is critical for normal brain function. Impaired OL function in the pathogenesis of numerous brain diseases originally attributed to neuronal defects, such as Alzheimer's disease and schizophrenia, has become increasingly recognized. Furthermore, signaling pathways performing critical functions in neuronal development, such as Cdk5, are found to play key roles in OL development. However, OL-specific regulators and functional targets of Cdk5 activity remain elusive. In this dissertation, mechanisms regulating Cdk5 activation in OLs and functional targets mediating Cdk5-dependent OL development, myelination, and myelin repair are explored.

We first examined whether neurons and OLs employ similar or distinct mechanisms to regulate Cdk5 activity. We showed that in contrast to neurons that harbor high levels of two Cdk5 activators, p35 and p39, OLs express abundant p39 but negligible p35. We found that specific knockdown of p39 by siRNA significantly attenuates Cdk5 activity and OL differentiation without affecting p35. Finally, we detected that expression of p39, but not p35, is increased during differentiation and myelin repair, and remyelination is impaired in $p39^{-/-}$ mice.

We next investigated whether and how p39-dependent Cdk5 activation targets key proteins known to promote OL differentiation. We discovered that the selective RNA-binding protein QKI, which is critical for OL and myelin development, is a target of Cdk5. Specifically, T243, shared by all QKI isoforms that display distinct nuclear-cytoplasmic distribution, is an important site of Cdk5-dependent phosphorylation. We further demonstrated that myristoylation of p39 restricts the majority of Cdk5 activity to the cytoplasm. Moreover, T243 phosphorylation of cytoplasmic QKI isoforms by Cdk5 induces nuclear translocation and enhanced QKI function in promoting OL differentiation.

Together, these studies uncovered novel mechanisms that specifically underlie Cdk5 activation in OLs during normal development and myelin lesion repair. Furthermore, we provided the first evidence connecting Cdk5 signaling with the posttranscriptional cascade under control of QKI. Further investigation of the functional interplay between Cdk5 and QKI pathway may ultimately help to develop new strategies against neurological disorders involving OL impairment.

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List of Abbreviation

AATYK1: apoptosis-associated tyrosine kinase 1

AD: Alzheimer's Disease

AIP-1: actin-interacting protein 1

ALK: anaplastic lymphoma kinase

ALS: amyotrophic lateral sclerosis

APP: amyloid precursor protein

Arp 2/3: actin-related protein 2/3

Aβ: amyloid-beta

BACE1: β secretase 1

bFGF: basic fibroblast growth factor

CA1: cornu Ammonis region 1 of hippocampus

CAK: Cdks-activating kinase

CC1: coiled-coil forming protein 1

Cdk2: cyclin-dependent kinase 2

Cdk5: cyclin-dependent kinase 5

CEBPα: CCAAT/enhancer binding protein alpha

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

CNS: central nervous system

DARPP-32: dopamine and cyclic AMP-regulated phosphoprotein 32 kDa

δFosB: delta FBJ murine osteosarcoma viral oncogene homolog B

DIV: days *in vitro*

DM: demyelination

DUSP: Dual-specificity phosphatase

eIF5α: eukaryotic translation initiation factor 5α

ELAV: embryonic lethal abnormal vision

EM: electron microscopy

endo-N: endoneuraminidase N

ENU: N-ethyl-N-nitrosourea

FAK: focal adhesion kinase

GCIs: glial cytoplasmic inclusions

GDNF: glial-derived neurotrophic factor

GFAP: glial fibrillary acidic protein

HBSS: Hank's balanced salt solution

HD: Huntington's Disease

How: held out wings

Htt: huntingtin protein

IGF-I: insulin-like growth factor-I

KH: hnRNP K homology

L-MAG: Myelin associated glycoprotein, long form

LIF: leukemia inhibitory factor

LTD: long-term depression

LTP: long-term potentiation

MAG: myelin-associated glycoprotein

MAP: microtubule-associated protein

MAP1B: microtubule associated protein 1B

MBP: myelin basic protein

MCT1: monocarboxylate transporter1

MEK1: mitogen-activated protein kinase kinase

MF: microfilaments

mHtt: mutant form of Htt

miR-103: microRNA-103

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS: multiple sclerosis

MSA: multiple system atrophy

MT: microtubules

Myt: membrane-associated tyrosine- and threonine-specific

NF155: neurofascin

NFs: neurofilaments

NGS: normal goat serum

NLS: nuclear localization signal

NMDAR: *N*-methyl D-aspartate receptor

NR2A: *N*-methyl D-aspartate receptor 2a

NRG1: neuregulin-1

NTF: neurotrophic factor

OL: oligodendroglia

Olig2: oligodendroglia-specific transcription factor

OPCs: oligodendroglia progenitor cells

PD: Parkinson's Disease

PDGF: platelet-derived growth factor

PKA: protein kinase A

PSD-95: postsynaptic density 95 kDa

PLP: proteolipid protein

PRMT5: protein arginine methyltransferase 5

PSA-NCAM: polysialylated neural cell adhesion molecule

PSF: polypyrimidine tract binding protein associated factor

QKI: quaking I

qk^{v}: quakingviable

QRE: QKI recognition element

RM: remyelination

RNPs: ribonucleoprotein particles

S-MAG: Myelin associated glycoprotein, short form

SAM68: Src-associated protein in mitosis 68 kDa

SH3: Src-homology 3

Sirt2: Silent information regulator 2

SNARE: soluble NSF attachment protein receptor

Sp1: specificity protein 1

Sp3: specificity protein 3

Src-PTKs: Src family protein tyrosine kinases

STAR: Signal Transduction Activation of RNA

Tag1: transient axonal glycoprotein 1

TDP-43: transactive response DNA binding protein 43 kDa

Tg-Cdk5: transgenic cyclin-dependent kinase 5

Tg-p35: transgenic p35

TPPP: tubulin polymerization-promoting protein

VSMCs: vascular smooth muscle cells

WASP: Wiskott Aldrich Syndrome protein

WAVE: WASP-family verprolin-homologous protein

Wee1: Wee family kinase 1

YY1: Yin Yang

Chapter 1: Introduction to Dissertation

A portion of this chapter is published as:

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Oligodendroglia (OLs) provide myelin on neuronal axons for fast and efficient saltatory conduction as well as neurotrophic and metabolic support for axonal integrity (Bankston, Mandler, and Feng 2013). A growing number of reports have identified defects of OLs in numerous neurodegenerative diseases, often appearing before neuronal loss in pre-symptomatic episodes, suggesting that OL pathology may be an important mechanism contributing to the initiation and/or progression of neurodegeneration. Cyclin-dependent kinase 5 (Cdk5), a well-known regulator of neuronal development and function whose dysregulation is associated with neurodegeneration and cognitive impairment, has emerged as a critical factor essential for OL and myelin development (He et al. 2010, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Su and Tsai 2011, Yang et al. 2013). Moreover, previous studies have reported increased Cdk5 activity during OL differentiation (Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998). Thus, understanding the mechanisms that regulate Cdk5 activity and identifying downstream targets that mediate Cdk5 function are prevailing challenges with the potential to yield novel strategies against neurological disorders involving OL impairment. To pursue this goal, **my dissertation focuses on the regulation and function of the Cdk5 activators, p35 and p39, in OL and myelin development as well as the role of the glial-specific protein QKI in mediating Cdk5 function in OLs**.

Traditionally, Cdk5 has been studied as a crucial regulator of neuronal development (Su and Tsai 2011). Emerging evidence has uncovered an equally important role for Cdk5 in OL and myelin development (Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013). In Chapter 2 of this dissertation, we questioned "**Do similar or distinct mechanisms regulate Cdk5 activation in neurons and OLs?"** We first compared the expression of Cdk5 activators between neurons, astroglia, and oligodendroglia and investigated molecular mechanisms that control expression profiles of Cdk5 activators during OL differentiation in culture, myelin development *in vivo*, and myelin repair after induction of demyelination.

Previous work using primary OL cultures demonstrated the essential role of Cdk5 in OL migration and differentiation (Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008). Subsequent studies using OL-specific knockout of Cdk5 in mice confirmed the role of Cdk5 in OL differentiation *in vivo* (Yang et al. 2013). Furthermore, OL-specific knockout of Cdk5 caused impaired myelination. In demyelinating disorders, failures of myelin repair are a prevailing issue that must be overcome in order to provide effective strategies against these debilitating diseases. Considering the critical roles for Cdk5 in *de novo* OL and myelin development, whether the Cdk5 pathway regulates myelin repair is an important question. Therefore, we next asked: **What is the role of Cdk5 activators in OL development, myelination, and myelin repair?** In Chapter 2 of this dissertation, we use conventional knockout mice and siRNA-mediated knockdown in cultured cells to determine whether the OL-expressed Cdk5 activator(s) are required for OL differentiation, myelin development, and myelin repair.

The functional influence of Cdk5 on cellular processes is mediated by phosphorylation of key target proteins. Few targets of Cdk5 have been identified in OLs (Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008). Moreover, whether Cdk5 targets specific proteins in OLs that are not shared in neurons to advance OL differentiation is an intriguing question that remains unanswered. One such protein is QKI, a selective RNA-binding protein essential for OL differentiation and myelination (Chen et al. 2007, Sidman, Dickie, and Appel 1964). QKI belongs to the signal transduction activator of RNA (STAR) family, which interacts with RNA and signaling proteins to connect RNA homeostasis to extracellular cues (Vernet and Artzt 1997). Analysis of the QKI sequence reveals putative Cdk5 target sties, leading us to ask: **Does Cdk5 phosphorylate QKI and can QKI mediate Cdk5's function in promoting OL development?** In Chapter 3 of this dissertation, we address this question using *in vitro* kinase assays, as well as QKI mutants to determine the potential impacts of phosphorylation on QKI function. Using our established method, we assess the potential functional connection between Cdk5 and QKI in

morphological differentiation of OL progenitor cells. We further discuss the possible mechanisms by which QKI would act downstream of Cdk5 during OL development in Chapter 4.

1.1 Oligodendroglial and myelin development and dysfunction

1.1.1 Myelin is essential for central nervous system function

One of the most apparent visual distinctions in the central nervous system (CNS) is between the gray matter, composed largely of neuronal cell bodies and their dendritic arbors, and the white matter, which contains glial cells and axons wrapped in specialized glia membrane lamellae called myelin. Myelination evolved in vertebrates for the need of increasing axonal conduction velocities while conserving space and energy. Conduction velocity along an unmyelinated axonal fiber is proportional to the square root of the axon diameter, while the velocity along a myelinated fiber is proportional to the axon diameter. Thus, the addition of myelin vastly increases the conduction velocity that can be achieved along a given axon without substantial increases in the size of the axon. Myelin imparts this advantage by acting as an electrical insulator that organizes ion channels in clusters at periodic interruptions in the myelin sheath known as nodes of Ranvier. Action potentials are then able to propagate by "leaping" from node to node through salutatory conduction.

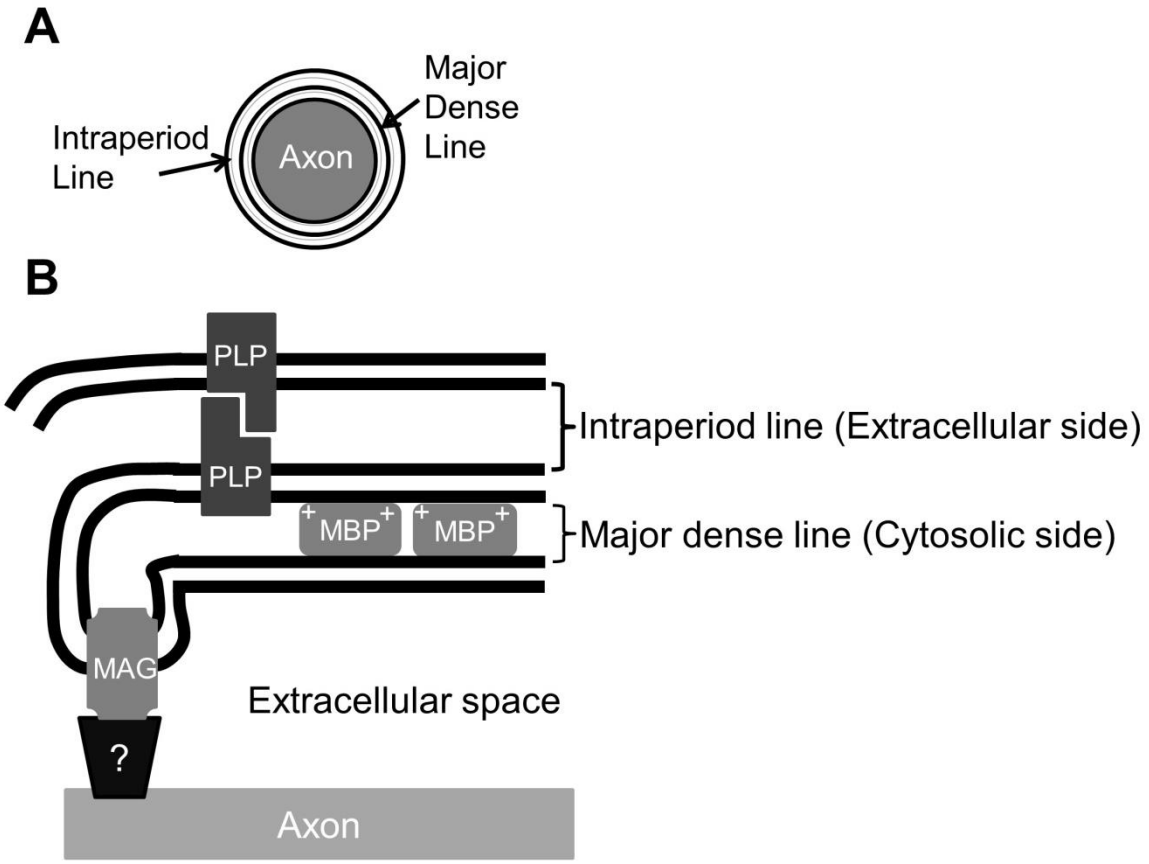
Visualization of myelin using electron microscopy (EM) reveals a highly organized, repeating structure of alternating major dense lines and intraperiod lines (Figure 1-1A). These lines are protein-coated interfaces between lipid bilayers, with the major dense line corresponding to apposing cytosolic sides of the plasma membrane and the intraperiod line indicating the boundary between the extracellular surfaces of two plasma membrane layers. The major dense and intraperiod lines form due to the overlap of concentrically wrapped compacted myelin layers. The cytoplasm has been extruded from these layers and is only retained at the paranodal loops, regions adjacent to the nodes of Ranvier that form contacts with the myelinated axon.

While myelin is primarily composed of lipids (70-85% of the total dry mass), there are a small number of myelin-specific proteins expressed at high levels in myelinating glia that play key roles in myelin compaction and maintenance (Figure 1-1B). Of these proteins, the two most abundant are the structural proteins myelin basic protein (MBP) and proteolipid protein (PLP) (Boggs

2006, Griffiths et al. 1998). MBP is a cytosolic protein that electrostatically interacts with the negatively-charged lipids to aid in formation of the major dense line (Boggs 2006). PLP has four transmembrane domains and plays keys roles in stabilizing the intraperiod line (Griffiths et al. 1998). Two other major proteins that localize to myelin include myelin-associated glycoprotein (MAG) and 2', 3'-cyclic nucleotide 3' phosphodiesterase (CNP). MAG is a member of the Ig superfamily with a single transmembrane domain that localizes to the periaxonal regions of the myelin sheath and associates with receptors on the axonal surface (Montag et al. 1994). CNP is highly enriched in CNS myelin, but is not a major component of compact myelin (Gravel et al. 2009). Instead, CNP localizes to cytoplasmic regions of myelin sheaths, where it is reported to associate with membranes and bind the cytoskeleton to regulate steps leading to myelination, including process outgrowth, through mechanisms that remain unclear (De Angelis and Braun 1996, Lee et al. 2005). Mouse models carrying mutation in myelin structural genes have helped distinguish their functions in myelin development. The *shiverer* mutant mice, which lost the MBP gene, display severe hypomyelination and failure of compaction at the major dense line (Rosenbluth 1980). In mice which lack or harbor mutations in the *PLP* gene, the intraperiod line is aberrantly compacted though multilamellar; however some compact myelin can still form (Nave 1994). While myelin formation does not appear to be affected in CNP- and MAG-null mice, defects in the underlying axons have been observed. CNP-null mice display axon swelling that leads to neurodegeneration and premature death (Lappe-Siefke et al. 2003). MAG-null mice have reduced axon caliber, neurofilament spacing, and neurofilament phosphorylation, followed by eventual axon degeneration (Yin et al. 1998).

Figure 1-1: Structure of central nervous system myelin (A) Cross-section of a myelinated neuronal axon. The major dense line forms with the compaction of opposing cytosolic membrane bilayers. The intraperiod line is the adhesion of overlapping extracellular surfaces. (B) Myelin structural proteins, MBP and PLP, function in myelin membrane compaction at the major dense line and intraperiod line, respectively. MAG functions mainly in non-compact myelin at the node to mediate myelin contact with the axolemma.

Figure 1-1


A
Major
Dense
Line
Intraperiod
Line
Axon
B
PLP
PLP
Intraperiod line (Extracellular side)
MBP+
MBP+
Major dense line (Cytosolic side)
MAG
Extracellular space
?
Axon

Failures in myelin development (dysmyelination) or damage of myelin that has already formed (demyelination) result in severe neurological disorders. The best-recognized demyelinating disorder in the CNS is multiple sclerosis (MS), affecting more than 400,000 people in the US and 2.5 million people worldwide. This debilitating disease stems from repetitive inflammatory autoimmune attacks on CNS myelin. The accumulation of damage from recurring inflammatory insults often leads to progressively worsening neurological symptoms, including visual and speech impairment and paralysis (Lassmann, van Horssen, and Mahad 2012). Although MS is clearly a myelin disorder, an increasing body of evidence suggests that the accompanying loss of axonal integrity and eventual neurodegeneration are the underlying causes of permanent neurological dysfunction. In fact, axonal transection is seen in active demyelinating lesions even early in the disease and appears in nearly all lesions (Trapp et al. 1998). It is important to note that long-term disability is not proportional to the degree of demyelination but rather to the secondary axonal loss (De Stefano et al. 1998). In addition, immunomodulatory therapies that effectively suppress inflammation fail to prevent axonal loss, which continues into the progressive phase of chronic MS (Coles et al. 1999). Thus, besides inflammatory insults, the loss of support by oligodendroglia (OLs) must also play key roles in the axonal loss and irreversible neuropathology in MS.

The protective role of the myelin sheath for axons has long been recognized (Nave 2010). In animal models with nonimmune-based demyelination, slow progressive axonal degeneration is also well-documented, which demonstrates not only the essential role of myelin in maintaining long term axonal integrity (Nave 2010, Wilkins et al. 2010), but also that loss of myelin can lead to axonal degeneration independent of immune insult. Oxidative damage is one detrimental factor for axons upon myelin loss. In MS lesions, oxidative damage is evident with oxidized lipids detected in axonal spheroids (Haider et al. 2011). The increases in reactive oxygen and nitrogen likely contribute to the mitochondrial damage seen in MS lesions and mouse models of demyelination (Campbell et al. 2012, Mahad et al. 2008, Mahad et al. 2009). Impaired

mitochondrial function may create an energy-deficiency that stimulates an increase in mitochondria in demyelinated axons in MS lesions as compared to age-matched controls (Frischer et al. 2009). It has been suggested that this mitochondrial damage results in diminished ATP levels, which impairs the function of the Na^+/K^+ ATPase. As these demyelinated axons are still transmitting action potentials, Na^+ accumulates in the axon and eventually causes a reversal of the Na^+/Ca^{2+} exchanger, leading to Ca^{2+} accumulation in the axon (Stys, Waxman, and Ransom 1991, Trapp and Stys 2009). The influx of Ca^{2+} can activate many Ca^{2+}-dependent proteases, such as calpains, that cause alterations in signaling pathways and degradation of cytoskeleton proteins, leading to the disruption of axonal transport (Trapp and Stys 2009, Worku Hassen et al. 2008). Interestingly, remyelination in mouse models restores ion channel clustering and alleviate mitochondrial accumulation in demyelinated axons to nearly normal levels (Franklin and ffrench-Constant 2008, Sathornsumetee et al. 2000).

Cytoskeletal abnormality within axons is another commonly-observed consequence of myelin defects, although the precise mechanism remains undefined. Aberrant neurofilament phosphorylation detected by SMI32 staining, a surrogate marker of axonal damage, occurs in the demyelinated corpus callosum of cuprizone-treated mice (Crawford et al. 2009), a well-characterized model of demyelination without T-cell activation (Matsushima and Morell 2001). Genetic mouse models that harbor mutations in myelin structural genes have also helped in elucidating the roles of OLs in governing axonal cytoskeletal architecture (Griffiths et al. 1998, Nave and Trapp 2008, Yin et al. 1998). For example, mice null for the periaxonal myelin protein, MAG, display normal-appearing myelin, but reduced axon caliber, neurofilament phosphorylation, neurofilament spacing, and microtubule stability, which together are thought to underlie axon degeneration (Yin et al. 1998). MAG interacts with the complex gangliosides GD1a and GT1b on the axon surface (Yang et al. 1996). Mice null for *Galgt1*, a gene responsible for the synthesis of complex gangliosides including GD1a and GT1b, display reductions in axon caliber and neurofilament spacing similar to the MAG-null mice (Pan et al. 2005). Moreover,

axons in mice double-null for MAG and *Galgt1* are phenotypically comparable to those in MAG-null and *Galgt1*-null mice, suggesting that MAG/ganglioside binding governs neurofilament integrity and long-term axon stability (Pan et al. 2005).

One clue for myelin regulation of the axonal cytoskeleton comes from the discovery that the axonal surface contactin/Caspr complexes interact with myelin membrane proteins NF155 (neurofascin) and Tag1 (transient axonal glycoprotein 1) at the paranode to form an axon–myelin junction (Poliak and Peles 2003). The intracellular domain of Caspr interacts with Protein 4.1B that connects this complex to the axon cytoskeleton (Poliak and Peles 2003). Interestingly, the localization of Caspr at these junctions appears to be myelin ligand dependent (Poliak and Peles 2003), suggesting possible roles of myelin proteins in regulating the axonal cytoskeleton. Another example suggesting that axon–myelin interaction, rather than merely shielding the axon from extracellular insults, is key to maintaining axonal health, comes from studies of proteolipid protein (PLP), the most abundant CNS myelin protein. Mice with PLP deficiency display axon swelling and degeneration despite the formation of nearly normal amounts of myelin (Griffiths et al. 1998). This finding uncouples the role of PLP as the major myelin structural component from its neuroprotective effect. Recent reports also uncovered the essential function of PLP in controlling the abundance of the NAD-dependent deacetylase Silent information regulator 2 (Sirt2) in the myelin compartment (Werner et al. 2007, Zhu et al. 2012). Considering the function of Sirt2 in regulating the acetylation of numerous proteins, including α-tubulin that supports the arborization of OL processes and myelin sheath formation (Li Wenbo et al. 2007), PLP-dependent axonal protection may involve a broad downstream pathway to be defined in the future.

Any strategies to correct impaired myelin development or promote myelin repair would involve progressing cells through the OL lineage. Oligodendroglia progenitor cells (OPCs) must advance to mature OLs before myelin formation can occur. Therefore, understanding the factors

that regulate OL lineage development is key to providing strategies to restore normal myelin function.

1.1.2 Oligodendroglial lineage development

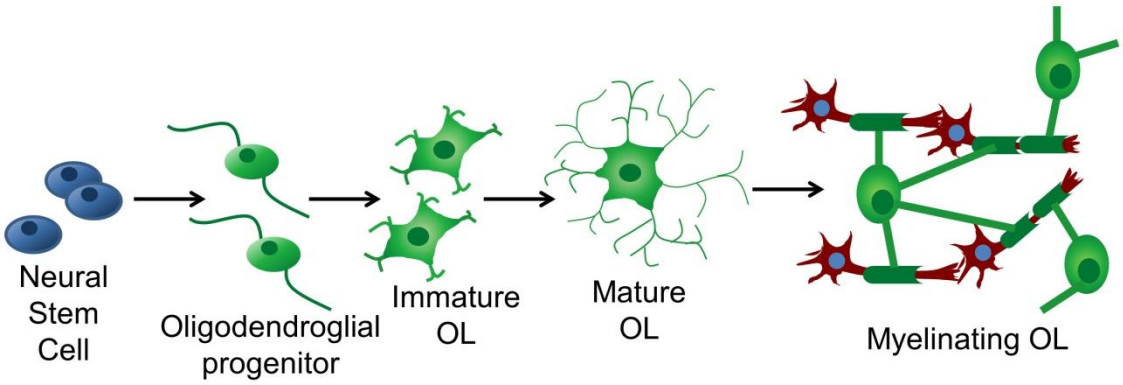
Oligodendroglia (OLs) were first identified as the myelin forming cells of the CNS early in the 20th century by Robertson (Compston et al. 1997). Bunge used electron microscopy to clearly demonstrate that oligodendroglial processes are contiguous with the myelin sheath (Bunge 1962). Development of the OL lineage largely occurs after most neuronal axons have been generated, with the majority of myelination occurring after birth (Altman and Bayer 1984, Skoff, Price, and Stocks 1976). The OL lineage in the brain derives from cells originating in the subventricular zone (Miller 2002, Ono et al. 1997). As illustrated in Figure 1-2, oligodendroglia progenitor cells (OPCs) specified from neural stem cells proliferate to produce a sufficient number of cells to myelinate the neuronal axons. These OPCs then migrate out of the subventricular zone to the axons that they will myelinate in distinct regions of the brain. As the OPCs migrate, they also differentiate to immature OLs, which extend many increasingly complex processes. As the immature OLs approach the axon, they increase expression of many myelin structural proteins, including CNP, MAG, PLP, and MBP described above (Pfeiffer, Warrington, and Bansal 1993). OL maturation and vigorous production of these key proteins then allows formation of myelin sheaths on the neuronal axon.

Not surprisingly, axons exert strong influence on developing OLs and myelination. For example, tetrodotoxin, which blocks sodium-dependent action potentials, inhibits myelination (Demerens et al. 1996). Additionally, activity-induced release of ATP from axons, which is hydrolyzed to form adenosine, stimulates myelination in culture (Stevens et al. 2002). An alternate pathway exists in which activity-dependent release of ATP stimulates astrocytes to secrete leukemia inhibitory factor (LIF), which promotes myelination (Ishibashi et al. 2006). Direct axon-OL contact may also regulate OL development and myelination. In the CNS, myelin

thickness is closely related to the axon diameter, resulting in a constant ratio of axon diameter to diameter of the entire myelinated fiber (Friede 1972). In fact, OLs transplanted from the optic nerve, which contains small diameter axons, to the spinal cord containing large axons form appropriately sized myelin sheaths (Fanarraga et al. 1998), suggesting that myelin thickness is regulated by unidentified extrinsic signals from the axon.

Figure 1-2: The oligodendroglial lineage Oligodendroglial lineage development begins with neural stem cell fate specification to oligodendroglial progenitors, which proliferate to produce a sufficient number of cells for later myelination. The progenitors migrate through much of the central nervous system as they differentiate to immature oligodendrocytes. These maturing cells begin extending many processes as they approach their target axons. Once fully mature, the cells form myelin around appropriate axons. Migration and differentiation are prerequisites to myelination.

Figure 1-2


Neural
Stem
Cell
Oligodendroglial
progenitor
Immature
OL
Mature
OL
Myelinating OL

One candidate is type III neuregulin-1 (NRG1), which controls initiation of myelination and myelin sheath thickness in the peripheral nervous system, but appears less essential for CNS myelination as mice with conditional knockout of NRG1 or its OL receptors, ErbB3 and ErbB4, form normal myelin (Brinkmann et al. 2008, Michailov et al. 2004, Roy et al. 2007, Taveggia et al. 2008). However, transgenic NRG1 overexpression causes hypermyelination, suggesting an involvement of NRG1 in promoting CNS myelination (Brinkmann et al. 2008). Regulation of myelin formation by laminin on the axon surface has also been proposed as stimulation of OLs with laminin-2 enhances myelin formation and OLs lacking the laminin receptor, β1 integrin, fail to myelinate axons (Barros et al. 2009, Buttery and ffrench-Constant 1999). On the other hand, polysialylated neural cell adhesion molecule (PSA-NCAM) on the axon surface has been suggested as an inhibitor of myelination. PSA-NCAM expression is negatively correlated with myelin formation in the spinal cord, embryonic human brains, and myelinating co-cultures (Charles et al. 2000, Jakovcevski, Mo, and Zecevic 2007, Oumesmar et al. 1995). Furthermore, removal of PSA moieties from myelinating co-cultures or rat optic nerve by treatment with endoneuraminidase N (endo-N) increased myelination, occurring only on PSA^- axons (Charles et al. 2000). Notch signaling represents another potent inhibitor of OL differentiation. Interaction of Notch on the OL membrane with Jagged on axons inhibits OPC differentiation, and Jagged expression decreases during myelin development on the optic nerve while Notch expression is unchanged (John et al. 2002, Wang et al. 1998). Interestingly, interaction of Notch with contactin, which is also expressed on axons, promotes OL differentiation (Cui et al. 2004, Hu et al. 2003), indicating that Notch may regulate the timing of OL maturation and myelination through sequential interaction with these axonal ligands. OL differentiation involves both changes in gene expression as well as morphological changes involving the extension of increasingly complex processes and myelin sheath formation that rely on extensive cytoskeletal rearrangements.

The cytoskeleton of OLs is composed of microtubules (MT) and actin microfilaments (MF), but lacking in intermediate filaments (Bauer, Richter-Landsberg, and ffrench-Constant

2009). Both cytoskeletal components not only facilitate process outgrowth and maintenance, but also provide tracks for transport of key cellular components needed in the formation of OL processes and myelin sheath. While MT in the OL soma form a web-like network, MT in extended OL processes orient in a direction parallel to the axes of the processes (Lunn, Baas, and Duncan 1997). Tubulin, the monomeric constituent of MT, undergoes extensive posttranslational modification that can regulate the balance between MT dynamics and stability as well as binding of MT-associated proteins. Acetylation of α-tubulin is correlated with increased MT stability in mature OLs (Song et al. 2001). In fact, acetylated α-tubulin is detected in cell soma and primary processes, but absent from the most distal tips of extended processes (Lunn, Baas, and Duncan 1997). The deacetylation of tubulin would therefore facilitate increased MT dynamics that could then participate in OL process extension and myelin formation. Silent information regulator 2 (Sirt2), a member of the sirtuin family of proteins, mediates tubulin deacetylation (Li W. et al. 2007). In differentiating OLs, Sirt2 is enriched in OL processes, where MT turnover is high (Li W. et al. 2007, Southwood et al. 2007). Moreover, Sirt2 expression is correlated with active morphological differentiation, with highly complex process arborization (Bauer, Richter-Landsberg, and ffrench-Constant 2009). Another family of proteins, the microtubule-associated protein (MAP) family, binds to and stabilizes MT. In addition, binding of MAPs to MT facilitate the interaction of MT with other cytoskeletal components, including MF. Of particular interest, MAP1B and Tau are expressed in OLs and regulate MT dynamics to mediate process extension and myelin formation (Fischer, Konola, and Cochary 1990, Richter-Landsberg 2001). In fact, MAP1B has been shown to bind both MT and MF to possibly couple their dynamics as MAP1B is required for MT retraction following lysophosphatidic acid-induced actin contraction (Bouquet et al. 2007). Expression of MAP1B is enriched in mature OLs at the initiation of myelin sheath formation (Vouyiouklis and Brophy 1993, Wu et al. 2001). Mice lacking MAP1B display defects in myelin development (Meixner et al. 2000). Tau colocalizes with MT in mature OLs and is particularly enriched at the ends and branching points of processes (Bauer, Richter-Landsberg,

and ffrench-Constant 2009). Both MAP1B and Tau binding to MT is regulated through extensive phosphorylation, and a downregulation of Tau phosphorylation is seen during OL differentiation (Gorath et al. 2001), allowing increased MT binding.

Actin microfilaments (MF) in OLs are located immediately below the plasma membrane and promote primary process formation and stability (Bauer, Richter-Landsberg, and ffrench-Constant 2009). In the OL soma, MF form a complex cortical mesh, but orient the growing end of filaments toward the membrane during filopodia formation. During process outgrowth in OLs, MF produce small membrane protrusions by filament polymerization and branching that eventually result in filopodia that are transformed to lamellipodia through MF branching. The mechanisms controlling MF nucleation are not clear, but the actin-related protein 2/3 (Arp 2/3) may catalyze the initiation of MF polymerization. Arp 2/3 also binds to MF sides and may cooperate with the Wiskott Aldrich Syndrome protein/WASP-family verprolin-homologous protein (WASP/WAVE) family to drive lamellipodia formation (Goley and Welch 2006). Indeed, inhibition of WAVE1 in OLs impaired process formation, elongation, and branching (Kim H. J. et al. 2006). Moreover, mice lacking WAVE1 had a significant reduction in the number of myelinated axons (Kim H. J. et al. 2006). The other WAVE protein expressed in OLs, WAVE2, is required for PDGF-dependent migration of OPCs (Miyamoto, Yamauchi, and Tanoue 2008). In astrocytes, loss of WAVE2-dependent Arp 2/3 activation led to reduced process branching complexity (Murk et al. 2013). Besides regulation of cytoskeletal dynamics by intracellular signals, extracellular cues exert potent influence on MF. For example, laminin binding to integrin in the OL plasma membrane promotes OL membrane production (Buttery and ffrench-Constant 1999). Integrins associate with focal adhesion complexes that relay extracellular signals to the cytoskeleton and include proteins such as focal adhesion kinase (FAK) and paxillin. FAK is required for OL process outgrowth, and paxillin is necessary for OL differentiation (Miyamoto et al. 2007).

During the myelination process, intersheet interactions between concentric layers of the myelin sheath may provide new signals promoting extensive reorganization of the cytoskeleton rather than the stabilization and polymerization that occur during process elaboration. Among many other responses to the new interaction between overlapping myelin layers, vigorous depolymerization of the MT and MF occurs, and much of this process is impaired by pharmacological stabilization of MF (Boggs, Gao, and Hirahara 2008a, Boggs, Gao, and Hirahara 2008b, Boggs and Wang 2004). As the myelin sheath becomes compacted, it seems likely that much of the cytoskeleton must be extruded to allow close apposition of the intracellular surfaces of overlapping myelin membranes (Bauer, Richter-Landsberg, and ffrench-Constant 2009). One possible mechanism for MT depolymerization involves Sirt2, which is targeted to the cytoplasmic loops of myelin sheaths. Further investigation is needed to define the pathways which regulate cytoskeletal dynamics and are localized to the myelin sheath. Identifying the factors which control OL lineage progression would provide strategies to restore OL function in neurological diseases involving OL impairment. The importance of proper OL lineage development is highlighted by the mounting evidence for the role of OL impairment in neurodegeneration.

1.1.3 Emerging evidence for oligodendroglia and myelin impairment in neurodegeneration

The majority of research on classic neurodegenerative disorders is centered around the view that neuronal death is the initial impetus to pathogenesis. However, accumulating studies have reported white matter alterations including loss of OLs and reduction of OL-specific proteins, even in the early phase of many such disorders (Bohnen and Albin 2011, Di Paola et al. 2012, Gold et al. 2012, Rosas et al. 2006). A well-recognized commonality among these diseases is axonal degeneration before the loss of the soma (Duyckaerts, Delatour, and Potier 2009, Jellinger 2012, Zheng and Diamond 2012). In addition, neurotrophic factors (NTFs) produced by OLs and/or acting on OLs to elicit neuroprotection and promote myelination, including BDNF,

CNTF, and LIF, are implicated in these diseases (Alberch, Perez-Navarro, and Canals 2004, Pezet and Malcangio 2004). Considering the role of OLs in trophic support and axonal health, whether and how OLs are involved in the onset and/or progression of neurodegenerative disorders are important questions attracting increasing attention.

1.1.3.1 Alzheimer's Disease

Alzheimer's Disease (AD) is the most common form of dementia, characterized by a progressive loss of cognition and the inability to perform simple tasks, with extensive neuronal death and atrophy in the cerebral cortex. Two major hypotheses have been postulated regarding the mechanisms underlying the neuronal loss in AD: (1) formation of amyloid-beta (Aβ) plaques; and (2) hyperphosphorylation of tau and formation of neurofibrillary tangles (Duyckaerts, Delatour, and Potier 2009). Interestingly, the neurons most affected in AD are those most recently myelinated (Lu et al. 2013). In fact, extensive white-matter loss has been reported in postmortem brains from AD patients (Gold et al. 2012). Further evidence supporting the involvement of OLs in AD pathology comes from the report that Aβ treatment induces OL death in culture (Roth et al. 2005). Injection of Aβ into rat white matter also causes loss of OLs and myelin (Jantaratnotai et al. 2003). In addition, mice carrying a mutation in presenilin-1, a gene frequently mutated in familial AD cases, display a heightened sensitivity to toxic insult of OLs and demyelination (Desai et al. 2011). Moreover, triple-transgenic mice with mutations in APP, presenilin-1, and tau have defects in OLs and myelin even before the signs of amyloid or tau malfunction (Desai et al. 2009). However, injection of vectors expressing intracellular antibodies against Aβ1-42 restores OL marker expression and myelin integrity (Desai et al. 2010), raising the intriguing question whether OL impairment is a causative factor in the early phase of AD pathology.

1.1.3.2 Huntington's Disease

Huntington's Disease (HD) is a genetic neurodegenerative disorder leading to loss of coordination and cognitive function due to autosomal dominant inheritance of an extended CAG trinucleotide repeat that produces an expanded poly-glutamine tract within the huntingtin protein (Htt) (Zheng and Diamond 2012). The mutant form of Htt (mHtt) aggregates and forms inclusions, which are thought to disrupt neuronal function and eventually lead to neuronal death. However, Htt expression is not restricted to neurons, but is universal in glial lineages as well (Hsiao and Chern 2010, Shin et al. 2005). In fact, the expression of mHtt specifically in astroglia of transgenic mice leads to neuronal degeneration (Bradford et al. 2009), suggesting the involvement of glia in HD pathology. Specifically, recent neuroimaging studies point to OL pathology in HD. For instance, MRI imaging revealed a reduction of white-matter volume in HD patients as compared to normal controls (Ciarmiello et al. 2006). Moreover, reduced white-matter volume has even been observed in presymptomatic HD brains and often correlates with measures of cognitive impairment, suggesting that OL dysfunction may precede neuronal loss (Beglinger et al. 2005, Ciarmiello et al. 2006, Di Paola et al. 2012, Rosas et al. 2006). Interestingly, myelin breakdown and neurodegeneration have been observed along axons myelinated during early development (Bartzokis et al. 2007). Increased OL density has also been observed in the damaged regions of postmortem HD brains (Gómez-Tortosa et al. 2001, Myers et al. 1991), possibly as a result of a failed attempt to repair lost myelin.

1.1.3.3 Parkinson's Disease

Parkinson's Disease (PD) is the second most common age-related neurodegenerative disorder and affects the dopaminergic neurons of the substantia nigra, resulting in early motor symptoms and later cognitive impairment (Jellinger 2012). Classified as a synucleinopathy, PD neurodegeneration is due to the accumulation of α-synuclein in Lewy bodies followed by cell death (Jellinger 2012). Current evidence suggests a lack of OL involvement in the initiation of PD

but does point to a role in PD progression (Halliday and Stevens 2011). While α-synuclein filaments have been reported in nonmyelinating OLs, they do not appear until clinical symptoms are already evident (Arai et al. 1999, Wakabayashi et al. 2000). However, neurons affected by α-synuclein often show decreased myelination and increased association with non-myelinating OLs (Braak and Del Tredici 2004, Braak and Del Tredici 2009). Moreover, complement-activated OLs, which are often seen in degenerating brains, have been detected in PD brains (Yamada, McGeer, and McGeer 1992). These data suggest that although OLs may not be involved in PD initiation, they likely contribute to the exacerbation of PD pathogenesis by removing a source of trophic factors for the survival of remaining neurons.

1.1.3.4 Neurodegenerative Diseases as a Result of OL Impairment

OL and myelin abnormalities in the above neurodegenerative disorders could be causative in the etiology of the diseases or a consequence of neuronal damage, and these are currently difficult to clearly distinguish. However, recent findings provide strong evidence that dysfunction of OLs causes neurodegeneration in the CNS, represented by the case of multiple system atrophy (MSA). MSA is a devastating neurodegenerative disease characterized by a unique synucleinopathy in which glial cytoplasmic inclusions (GCIs) with α-synuclein as the major component, rather than neuronal synucleinopathy, dominate the affected brain regions (Ahmed et al. 2012). In particular, GCIs in oligodendroglia is the pathological hallmark of MSA. The lifespan of MSA patients is ~8 years after the onset of symptoms (Stefanova et al. 2009), and the pathogenesis is considered to be primary OL dysfunction in the affected brain regions responsible for the clinical phenotypes, including autonomic dysfunction, muscle rigidity, and ataxia (Wenning et al. 2008). The formation of GCIs is predicted to increase OL susceptibility to the inflammatory cytokine TNF-α and to oxidative stress (Riedel, Goldbaum, and Richter-Landsberg 2009, Stefanova et al. 2005, Stefanova et al. 2003). *In vitro* studies suggest that α-synuclein aggregation is enhanced by phosphorylation at serine-129 and can be induced by the

oligodendroglia-specific protein tubulin polymerization-promoting protein (TPPP) (Hasegawa et al. 2010). Overexpression of α-synuclein and TPPP in cultured OLs causes OL apoptosis in a serine-129-dependent manner (Kragh et al. 2009), although which of the kinases known to phosphorylateserine-129 is responsible for the pathogenic accumulation of α-synuclein in OLs of the MSA brain remains elusive. Perhaps the most definitive evidence for the causative role of OL-specific synucleinopathy in MSA comes from transgenic mice that express human α-synuclein specifically in OLs under the PLP, CNP, or MBP promoters that drive transcription in OLs (Kahle et al. 2002, Shults et al. 2005, Yazawa et al. 2005). These mice display extensive myelin impairment and persistent neuronal degeneration in many brain areas, recapitulating those affected in MSA patients. Interestingly, overexpression of human α-synuclein under the control of neuronal or OL promoters leads to reduced expression of NTFs derived from OLs (Ubhi et al. 2010). Furthermore, specific reduction of glial-derived neurotrophic factor (GDNF) is seen in transgenic mice that overexpress human α-synuclein specifically in OLs, and infusion of GDNF partially rescues the behavioral deficits and neuronal loss seen in these mice (Ubhi et al. 2010). Finally, reduced GDNF levels have also been detected in the frontal cortex white matter and cerebellum of human MSA samples (Ubhi et al. 2010). Thus, reduced NTF production in OLs as a result of synucleinopathy may be an underlying mechanism for the neurodegeneration in MSA. OL abnormality as a cause of axonal degeneration is reinforced by a recent discovery by Lee and colleagues, who reported that deficiency of monocarboxylate transporter1 (MCT1, also known as SLC16A1) in OLs results in axonal damage and neuronal loss independent of demyelination (Lee et al. 2012). MCT1 is primarily expressed in OLs, with a defined function to export lactate from OLs and provide energy metabolites to neurons. The authors demonstrated that axonal toxicity can be achieved by reduced lactate release from OLs upon MCT1 disruption, suggesting that OL-dependent axonal energy metabolism is a fundamental mechanism by which OLs support axons and neurons. It is important to mention that the same MCT1 transporter is reduced in patients and mouse models of amyotrophic lateral sclerosis (ALS) (Lee et al. 2012), another devastating

neurodegenerative disorder that involves TDP-43 inclusion besides frontotemporal lobar degeneration, raising the possibility that insufficient axonal energy support through OLs may be an underlying mechanism for ALS as well as axonal degeneration in other diseases yet to be elucidated. Findings of OL and myelin impairment in various neurological disorders, whether they are primary or secondary features of the disease pathogenesis, point to the need for methods to promote the restoration of OL and myelin function.

1.1.4 Enhancing OL lineage progression for myelin repair

In disorders involving a loss of myelin, such as multiple sclerosis, the resulting axonal degeneration can lead to severe neurological impairment. Upon such demyelinating insults, an attempt to repair the lost myelin is made through a process termed remyelination (Franklin and ffrench-Constant 2008). This myelin repair process begins with proliferation of adult quiescent OPCs that migrate to the demyelinated lesion. Upon differentiation to mature OLs, myelin is produced and ensheathment of the demyelinated axon occurs. These remyelinated axons regain the ability to transmit signals through saltatory conduction and are often protected from more severe axonal damage (Smith, Blakemore, and McDonald 1979). In multiple sclerosis, remyelination in the early stages is associated with improvement of neurological symptoms; however, once the disease reaches later stages, failure of remyelination becomes more frequent, leading to a progressive loss of neurological function (Hagemeier, Bruck, and Kuhlmann 2012). Therefore, novel strategies are needed to enhance myelin repair in demyelinating disorders. Such strategies may come from answering such questions as: Where do the OLs that participate in myelin repair come from? Do our studies of *de novo* myelin development offer any clues to the mechanisms guiding myelin repair? What conditions are required in the brain, in particular presence or absence of other cell types and the functional state of the demyelinated axon, to allow remyelination to occur? Remyelinating OLs appears to most likely derive from OPCs that migrated from the subventricular zone, rather than local OLs (Franklin and ffrench-Constant

2008). This is supported by evidence that the number of OLs in an area of remyelination is increased compared to undamaged brain regions (Prayoonwiwat and Rodriguez 1993). Additionally, remyelination still occurs in areas in which OLs have been depleted, both suggesting a distal source of remyelinating OLs (Sim et al. 2002). Moreover, when mature OLs are transplanted into demyelinating experimental models, remyelination does not occur (Targett et al. 1996). In experimental models where OPCs are removed by X-irradiation, no remyelination occurs upon induction of demyelination despite the retention of intact OL cell bodies (Keirstead and Blakemore 1997). Failures of remyelination appear to be the result of failed maturation of OPCs to OLs capable of remyelination. A recent study reported that myelin remodeling occurs in the adult brain through maturation of adult OPCs to mature OLs that then intercalate myelin sheaths within existing myelinated tracts or replacing OLs undergoing cell death, suggesting that the adult brain may provide a suitable environment for myelin production (Young et al. 2013). It is possible that factors which act as inhibitors of OPC differentiation are present in the region surrounding the demyelinating lesion. Evidence suggesting the interaction of OPC Notch with Jagged on astrocytes in MS lesions indicates the potential for this pathway, which negatively regulates OPC differentiation, to inhibit remyelination (John et al. 2002). Additionally, detection of polysialylated neural cell adhesion molecule (PSA-NCAM), an inhibitor of myelination in cell culture, on axons raised the possibility that the axon itself inhibits remyelination (Charles et al. 2002). Whether inhibitory signals from the demyelinated lesion environment can be blocked or whether intrinsic factors can be activated to overcome these extracellular inhibitions is an area of active study with the potential to yield effective therapeutics for patients suffering with demyelinating disorders. A deeper understanding of the pathways that regulate OL development is needed to yield therapeutic strategies. Interestingly, key components of cell signaling pathways traditionally thought to control neuronal development and function have now been found expressed and playing critical roles in OLs as well. One such signaling molecule is Cyclin-

dependent kinase 5 (Cdk5). However, it remained unclear whether the mechanisms controlling Cdk5 activity in neurons also regulate Cdk5 in OLs.

1.2 Cyclin-dependent kinase 5 (Cdk5) is essential for neuronal and oligodendroglial development

1.2.1 Regulation of Cdk5 activity

The discovery of Cdk5 in 1992 was reported by four separate groups. Hellmich et al. reported the cloning from rat brain cDNA of a neuronal Cdc2 like kinase that phosphorylates neurofilaments and shares 61% amino acid sequence homology with human Cdk2 (Hellmich et al. 1992). RT-PCR of HeLa cell mRNA using degenerate primers targeting conserved regions of cdc2 uncovered several Cdks, including Cdk5 (Meyerson et al. 1992). Another study noted the functional similarity to cdc2 for a proline-directed kinase isolated from bovine brain (Lew et al. 1992). The final group reported the discovery of a Tau protein kinase that associated with microtubules (Ishiguro et al. 1992). The next year, the 30 kDa enzyme was identified and named Cdk5 (Kobayashi et al. 1993). As a member of the Cdk family, Cdk5 has many structural features which are similar to other family members. The catalytic domain contains N-terminal and C-terminal lobes flanking an ATP binding site and a twenty amino acid activation loop (T-loop) that can change conformation to allow phosphate transfer to substrates (Figure 1-3A and B), preferring sequences of (S/T)Px(KRH) (Dhariwala and Rajadhyaksha 2008).

However, beyond these structural similarities, few parallels exist between the regulation of Cdk5 and other Cdk family members. In fact, Cdk5 activation occurs in post-mitotic cells. As with other Cdk family members, monomeric Cdk5 is not catalytically active, but requires binding to an activator subunit for catalytic activity (Ko et al. 2001). Cdk5 activity is largely detected in the cytoplasm in contrast to the function of classic Cdks in the nucleus. Full activation of classic Cdks requires binding to an activator protein and phosphorylation at Thr160 in the T-loop of the Cdks by Cdks-activating kinase (CAK). Phosphorylation at Thr160 allows interaction between

the phosphate group and the basic residues at the +3 position relative to the (S/T) residue in the substrate. This extends the T-loop into a conformation that favors recognition of the +1 Pro in substrates followed by substrate phosphorylation (Brown et al. 1999, Russo, Jeffrey, and Pavletich 1996). However, phosphorylation of the equivalent site, Ser159 (shown in Figure 1-3C), in the T-loop of Cdk5 is not required for Cdk5 activation (Tarricone et al. 2001). Binding of Cdk5 to its activator is sufficient to fully activate the kinase (Figure 1-3B), as demonstrated by structural studies of Cdk5 binding to its activator p25, which causes extension of the T-loop to an active conformation (Tarricone et al. 2001). Further modulation of Cdk activity stems from phosphorylation of Thr14 and Tyr15 (shown in Figure 1-3C and D) in the glycine-rich G-loop by the dual-specificity kinases, membrane-associated tyrosine- and threonine-specific (Myt) and Wee1, named for the small cell size of dividing fission yeast harboring a mutation in the *wee1* gene (Gu, Rosenblatt, and Morgan 1992). Phosphorylation of these residues inhibits the activity of classic Cdks by causing a conformational shift that leads to ATP misalignment and increased distance between ATP and the (S/T) target residue (Bártová et al. 2005). While Cdk5 phosphorylation at Thr14 is inhibitory, as with classic Cdks, phosphorylation of Tyr15 appears to stimulate Cdk5 activity (Zukerberg et al. 2000). The mechanism of this stimulatory effect remains unclear, though enhanced substrate binding has been proposed. Studies using molecular dynamics simulations indicate that Tyr15 phosphorylation of Cdk5 actually repositions ATP for enhanced interaction with the substrate and a more efficient Cdk5 kinase reaction (Zhang et al. 2007). Additionally, rather than Wee1-mediated phosphorylation, Tyr15 of Cdk5 is phosphorylated by c-Abl and Fyn (Sasaki et al. 2002, Zukerberg et al. 2000). Another mode of Cdk inhibition requires binding of the Cdk/cyclin complex by the cyclin-dependent kinase inhibitors, p21 and p27, causing Cdk inactivation. In contrast, p21 and p27 do not appear to affect Cdk5 activity (Lee et al. 1996).

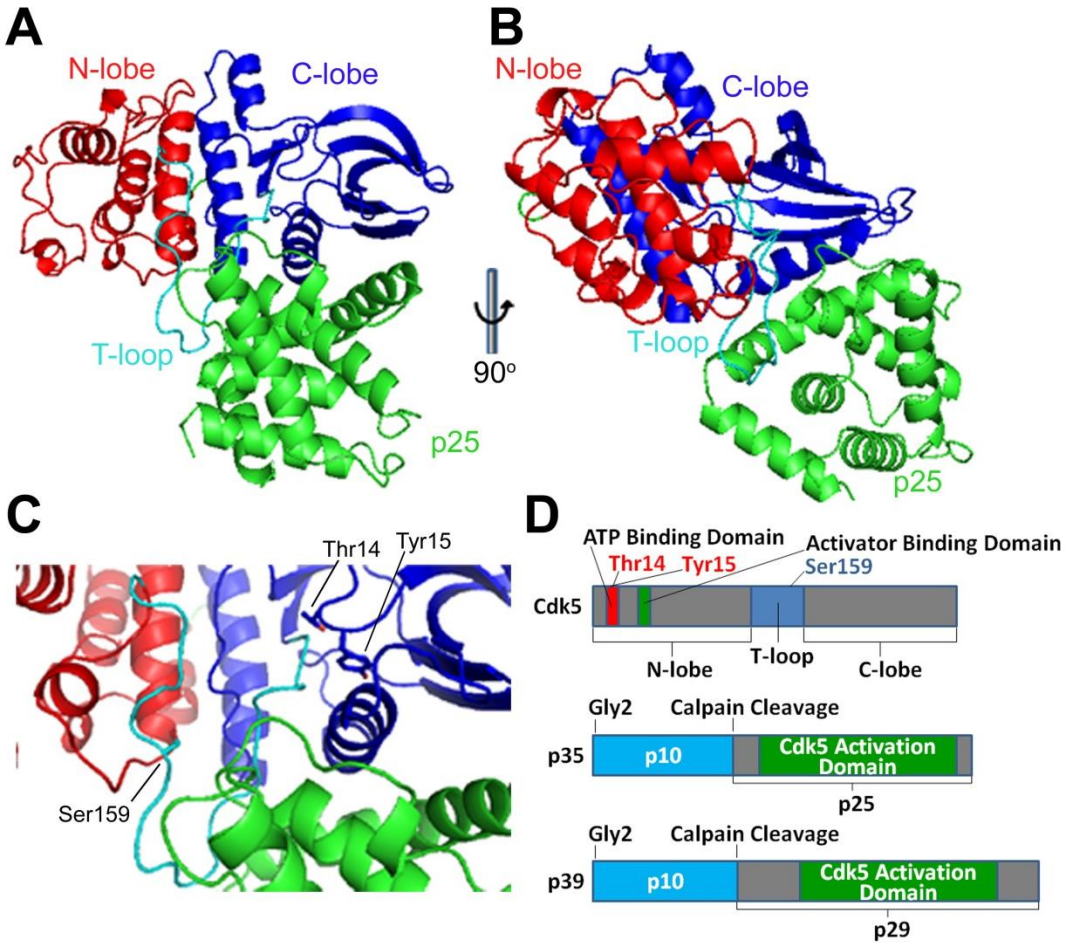
Perhaps the most intriguing difference between Cdk5 and classic Cdks is their activator proteins. While activation of classic Cdks requires binding of specific cyclin proteins, Cdk5

activation is mediated by two non-cyclin proteins, p35 and p39, which are believed to be the sole activators of Cdk5 in the brain (Ko et al. 2001). The initial isolation of p35 from brain extract in 1994 was based on its binding and activation of Cdk5 (Tsai et al. 1994). A second activator, p39, was identified due to its 57% amino acid sequence homology to p35 (Tang et al. 1995). The unique Cdk5 activators share very little sequence similarity to cyclins, but appear to form a cyclin-like fold that binds and activates Cdk5 (Tarricone et al. 2001). Binding of Cdk5 by either p35 or p39 is sufficient to fully activate the kinase, possibly because p35 and p39 actually participate in binding to basic residues in the substrate, eliminating the need for S159 phosphorylation (Humbert, Dhavan, and Tsai 2000, Tang et al. 1995, Tarricone et al. 2001, Tsai et al. 1994). Both classic Cdks and Cdk5 are inactivated through ubiquitylation and degradation of their activator proteins. Mutation of Cdk5 target sites in p35 increased the half-life of p35 (Kamei et al. 2007, Patrick et al. 1998), demonstrating that phosphorylation of the Cdk5 activators by Cdk5 stimulates their degradation and introducing a novel mechanism of self-regulation by Cdk5.

Figure 1-3: Structure of Cdk5/p25 complex and domains of Cdk5 and its activators

(A) Binding of Cdk5 to p25 (green), or the activators p35 or p39, opens the T-loop (cyan) into an extended conformation. The T-loop is flanked by a N-terminal lobe (red) and a C-terminal lobe (blue). (B) 90º rotation of (A) on the vertical axis. The close proximity of the interaction of p25 with the region between the T-loop and C-terminal lobe of Cdk5 and the extension of the T-loop can be seen. (C) Zoomed view of same orientation as in (A) with key residues labeled and side chains shown. (D) Schematic representations of Cdk5, p35, and p39 proteins. Cdk5 harbors an ATP-binding domain containing regulatory sites of phosphorylation, Thr14 and Tyr15, an activator-binding domain, and a T-loop which adopts an extended conformation in the active kinase. The T-loop contains Ser159, which is equivalent to Thr160 in classic Cdks, but does not require Ser159 phosphorylation for full extension. p35 and p39 contain a C-terminal Cdk5 activating domain. The N-terminal p10 regions of these activators contain regulatory sites, including the myristoylation site Gly2, and can be removed by calpain cleavage.

Figure 1-3


A
N-lobe
C-lobe
T-loop
p25
90°
B
N-lobe
C-lobe
T-loop
p25
C
Thr14
Tyr15
Ser159
D
ATP Binding Domain
Activator Binding Domain
Thr14
Tyr15
Ser159
Cdk5
N-lobe
T-loop
C-lobe
Gly2
Calpain Cleavage
p35
p10
Cdk5 Activation Domain
p25
Gly2
Calpain Cleavage
p39
p10
Cdk5 Activation Domain
p29

In cortical and cerebellar granule neurons, the half-life of p35 is around one hour, while the half-life of p39 is greater than two hours (Minegishi et al. 2010). The sequence within the N-terminal p10 region of the Cdk5 activators appears to contain the major determinants of Cdk5 activator stability (Minegishi et al. 2010). Exchange of the p35 and p39 N-terminal p10 regions increases the half-life of p35 but decreases the half-life of p39. Association of p35 and p39 with the plasma membrane appears to facilitate their degradation. Membrane-binding is conferred to p35 and p39 by myristoylation at Gly2 (Figure 1-3D), and mutation of Gly2 to alanine increases the half-life of the Cdk5 activators. Importantly, the membrane association of p35 and p39 helps to restrict the kinase activity of Cdk5. Myristoylated p35 and p39 recruit Cdk5 activity to those targets present on the membrane or in the cytoplasm. Blockade of Cdk5 activator myristoylation likely causes increased Cdk5 activation in the nucleus, which is often associated with cell death (Barnett and Bibb 2011, Hisanaga and Endo 2010). This is further supported by the effects of cleavage of p35 to p25 by calcium-dependent calpains (Kusakawa et al. 2000). The increased half-life of p25 compared to p35, due to loss of the regulatory p10 region of p35 (Figure 1-3D), causes hyperactivation and dysfunction of Cdk5 (Cruz et al. 2003, Noble et al. 2003, Patrick et al. 1999). Calpain-dependent cleavage of p39 to form p29 can also occur (Figure 1-3D), but the effects of p29 generation are not clear (Patzke and Tsai 2002). Interestingly, formation of p25 and p29 can occur in response to neuronal stimulation with excitotoxic glutamate (Miao et al. 2012). Glutamate stimulation also causes proteasomal degradation of p35 and p39 (Minegishi et al. 2010). An interesting model was proposed by Hisanaga and Saito in which rapid degradation of p35 by the ubiquitin proteasome upon glutamate stimulation may prevent p25 generation by the increased calcium levels that follow (Hisanaga and Saito 2003). They noted that p35 in the developing brain appeared to be less stable and more heavily phosphorylated than p35 in the adult brain. Cdk5-dependent phosphorylation of p35 was shown to prevent calpain-mediated cleavage of p35 to p25. Moreover, p25 generation was observed in the adult brain but not the developing brain, despite higher calpain activity levels in the developing brain. Therefore, phosphorylation of

p35 by Cdk5 may protect developing neurons from p25-induced Cdk5 dysfunction upon excitation-triggered increase of intracellular Ca^{2+}. This protective mechanism may be less critical in the adult brain, where calpain activity is decreased.

Considering the short half-lives of p35 and p39, the mechanisms controlling production of the Cdk5 activators are critical determinants of Cdk5 activity levels. This idea is highlighted by evidence obtained from genetic manipulation of Cdk5 activator expression in mice. Mice with the genotypes $p35^{+/-}$, $p35^{-/-}$, $p39^{+/-}$, and $p39^{-/-}$ had a proportional decrease in p35 or p39 levels and Cdk5 activity compared to wildtype mice (Ko et al. 2001, Takahashi et al. 2005). Moreover, transgenic overexpression of p35 (Tg-p35) increased Cdk5 activity, while $Cdk5^{+/-}$ and Tg-Cdk5 mice displayed no change in Cdk5 activity compared to wildtype, indicating that the available amounts of p35 and p39 determine Cdk5 activity. During development, the peak of p35 expression is observed in the embryonic brain, while p39 expression is highest after 2-3 weeks of postnatal development, suggesting distinct roles of the Cdk5 activators in regulating brain development (Cai et al. 1997, Delalle et al. 1997, Honjyo et al. 1999, Wu et al. 2000, Zheng, Leung, and Liem 1998). Laminin binding to neuronal integrins stimulates increased levels of p35 mRNA and protein as well as Cdk5 activity (Paglini et al. 1998). NGF and BDNF have also been reported to induce p35 expression, which could be blocked by pharmacological inhibition of the Erk pathway (Harada et al. 2001, Tokuoka et al. 2000). Furthermore, Erk-mediated expression of p35 appears to require the transcription factor early growth response-1 (EGR-1) (Harada et al. 2001). Interestingly, MEK1 is targeted by Cdk5, which represses MEK1 activity and the downstream pathway responsible for p35 upregulation, providing a negative feedback loop for MAPK-dependent Cdk5 activation. Additionally, GC-box binding transcription factors, Sp1 and Sp3, bind the promoters of p35 and p39 to stimulate expression in neurons (Ross et al. 2002, Valin et al. 2009). However, most studies have focused on pathways that cause transcriptional activation of p35 expression, with limited literature addressing p39 transcription. Transcription of Cdk5 is induced by δFosB (Chen et al. 2000), which is often increased after dopamine or cocaine

administration or electroconvulsive seizures. Posttranscriptional regulation of p35 protein production also contributes to the final levels of available Cdk5 activators. The p35 mRNA contains an AU-rich element in its 3'UTR that mediates binding and transcript stabilization by members of the embryonic lethal abnormal vision (ELAV) family in a neuronal cell line (Moncini et al. 2007). Additionally, the p35 3' UTR is targeted by miR-103 and miR-107, which suppress p35 expression (Moncini et al. 2011). So far, no studies have reported post-transcriptional regulation of p39 expression, which must be addressed to obtain a full understanding of mechanisms regulating Cdk5 function.

Recent work has also demonstrated binding of Cdk5 to some cyclins. Cyclin E binding to Cdk5 inhibits Cdk5 activity (Odajima et al. 2011), possible by preventing Cdk5 binding to p35 or p39. In contrast, Cyclin I binding to Cdk5 activates the kinase. Cyclin I is an atypical kinase due to its predominant expression in postmitotic cells. Upon activation, the Cdk5/Cyclin I complex appears to promote cell survival and mainly function in podocytes (Brinkkoetter et al. 2009). However, Cdk5 function is best understood in the context of neuronal development.

1.2.2 Cdk5 is essential for neuronal development

Cdk5 has been most extensively studied in brain neurons due to the high levels of Cdk5 activity and Cdk5 activator expression there (Su and Tsai 2011, Tsai et al. 1993). Cdk5-null mice display perinatal lethality and severe disruption of neuronal layering of the cerebral cortex, hippocampus, cerebellum, and olfactory bulb (Ohshima et al. 1996). In contrast, mice null for p35 are viable and have only minor disruption of neuronal layering in the hippocampus and cerebellum, though layering in the cerebral cortex is similar to that in Cdk5-null mice (Chae et al. 1997). No obvious defects were observed in p39-null mice. However, when mice deficient for both p35 and p39 were studied, a layering phenotype nearly identical to the Cdk5-null phenotype was observed (Ko et al. 2001). Moreover, lack of Cdk5 kinase activity and perinatal lethality were also reported. Together, these results suggested that p35 and p39 were the sole activators of

Cdk5 in the brain and that some functional redundancy may exist between p35 and p39. These studies also established a model in which p35 was considered the more critical Cdk5 activator in the brain.

The laminar defects in Cdk5-null and p35/p39-null mice pointed to a failure of the developing neurons to migrate through the subplate of the cerebral cortex (Ko et al. 2001, Ohshima et al. 1996). Loss of Cdk5 in the developing spinal cord also impedes the ability of neuronal subpopulations to migrate to the appropriate position (Yip et al. 2007). An additional study demonstrated that PKCδ stimulates radial migration of cortical neurons by stabilizing p35 (Zhao et al. 2009). Besides migration, Cdk5 is also an important regulator of neurite outgrowth. Similar to OLs, maturing neurons extend specialized processes, axons and dendrites, with growth cones at their ends. The leading edge of the growth cone is composed of a mix of filopodia that sense the environment and lamellipodia that move the growth cone. These unstable structures consist of actin along the edge of the growth cone and microtubules filling the cone that mediate their frequent advance and retraction along the surface of the growth cone. Colocalization of Cdk5 and p35 with the actin domain has been detected at axonal growth cones (Nikolic et al. 1996). Moreover, inhibiting Cdk5 activity causes reduced neurite outgrowth, while p35 overexpression to increase Cdk5 activity results in longer neurites. Cdk5 function in migration and neurite outgrowth is mediated by target phosphorylation. Specifically, much of Cdk5 function in cell migration and maturation is mediated by association with and phosphorylation of cytoskeleton-associated proteins. Sirt2 activity is inhibited by Cdk5, which facilitates neuronal migration and neurite extension (Pandithage et al. 2008). Phosphorylation of the microtubule-associated proteins Tau and MAP1B by Cdk5 mediate its effect on neurite outgrowth (Baumann et al. 1993, Paglini et al. 1998, Paudel et al. 1993, Pigino et al. 1997). Cdk5 is also a key regulator of synaptic plasticity. Phosphorylation of many regulators of the actin cytoskeleton mediates Cdk5 modulation of dendritic spine morphogenesis. Spine formation is attenuated in Cdk5-null mice. Phosphorylation of WAVE1 by Cdk5 inhibits the ability of WAVE1 to activate Arp 2/3 and

actin branching, which is critical to spine development (Kim Y. et al. 2006). In fact, loss of WAVE1 in mice results in a reduced number of mature dendritic spines.

In addition to neuronal process growth, Cdk5 and its activators, p35 and p39, localize to the pre- and post-synaptic compartments. In the presynaptic compartment, Cdk5 targets many proteins that are required for vesicle exocytosis, including components of the synaptic vesicle exocytosis machinery (Floyd et al. 2001, Lee et al. 2004, Matsubara et al. 1996), regulators of SNARE complexes that are necessary for neurotransmitter release (Fletcher et al. 1999), and a voltage-gated calcium channel that mediates calcium influx to trigger vesicle exocytosis (Tomizawa et al. 2002). Postsynaptic Cdk5 phosphorylates dopamine cyclic-AMP regulated phosphoprotein (DARPP-32) to dampen dopamine D1 receptor/protein kinase A (PKA) signaling upon chronic cocaine exposure (Bibb J. A. et al. 1999). Cdk5 in the postsynaptic compartment also regulates the synaptic recruitment and clustering of ion channels through phosphorylation of PSD-95 and NR2A (Morabito, Sheng, and Tsai 2004, Wang et al. 2003), a subunit of the *N*-methyl D-aspartate (NMDA) receptor. Therefore, Cdk5 is positioned to regulate synaptic plasticity from both pre- and post-synaptic compartments, as reviewed by Su et al. and Lai et al. (Lai and Ip 2009, Su and Tsai 2011).

Increasingly, studies are defining novel roles for Cdk5 in cells outside of the brain. Treatment with roscovitine, a pharmacological Cdk5 inhibitor, or siRNA-mediated Cdk5 knockdown reduces endothelial cell migration in culture and angiogenesis *in vivo* (Liebl et al. 2010). Treatment of lens epithelial cells with olomoucine, another pharmacological Cdk5 inhibitor, relieves Cdk5-dependent inhibition of Src, which likely mediates the increase in migration (Tripathi et al. 2008). Inhibition of Cdk5 using roscovitine induces apoptosis in many cells including basic fibroblast growth factor treated bovine aortic endothelial cells and retinal pigment epithelial cells (Sharma, Tuszynski, and Sharma 2004). Increased apoptosis is observed when p35-null podocytes are exposed to stress conditions, which can be rescued by p35 overexpression (Brinkkoetter et al. 2010). Overexpression of p35 in pancreatic β cells exposed to

high glucose decreased insulin secretion but increases apoptosis, which can be prevented by roscovitine treatment (Lilja et al. 2001, Ubeda, Kemp, and Habener 2004, Zheng et al. 2010b). In skeletal muscle, Cdk5 promotes myogenesis and phosphorylates titin, a giant elastic protein, to promote proper myofiber assembly and sarcomere structure (Fernando et al. 2009). In the immune system, p35-dependent Cdk5 activation is required for T cell receptor stimulated T cell activation. Inhibition of Cdk5 alters T cell proliferation after CD3/CD28 stimulation (Pareek et al. 2010). Roles for Cdk5 in cancer cell migration have also emerged, including human glioblastoma cells, pancreatic cancer cells, and prostate cancer cells (Feldmann et al. 2010, Liu et al. 2008, Strock et al. 2006). However, increasing interest has centered around the role of Cdk5 in OLs, resulting in work that has uncovered novel functions for Cdk5 in the brain.

1.2.3 Emerging evidence of crucial roles for Cdk5 in oligodendroglial and myelin development

Compared to the well-established functions of Cdk5 in neurons, the role of Cdk5 in OL development is much less understood. A recent report established the requirement of Cdk5 activity for gliogenesis (Petrik et al. 2013), the generation of astroglia and oligodendroglia along lineages involving a common glial-restricted progenitor cell, in early postnatal development. Cdk5 activity was first reported in OLs in 1998 by Tang et al. as they described the changes in Cdk2 and Cdk5 activity during OL differentiation (Tang, Strocchi, and Cambi 1998). This study and a 2007 report by Miyamoto et al. demonstrated increased Cdk5 activity during OL differentiation (Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998). Pharmacological and siRNA-mediated inhibition of Cdk5 demonstrated the requirement of Cdk5 for morphological differentiation of OLs and MBP expression. The same report also showed that siRNA-mediated knockdown of p35 inhibited OL differentiation. p39 was not studied in OLs by this study, likely due to the lack of commercially available antibodies against p39. However, our analysis revealed that the p35 siRNA used in this study also targeted p39 with equal efficiency (Bankston et al.

2013). Cdk5-dependent OL differentiation was also demonstrated to be mediated by phosphorylation of the focal adhesion protein paxillin (Miyamoto et al. 2007). The following year, Miyamoto et al. demonstrated that Cdk5 is also required for PDGF-stimulated OL migration using cell aggregates and Boyden chambers (Miyamoto, Yamauchi, and Tanoue 2008). As they now detected p39 in OLs, the effect of siRNA-mediated knockdown of p35 and p39 on migration was tested. Knocking down of p39, but not p35, significantly reduced PDGF-stimulated migration. Fyn mediated PDGF-stimulated OL migration and phosphorylated Tyr15 of Cdk5 as demonstrated by pharmacological and siRNA-mediated inhibition of Fyn. Activated Cdk5 phosphorylated WAVE2, which was required for PDGF-stimulated OL migration. However, these studies failed to clearly define the activator(s) responsible for Cdk5 function in OLs, leaving the question of whether neurons and OLs utilize similar mechanisms for Cdk5 activation unanswered. We address this question in Chapter 2 of this thesis. Two recent studies have demonstrated that conditional knockout of Cdk5 from cells of the forebrain or from OLs impairs OPC differentiation and myelin formation in mice. These studies presented evidence for utilization of related Cdk5 targets in regulation of neuronal and OL development by Cdk5. In Chapter 3, we present evidence that Cdk5 targets the glial-specific protein, QKI, which plays a critical role in OL and myelin development. The precise regulation and targeting of Cdk5 activity are crucial to the normal development and function of both neurons and OLs. Considering the increasing evidence of OL impairment in neurodegeneration, the role of Cdk5 in normal development of neurons and OLs as well as dysregulation of Cdk5 in neuronal and OL impairment are important questions warranting further study.

1.2.4 Cdk5 in neurodegeneration

Since its discovery, aberrant Cdk5 activation has been associated with a growing number of neurodegenerative disorders. The detection of the cytotoxic p25 in neurological disorders raises important questions about the role of Cdk5 dysfunction in the pathogenesis of these

diseases. Furthermore, the growing recognition of the importance of OL dysfunction in the etiology of many neurodegenerative disorders opens the possibility that Cdk5 in one or more cells of the brain may play a role in disease progression.

The detection of p25 accumulation in neurons containing neurofibrillary tangles in Alzheimer's disease (AD) brains suggests that Cdk5 dysregulation may be involved in AD pathology (Patrick et al. 1999, Tseng et al. 2002). Though there are conflicting reports on the levels of p25 in AD brains, data from animal models confirm the link between Cdk5 dysregulation and neuronal loss in AD (Cruz and Tsai 2004). In fact, selective inhibition of Cdk5/p25 complexes using a p5 fragment from p35 reduces Aβ-induced cortical neuron death (Zheng et al. 2010a). Generation of Aβ occurs by cleavage of amyloid precursor protein (APP) by β-secretase BACE1 and γ-secretase. Cdk5 activity can increase Aβ production by phosphorylation of STAT3, causing increased BACE1 transcription (Cruz et al. 2006, Fu et al. 2004, Wen et al. 2008a, Wen et al. 2008b). Besides generation of neurofibrillary tangles, Cdk5 also phosphorylates Tau, leading to microtubule destabilization (Baumann et al. 1993, Wen et al. 2008a). Moreover, knockdown of Cdk5 in triple-transgenic Alzheimer's mice reduces tau phosphorylation and neurofibrillary tangles (Piedrahita et al. 2010). Both APP and Tau are expressed in OLs (Richter-Landsberg 2001, Skaper et al. 2009). In addition, secretion of Aβ is detected from primary cultured OLs, which can be attenuated by treatment with α-, β-, or γ-secretase inhibitors (Skaper et al. 2009). Considering the abnormalities of OLs and white matter in AD patients and mouse models of AD (Desai et al. 2010, Desai et al. 2009, Gold et al. 2012, Jantaratnotai et al. 2003), whether dysregulation of APP and Tau in OLs contributes to AD pathology is an important question for future studies.

In Huntington's disease (HD), Cdk5 and p35 are reduced in late stage patients (Anne, Saudou, and Humbert 2007). Normally, Cdk5 phosphorylates the Huntington protein, htt, at Ser434 in the cytoplasm, which inhibits caspase cleavage of htt and lessens formation of toxic aggregates. DNA damage induces phosphorylation of htt at Ser1181 and Ser1201 by Cdk5 (Anne,

Saudou, and Humbert 2007). This phosphorylation protects striatal neuron cultures from mutant htt toxicity. Moreover, Cdk5-dependent disruption of microtubules inhibits mutant htt aggregate formation (Kaminosono et al. 2008). Striatal cultures also revealed that the p25/p35 ratio increases with expression of mutant htt (Paoletti et al. 2008). It is possible that formation of p25 favors nuclear Cdk5 activity, which cannot protect against the detrimental effects of mutant htt. Therapeutics to stimulate Cdk5 activity have been proposed as a strategy to counter neurodegeneration in HD. Since htt is expressed in neurons and glia, it is important to determine which cells are responsible for the pathogenesis of HD. Furthermore, understanding the mechanisms that regulate Cdk5 activity in neurons and OLs would represent a significant advance toward stimulating therapeutic Cdk5 activation to alleviate HD.

The neuronal loss in Parkinson's disease (PD) was attenuated by inhibition of Cdk5 activity in animal models of PD (Smith et al. 2003). Injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes Parkinson's-like symptoms in the brain, increases p25 levels and Cdk5 activity (Smith et al. 2003). Cdk5 deregulation appears to contribute to PD pathology through disruption of processes that would normally protect against PD. Cdk5 phosphorylates and inhibits antioxidative peroxidases and enzymes involved in DNA damage repair, making cells more vulnerable to oxidative stress in response to MPTP toxicity (Huang et al. 2010, Qu et al. 2007, Tian, Yang, and Mao 2009). Additionally, Cdk5 phosphorylates proteins regulating mitochondrial transmembrane potential and mitochondrial fission (Avraham et al. 2007, Fitzgerald et al. 2012, Rubio de la Torre et al. 2009). These data suggest that Cdk5 deregulation may play a role in creating the environment of high oxidative stress in PD. Finally, Cdk5 phosphorylates the prosurvival transcription factor MEF2D, which is associated with neuronal loss in MPTP toxicity, to inhibit its activity (Smith et al. 2006), leading to neuronal loss. Whether these Cdk5-dependent mechanisms contribute to the initial onset of PD is unknown. Additionally, whether Cdk5 deregulation in non-neuronal cells contributes to PD progression remains to be determined.

In multiple system atrophy (MSA), glial cytoplasmic inclusions (GCIs) form in OLs and cause OL abnormality and axonal damage. Cdk5 and p39 have been detected in GCIs, but the function of the Cdk5 pathway in MSA progression is unclear (Honjyo et al. 2001, Nakamura et al. 1998). In amyotrophic lateral sclerosis (ALS), loss of motor neurons in the spinal cord causes muscle atrophy, paralysis, and death within five years of disease onset (Walling 1999). Copper/zinc superoxide dismutase was identified as a gene with point mutations associated with ALS (Bowling et al. 1993). In a mouse model of ALS, p25 levels are increased in the spinal cord (Nguyen, Larivière, and Julien 2001). ALS is characterized by accumulation of neurofilaments (NFs) in the cell bodies. These NFs are highly phosphorylated, presumably by the increased Cdk5 activity. What role Cdk5 plays in ALS pathogenesis is an interesting question to be addressed in the future.

Recently, studies of the synaptic function of Cdk5 have revealed the requirement of Cdk5 for proper cognitive function. Initial studies demonstrated that roscovitine inhibited induction of long-term potentiation (LTP) in the CA1 region of the hippocampus (Li et al. 2001). Additionally, treatment with butyrolactone I, another Cdk5 inhibitor, attenuated context-dependent fear conditioning (Fischer et al. 2002). Later studies revealed that Cdk5 raises the threshold for LTP induction, but is not required for LTP induction (Hawasli et al. 2007, Wei et al. 2005). Spatial learning is enhanced in mice with conditional knockout of Cdk5 in the adult brain as well as transgenic mice expressing p25 (Angelo et al. 2003, Fischer et al. 2005, Hawasli et al. 2007). In contrast, spatial leaning was impaired in mice with conditional knockout of Cdk5 in the forebrain (Su et al. 2013). p35-null mice also have impaired long-term depression (LTD) and spatial learning (Ohshima et al. 2005). The apparent discrepancy in the role of Cdk5 activity in learning between p35-null and p25 overexpressing mice may be explained by differences in target selection between p35- and p25-activated Cdk5. Cdk5-dependent modulation of learning and synaptic plasticity appears to be mediated mainly by NMDA receptor (NMDAR) regulation. Cdk5 can promote the proteolysis of the NMDAR subunit NR2B and NMDAR endocytosis

through phosphorylation of the NMDAR scaffold PSD-95 (Hawasli et al. 2007, Zhang et al. 2008). Cdk5 also directly phosphorylates NR2A, another NMDAR subunit, to increase NMDAR activity (Li et al. 2001). Recent work uncovered a reduction of p35 in schizophrenia, a disorder involving cognitive impairment (Engmann et al. 2011). p35 heterozygous mice displayed cognitive phenotypes reminiscent of schizophrenia as well as protein expression changes similar to those found in post-mortem schizophrenic brains. Identifying the changes in Cdk5 target phosphorylation may aid our understanding of the mechanisms underlying impairment of cellular function as the result of Cdk5 dysregulation. Few targets of Cdk5 have been identified in OLs. OL development and function involves many proteins that are unique to the OL lineage. Whether any OL-specific Cdk5 targets exist that regulate OL/myelin development and/or myelin repair is unknown. The RNA-binding protein QKI, which is essential for OL and myelin development is on candidate protein. QKI belongs to the signal transduction activator of RNA (STAR) family, which interacts with RNA and signaling proteins to connect RNA homeostasis to extracellular cues. Whether QKI mediates the function of Cdk5 in regulating OL and myelin development is an intriguing question.

1.3 The function of the selective RNA-binding protein QKI in oligodendroglial and myelin development

1.3.1 QKI is essential for oligodendroglial lineage development and CNS myelinogenesis

Uncovering the important role of QKI in myelinogenesis began with the identification of the CNS dysmyelination mutant, *quakingviable* (*qk*^{v}), by Sidman, Dickie, and Appel in 1964 (Sidman, Dickie, and Appel 1964). Mice homozygous for the spontaneous recessive *qk*^{v} mutation present with severe tremors at about postnatal day 11 or 12, while heterozygous mice are phenotypically normal. Many mRNAs encoding myelin structural proteins, including MBP, are severely reduced in the brain and spinal cord of the *qk*^{v}/*qk*^{v} mice, which causes a 90% loss of myelin compared to wild type mice (WT) (Friedrich 1974, Li et al. 2000, Zhao et al. 2006b).

Furthermore, the minimal amount of myelin that does form fails to compact. Importantly, the number of OLs appears to be normal in areas of severe dysmyelination, indicating that loss of myelin is not due to the lack of oligodendrogliogenesis (Friedrich 1975).

It was not until 1992 that the qk^v mutation was finally identified as 1.2 mega base-pair deletion on chromosome 17 (Ebersole, Rho, and Artzt 1992, Ebersole et al. 1996). While not affecting the exons or introns of the *qkI* gene, the deletion in qk^v mice appears to remove key regulatory sequences that support *qkI* transcription specifically needed in the OL lineage. The diminished QKI expression specifically in qk^v/qk^v OLs as well as similar dysmyelination in mice carrying N-ethyl-N-nitrosourea (ENU)-induced mutations in the *qkI* gene suggested that deficiency of QKI results in CNS dysmyelination (Ebersole et al. 1996, Justice and Bode 1986). Although the qk^v lesion also deletes the *pacrg* and part of the *parkin* genes, the fact that expression of a *qkI*-transgene in OLs was sufficient to rescue the failure in myelination clearly demonstrated that dysregulation of the *qkI* gene underlies the dysmyelination phenotype and crucial function of QKI in CNS myelin development (Ebersole et al. 1996, Zhao et al. 2006b). More recent discoveries demonstrated the essential roles of QKI in controlling proliferation, differentiation and maturation of OL progenitor cells (OPCs) and Schwann cells (Chen et al. 2007, Larocque et al. 2009). The precise balance of proliferation and differentiation of OPCs ensures a sufficient number of myelinating cells and the proper timing of myelination (Miller 2002). During brain development, *qkI* gene products are initially detected in neural stem cells that give rise to both the neuronal and glial (astroglia and oligodendroglia) lineages (Hardy 1998). Upon lineage specification, *qkI* expression is diminished in the neuronal lineage, but maintained in glia. It was postulated that QKI governs neural cell fate decisions. However, more studies are needed to understand the functional requirement of QKI in this process. Together, these data reveal that QKI is a critical factor regulating OL and myelin development at multiple stages (more details are reviewed in (Bockbrader and Feng 2008)). Consistent with the critical function of QKI for OL/myelin development in mice, QKI dysfunction in white matter appears to be

involved in impairment in cognitive diseases such as schizophrenia and depression (Aberg et al. 2006a, Aberg et al. 2006b, Haroutunian et al. 2006, Klempan et al. 2009, Lauriat et al. 2008).

Moreover, the *qkI* gene is widely expressed in various tissue types (Ebersole et al. 1996, Li et al. 2003). QKI could therefore exert profound influence on cell development, including functions outside its well-established role in the nervous system. Indeed, conventional knockout of the *qkI* gene in mice results in early embryonic lethality, due to severe developmental failure in many tissues including neural tube, cardiovascular system and smooth muscle (Ebersole et al. 1996, Li et al. 2003). As a matter of fact, QKI is a key regulator of the contractile phenotype of vascular smooth muscle cells (VSMCs) (van der Veer et al. 2013). Previous work has also revealed the essential role of QKI in muscle cell differentiation (Hall et al. 2013, Li et al. 2003). In astrocytes, QKI regulates the expression of glial fibrillary acidic protein (GFAP) (Radomska et al. 2013). QKI delays macrophage differentiation upon stimulation by CCAAT/enhancer binding protein (CEBPα) (Fu et al. 2012). More recent studies suggest that QKI not only plays important roles in controlling normal cell growth and development, but may also contribute to tumorigenesis in various cell types, including human glioma and colon cancer (Ichimura et al. 2006, Li et al. 2002, Mulholland et al. 2006, Yang et al. 2010, Yin et al. 2009). How QKI may govern normal development and furthermore how QKI abnormality may be involved in pathogenesis of cancer and developmental diseases are intriguing questions that warrant rigorous investigation.

1.3.2 The balance of nuclear and cytoplasmic QKI isoforms regulates oligodendroglial development

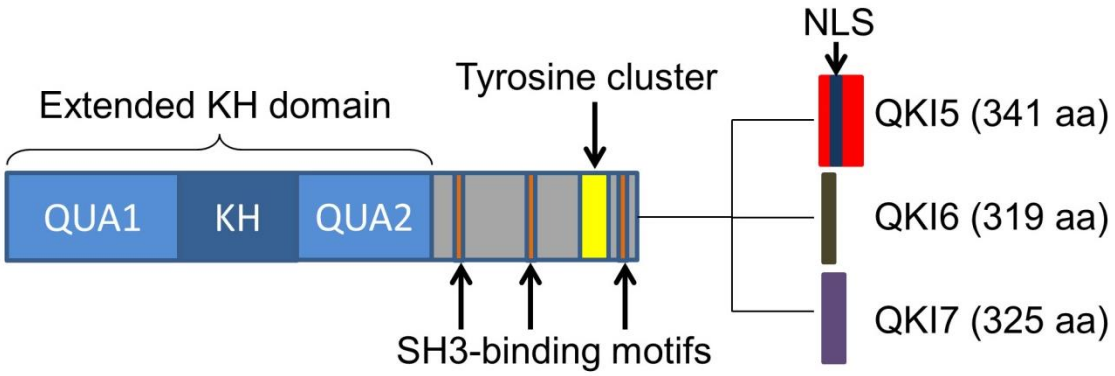
In 1996, the Artzt group cloned the mouse *qkI* gene (Ebersole et al. 1996). A *qkII* gene was suggested from the same locus, but never proved. Soon after, the Xenopus *QkI* homologue and Drosophila *QkI* homologue named held out wings (*How*) were cloned, followed by the isolation of human *QKI* several years later (Li et al. 2002, Zaffran et al. 1997, Zorn and Krieg

1997). The coding sequence and genetic structure of the *qkI* gene (Figure 1-4) are highly conserved in mammals (Kondo et al. 1999, Li et al. 2002). Alternative splicing of the 3′ coding exons produces three major *qkI* isoforms, named *qkI5*, *qkI6*, and *qkI7* based on the length of the corresponding mRNAs, which encode the corresponding QKI proteins (Ebersole et al. 1996). The shared N-terminus of all QKI isoforms contains an extended hnRNP K homology (KH) domain responsible for RNA-binding. This extended KH domain is composed of a core KH domain flanked by a dimerization (QUA1) domain and an RNA binding stabilization domain (QUA2). The subcellular localization of the QKI isoforms is determined by their alternative C-terminal tails. QKI5 displays predominantly nuclear localization at steady-state, due to a nuclear localization signal (NLS) found in its C-terminal sequence, despite its ability for nuclear-cytoplasmic shuttling (Wu et al. 1999). QKI6 and QKI7 are mainly detected in the cytoplasm. Thus, although the shared extended KH domain imparts similar selectivity and affinity to the mRNA ligands *in vitro* (Galarneau and Richard 2005), the nuclear and cytoplasmic QKI isoforms are thought to exert differential and perhaps even opposing influence on the same mRNA ligands.

During postnatal development, the mRNAs encoding cytoplasmic QKI isoforms are gradually increased during accelerated myelin formation (Hardy et al. 1996). Meanwhile, the nuclear QKI5 declines. The reciprocal regulation of the nuclear and cytoplasmic QKI isoforms suggests their differential function, which is carried out by controlling protein expression from its downstream mRNA targets via multiple post-transcriptional mechanisms. In the qk^{v}/qk^{v} mice, the reduction of QKI6 and QKI7 is much more severe than the reduction of QKI5 (Hardy et al. 1996). A working hypothesis postulates that QKI5 acts to maintain OL progenitor cells in proliferation and early differentiation state while QKI6 and QKI7 drive OL maturation and myelin synthesis.

Figure 1-4: QKI protein structure and isoform generation. The majority of QKI sequence is shared between all three major isoforms. This includes the extended hnRNPK homology (KH) domain that mediates RNA-binding and is composed of the KH domain with flanking QUA1 and QUA2 domains. QUA1 contains sequence necessary for QKI dimerization while QUA2 participates in binding of the RNA. Between the extended KH domain and the end of shared sequence are sequences matching the PxxP SH3-domain binding motifs and a cluster of tyrosines that can be phosphorylated by Src-PTKs to attenuate the RNA-binding ability of QKI. Alternative splicing generates unique C-termini for the three QKI isoforms. The C-terminal sequence of QKI5 contains a nuclear localization sequence (NLS) that leads to nuclear QKI5 localization at steady-state compared to the cytoplasmic localization of QKI6 and QKI7.

Figure 1-4


NLS
Tyrosine cluster
Extended KH domain
QUA1
KH
QUA2
QKI5 (341 aa)
QKI6 (319 aa)
QKI7 (325 aa)
SH3-binding motifs

A consensus RNA motif identified for QKI binding *in vitro*, named QKI recognition element (QRE), is found in over 1000 mRNA species (Galarneau and Richard 2005). The QRE was defined as NACUAAY-N_{1-20}-UAAY, where Y represents pyrimidines. Many of these putative QKI targets encode proteins that play key roles in cell growth and differentiation, such as $p27^{kip1}$ that controls cell cycle exit (Larocque et al. 2005). In qk^{v}/qk^{v} mice, the mRNA and protein levels of MBP are severely reduced compared to WT (Delassalle et al. 1981, Li et al. 2000). Aberrant posttranscriptional regulation of the MBP mRNA is thought as an important mechanism that underlies the dysmyelination phenotype in qk^{v}/qk^{v} mice, as suggested by the normal transcription of the MBP gene in qk^{v}/qk^{v} mice (Li et al. 2000). Indeed, QKI associates with the 3' UTR of MBP mRNA, which increases MBP mRNA stability (Li et al. 2000, Zhang et al. 2003). The mRNA encoding MAP1B is another target of QKI with important roles in OL process development and myelin sheath formation (Zhao et al. 2006a). QKI binds the 3'UTR of MAP1B and siRNA-mediated knockdown of QKI reduces MAP1B mRNA stability. MAP1B expression is upregulated during OL differentiation, and loss of MAP1B causes hypomyelination in the CNS (Meixner et al. 2000, Wu et al. 2001, Zhao et al. 2006a).

Other key regulators of myelin development, which are not direct targets of QKI, still rely on QKI for proper regulation of their expression. The gene encoding MAG produces two alternatively spliced isoforms, the long isoform (L-MAG) and the short isoform (S-MAG) (Tropak et al. 1988). S-MAG is produced by inclusion of alternative exon 12, which contains an early stop codon. Normally, S-MAG is expressed at low levels in early development and L-MAG is the predominant isoform (Quarles 2007). In qk^{v}/qk^{v} mice, the isoform ratio is reversed, with S-MAG being more abundant that L-MAG (Fujita et al. 1990). Nuclear QKI5 was demonstrated to associate with an intronic splicing element in MAG pre-mRNA to inhibit exon 12 inclusion in Cos7 cells (Wu et al. 2002). However, more recent studies demonstrated that it was the QKI cytoplasmic isoforms that play major roles to regulate MAG alternative splicing by controlling the expression levels of splicing factors which target MAG pre-mRNA (Zhao et al. 2010).

Alternative splicing of PLP is also regulated by QKI. The *plp* gene generates two isoforms, PLP and DM20. DM20 appears earlier in development that PLP, PLP is present at higher concentrations than DM20 in CNS myelin (Nave and Milner 1989). As with MAG, the isoform ratio of PLP/DM20 is altered in qk^{v}/qk^{v} mice, but the PLP/DM20 isoform ratio is rescued upon expression of a QKI6 transgene in OLs (Zhao et al. 2006b). Whether PLP/DM20 alternative splicing is regulated by similar mechanisms controlling MAG alternative splicing will be addressed by future studies.

The importance of maintaining a functional balance between the nuclear and cytoplasmic QKI isoforms is suggested by molecular defects in the qk^{v}/qk^{v} mice. The preferential reduction of cytoplasmic QKI isoforms in OLs of these mice leaves QKI5 as the predominant isoform. This leads to nuclear retention of MBP mRNA as observed by *in situ* hybridization (Larocque et al. 2002). Furthermore, expression of exogenous QKI5 also causes nuclear retention of MBP mRNA in COS cells and reduced MBP protein in primary OLs (Larocque et al. 2002). On the other hand, coexpression of cytoplasmic QKI6 and QKI7 returns MBP mRNA to the cytoplasm. These experiments highlight the need for the functional balance and perhaps cooperation between nuclear and cytoplasmic QKI isoforms to regulate the subcellular localization and eventual translation of target mRNAs. Besides the balance of isoforms, QKI function is also regulated by cellular signaling molecules, notably the Src family of protein tyrosine kinases (Src-PTKs).

1.3.3 QKI as a Signal Transduction Activation of RNA (STAR) family member

QKI and Src-associated protein in mitosis 68 kDa (SAM68) contain predicted features that are typical of RNA-binding proteins as well as signaling molecules, which prompted the idea that these proteins may function to connect cell signaling directly to mRNA homeostasis. A growing list of proteins sharing the same feature has been discovered. This family is named the Signal Transduction Activation of RNA (STAR) (Vernet and Artzt 1997). QKI contain several putative Src-homology 3 (SH3)-binding motifs that may interact with signaling factors, as well as

a tyrosine cluster that serves as the phosphorylation sites for Src-PTKs. Indeed, phosphorylation of QKI was observed in transfected cells, brain lysates, and compact myelin membrane isolations (Zhang et al. 2003). Additional experiments determined that the C-terminal tyrosine cluster of QKI mediated phosphorylation by Src-PTKs. Sam68 can also be tyrosine-phosphorylated by Src-PTKs, which alters the RNA binding activity of Sam68 (Shen et al. 1999). Unlike SAM68, QKI did not form a stable complex with Src-PTKs (Zhang et al. 2003). However, mutation of one of the SH3-binding motifs in QKI reduced Src-dependent QKI phosphorylation. Functionally, Src-dependent phosphorylation reduced the ability of QKI to bind MBP mRNA. In addition, phosphorylation of SAM68 by Erk1/2 induces nuclear export of SAM68, which then binds polysomes in the cytoplasm (Paronetto et al. 2006). Phosphorylation of the QKI homolog, held out wings (HOW) by Erk enhances HOW dimer formation (Nir et al. 2012). Whether QKI can be phosphorylated by serine-threonine kinases in mammals, especially in myelinating glia, still remains elusive.

Several Src-PTKs are expressed in OL progenitor cells. However, Fyn is the only Src-PTK member whose activity and expression are increased upon OL differentiation (Osterhout et al. 1999), accompanied with a general down-regulation of the rest of the Src-PTKs (Lu et al. 2005). Pharmacological inhibition and siRNA-mediated knockdown of Fyn attenuate OL differentiation (Colognato et al. 2004, Osterhout et al. 1999), indicating the essential role of Fyn in early OL development. However, upon the initiation of active myelin formation, Fyn activity markedly declines (Umemori et al. 1994), which is important for accelerated expression of myelin structural proteins such as MBP (Lu et al. 2005). In mutant mice that lack Fyn activity, a significantly slower accumulation of MBP and hypomyelination are observed, regardless of the normal MBP expression at the early phase of myelination. During the early phase of OL progenitor cell development, the nuclear QKI5 is the predominant isoform (Chen et al. 2007, Hardy et al. 1996), that stabilizes the bound mRNA ligands. Known QKI targets include mRNAs that encode key proteins for controlling cell cycle progression and morphogenic differentiation,

represented by $p27^{kip1}$ and the microtubule associated protein 1B (MAP1B), respectively (Larocque et al. 2005, Zhao et al. 2006a). Many of these QKI targets decline in later development, yet accurate timing for their transient expression is critical in governing proliferation, early differentiation and migration of OL progenitor cells. QKI targets that function later in myelination, such as the MBP mRNA, are either at low levels or not expressed. The vigorous increase of Fyn activity upon OL differentiation may help to release mRNAs from QKI5-dependent nuclear retention, which allows increased production of the corresponding proteins in the cytoplasm.

During OL maturation and active myelin formation, the nuclear QKI5 is markedly reduced, concomitant with increased cytoplasmic QKI isoforms (Hardy et al. 1996). This should attenuate the nuclear retention of QKI target mRNAs. At this stage, QKI target mRNAs encode many myelin structural proteins. Transcription of these myelin structural genes is markedly upregulated and a rapid accumulation of these QKI target mRNAs to exceptionally high levels is essential for accelerated myelin synthesis (Campagnoni and Macklin 1988). Despite the markedly enhanced transcription, these myelin structural mRNAs require protection by cytoplasmic QKI for survival from nuclease attack. QKI deficiency destabilizes these mRNAs, which in turn abrogates myelination in the qk^{v}/qk^{v} mice (Li et al. 2000). Expression of the cytoplasmic QKI6 isoform in OLs can rescue the aforementioned defects (Zhao et al. 2006b), indicating the functional requirement of cytoplasmic QKI in later development. Importantly, Fyn activity markedly declines in the developing myelin (Lu et al. 2005, Umemori et al. 1994). Because Fyn negatively regulates QKI-MBP mRNA interaction (Zhang et al. 2003), the developmentally programmed down-regulation of Fyn offers a mechanism to increase the RNA-binding activity of QKI, which protects the MBP mRNA and accelerates the expression of myelin basic protein and myelin synthesis. Consistent with this idea, lack of the Fyn-QKI mediated acceleration mechanism leads to slow accumulation of the MBP mRNA, delayed myelin development and

hypomyelination in both the fyn knockout mice and the qk^{v}/qk^{v} mutant (Li et al. 2000, Lu et al. 2005), with a more severe phenotype in the qk^{v}/qk^{v} mutant. Besides stabilization of mRNA ligands, the cytoplasmic QKI6 isoform can also act as a translation suppressor (Lakiza et al. 2005, Saccomanno et al. 1999). However, whether Fyn also regulates translation of QKI targets still remains unknown.

Src-PTKs represent the largest family of non-receptor tyrosine kinases, which play crucial roles to control a diverse array of biological functions including proliferation, differentiation, cell shape, motility, migration, angiogenesis and survival (Frame 2002, Wheeler, Iida, and Dunn 2009, York and O'Malley 2010). Numerous extracellular signals directly or indirectly lead to Fyn activation in OLs, which in turn controls multiple aspects of OL and myelin development (Feng and Bankston 2010). Many growth factors required for OL progenitor cell survival trigger Fyn activation, such as the insulin-like growth factor-I (IGF-I) and the platelet-derived growth factor (PDGF) (Cui et al. 2005, Miyamoto, Yamauchi, and Tanoue 2008). In addition, PDGF is well characterized for its function in Fyn-dependent migration of OL progenitor cells (Wu et al. 2002). During normal brain development as well as lesion repair, developing OLs migrate long distances to reach the site of myelin formation (Jarjour and Kennedy 2004, Miller 2002). Upon binding to its receptor tyrosine kinase, PDGF triggers phosphorylation-dependent Fyn activation, which in turn activates the serine/threonine kinase Cdk5 (Miyamoto, Yamauchi, and Tanoue 2008). Cdk5 is capable of phosphorylating actin and microtubule associated proteins (Miyamoto, Yamauchi, and Tanoue 2008, Su and Tsai 2011), including the microtubule associated protein 1B (MAP1B) (Hahn et al. 2005), a key factor that controls microtubule assembly and stabilization to drive early differentiation and migration of neurons and OLs (Gonzalez-Billault et al. 2005, Zhao et al. 2006a). Interestingly, our previous studies indicate that *MAP1B* mRNA is a target of QKI. Stabilization of *MAP1B* mRNA by QKI is an important mechanism for the vigorous upregulation of MAP1B in OL differentiation (Zhao et

al. 2006a). Presumably, Fyn-mediated QKI phosphorylation may contribute to MAP1B regulation. Thus, MAP1B may be a common target for PDGF-Fyn signaling via multiple mechanisms involving Cdk5 and QKI.

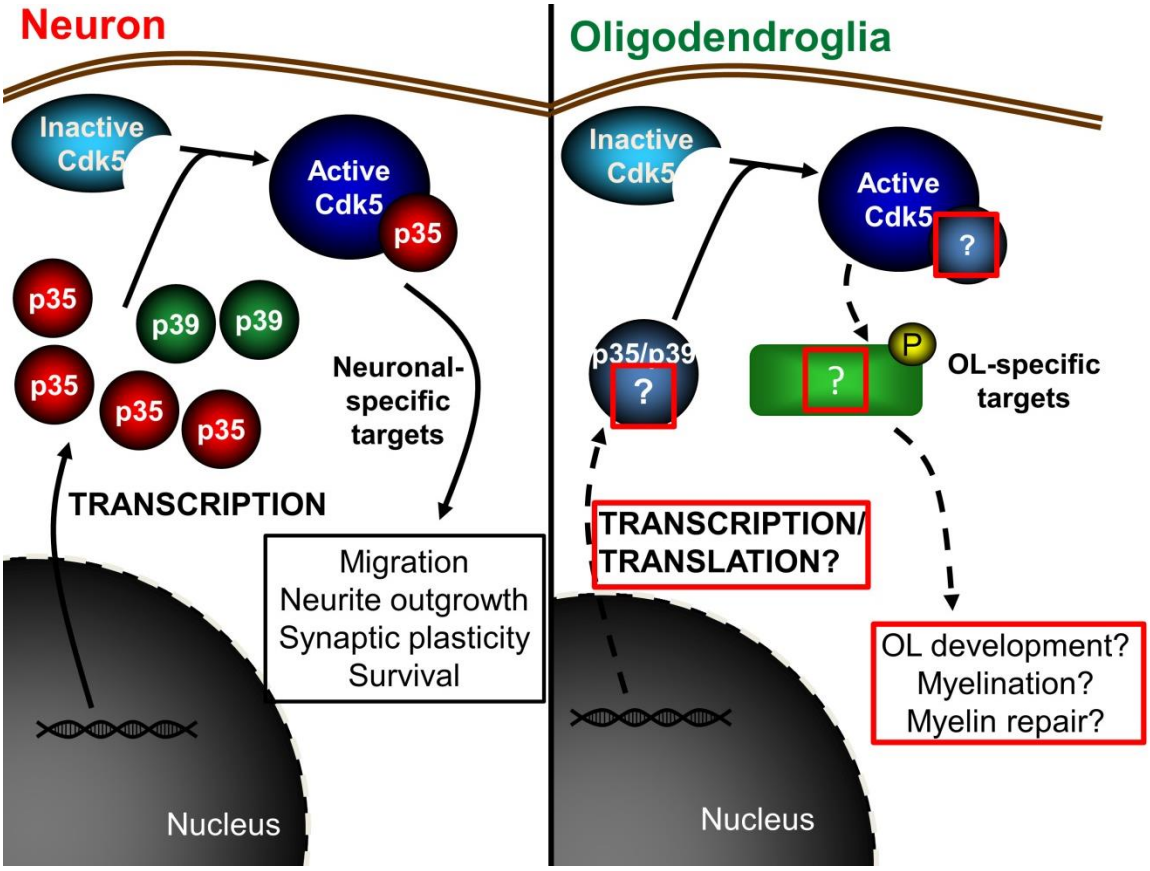
1.4 Summary

Proper development of the OL lineage is required the formation of myelin, which facilitates fast and efficient saltatory conduction along neuronal axons and neurotrophic and metabolic support for axonal integrity. Besides classic demyelinating disorders, such as multiple sclerosis, impaired OL development and function is an increasingly recognized contributor to the pathogenesis of neurodegenerative disorders (Bankston, Mandler, and Feng 2013). Furthermore, signaling pathways performing critical functions in neuronal development, such as Cdk5, are found to play key roles in OL development(Bankston et al. 2013, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013). However, OL-specific regulators and functional targets of Cdk5 activity remain elusive. This dissertation focuses on elucidating the mechanisms regulating Cdk5 activation in OLs and identifying functional targets that mediate Cdk5-dependent OL development, myelination, and myelin repair by answering the questions diagrammed in Figure 1-5. Cdk5 activity is essential for OL migration and differentiation as well as myelin development(Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013). However, whether similar or distinct mechanisms to those in neurons control Cdk5 activation in OLs is unknown. Moreover, few protein targets that mediate Cdk5 function in OLs have been identified. Many targets of Cdk5, which were identified in neurons, are also expressed in OLs, including many cytoskeleton-associated proteins. Whether Cdk5 function in OLs involves targeting of OL-specific proteins, which are not shared by neurons requires further investigation. OL and myelin development relies on many proteins which are unique to the OL or glial lineages with limited expression in other cells of the brain. One such protein is QKI, an RNA-binding protein playing crucial roles in OL and myelin development (Chen et al. 2007,

Zhao et al. 2006b). QKI contains sequences typical of proteins involved in both RNA-binding and signal transduction, allowing QKI to link cellular signaling cascades to RNA homeostasis (Vernet and Artzt 1997). Determining whether QKI can mediate the function of Cdk5 in OL and myelin development is an interesting question that would link two critical pathways in the OL lineage and provide new areas of research into the role of Cdk5 in modulating expression of QKI targets and to identify other OL-specific targets of Cdk5. Overall, a deeper understanding of the factors that regulate Cdk5 activation and mediate Cdk5 function in OLs could provide novel strategies for lineage-specific modulation of the Cdk5 pathway in neurological disorders involving OL impairment.

Figure 1-5: Questions to be addressed by this dissertation In neurons (left), predominantly transcription-based mechanisms are demonstrated to stimulate Cdk5 activator expression, which in turn increases Cdk5 activity. Active Cdk5, thought to be predominantly controlled by p35, phosphorylates downstream targets, including many neuronal-specific targets to regulate migration, neurite outgrowth, synaptic plasticity, and cell survival. In oligodendroglia (OL), Cdk5 is essential for differentiation, myelin formation, and possible myelin repair (right). However, many questions remain that this dissertation will attempt to address: What activator(s) mediate Cdk5 activation in OLs? What mechanisms control Cdk5 activator expression and thus Cdk5 activity? Can any OL-specific Cdk5 targets be identified that mediate Cdk5 function in OL/myelin development and/or myelin repair?

Figure 1-5


Neuron
Oligodendroglia
Inactive Cdk5
Active Cdk5
p35
p39
p39
p35
p35
p35
p35
Neuronal-specific targets
TRANSCRIPTION
Migration
Neurite outgrowth
Synaptic plasticity
Survival
Nucleus
Inactive Cdk5
Active Cdk5
?
p35/p39
?
?
P
OL-specific targets
TRANSCRIPTION/ TRANSLATION?
OL development?
Myelination?
Myelin repair?
Nucleus

Chapter 2: p39, the primary activator for cyclin-dependent kinase 5 (Cdk5) in oligodendroglia, is essential for oligodendroglial differentiation and myelin repair

A portion of this chapter is published as:

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2.1 Introduction

Normal brain function relies upon proper development and integrity of neurons and oligodendroglia (OLs), the myelinating glia that ensheath neuronal axons and enable rapid information flow in the CNS. OL impairment has become an increasingly recognized pathophysiological feature of numerous neurological disorders originally believed to result from neuronal defects alone, including Alzheimer's disease and schizophrenia (Bankston, Mandler, and Feng 2013, Fields 2008, Gold et al. 2012, Ren, Wang, and Xiao 2012). Moreover, key components of cell signaling pathways traditionally thought to control neuronal development and function have now been found expressed and playing critical roles in OLs as well. Thus, whether neuronal and OL lineages share common molecular pathways that govern their development and cooperation is an intriguing question and a growing area of research in neuroscience.

One such signaling molecule is Cyclin-dependent kinase 5 (Cdk5), an unconventional Cdk member that does not control cell cycle progression, but primarily functions in post-mitotic cells (Heuvel and Harlow 1993, Lew et al. 1992, Meyerson et al. 1992, Su and Tsai 2011, Zheng, Leung, and Liem 1998). Despite the ubiquitous expression and increasingly recognized broad function of Cdk5 in numerous cell types, the highest activity of this kinase is detected in the brain (Su and Tsai 2011, Tsai et al. 1993). Earlier studies demonstrated essential functions of Cdk5 in neuronal migration, neural network formation, and synaptic plasticity in mammalian brains (Su and Tsai 2011). Besides the traditional view of neuronal centric roles of Cdk5, emerging evidence suggests that Cdk5 also governs the development of oligodendroglia progenitor cells (OPCs) (Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Tang, Strocchi, and Cambi 1998). Similar to brain neurons, OPCs migrate to distant brain areas and become mature OLs to myelinate neuronal axons (Miller 2002). However, unlike neuronal development in the embryonic brains, the most vigorous OPC development occurs postnatally (Miller 2002). Loss of Cdk5 function affects OPC migration and differentiation in culture and results in CNS hypomyelination (He et al. 2010, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008,

Yang et al. 2013). However, molecular mechanisms that regulate Cdk5 function in neurons and OLs remain elusive.

The activity of Cdk5 is controlled by the available amounts of two activator homologs, p35 and p39 (Humbert, Dhavan, and Tsai 2000, Tsai et al. 1994, Zheng, Leung, and Liem 1998). Mice lacking both p35 and p39 displayed nearly identical defects in embryonic brain development and perinatal lethality as those found in the Cdk5-null mice (Ko et al. 2001, Ohshima et al. 1996), indicating that p35 and p39 together are solely responsible for Cdk5 activity and function in the brain. However, the Cdk5 activators display distinct temporal expression profiles, suggesting non-redundant roles for each activator over the course of brain development that have yet to be identified (Cai et al. 1997, Delalle et al. 1997, Honjyo et al. 1999, Wu et al. 2000, Zheng, Leung, and Liem 1998). p35 is abundantly expressed in embryonic neurons and is essential for Cdk5 activation in these cells which governs corticogenesis (Ohshima et al. 1996, Tsai et al. 1994). Conventional p35 knockout mice display impaired neuronal migration and cortical organization during embryonic development, similar to but less severe than that in Cdk5 null mice (Chae et al. 1997, Ohshima et al. 1996). In contrast to the early function of p35 in the embryonic brain, p39 levels are elevated and most prominently expressed in the postnatal brain (Humbert, Lanier, and Tsai 2000). Nonetheless, mice lacking p39 alone showed no overt neuronal abnormalities (Ko et al. 2001). Thus, p35 is considered the most important Cdk5 activator in neurons whereas the functional importance of p39 remains undetermined.

We report here that neurons and OLs display distinct profiles of Cdk5 activator expression and regulation. In contrast to the major role of p35 in activating Cdk5 in neurons, p39 is the primary Cdk5 activator in OLs where p35 expression is negligible. In addition, selective upregulation of p39, but not p35, accompanies the increased Cdk5 activity during normal OPC differentiation in culture and CNS myelin development *in vivo*. Upregulation of p39 is also detected in OLs during myelin repair upon induced demyelination. Finally, we show that p39 plays essential roles in advancing OPC differentiation, and the loss of p39 leads to impairment in

myelin repair in the brain. Together, these studies suggest that p39 is the primary activator responsible for Cdk5 function in CNS myelinogenesis in normal and diseased brains.

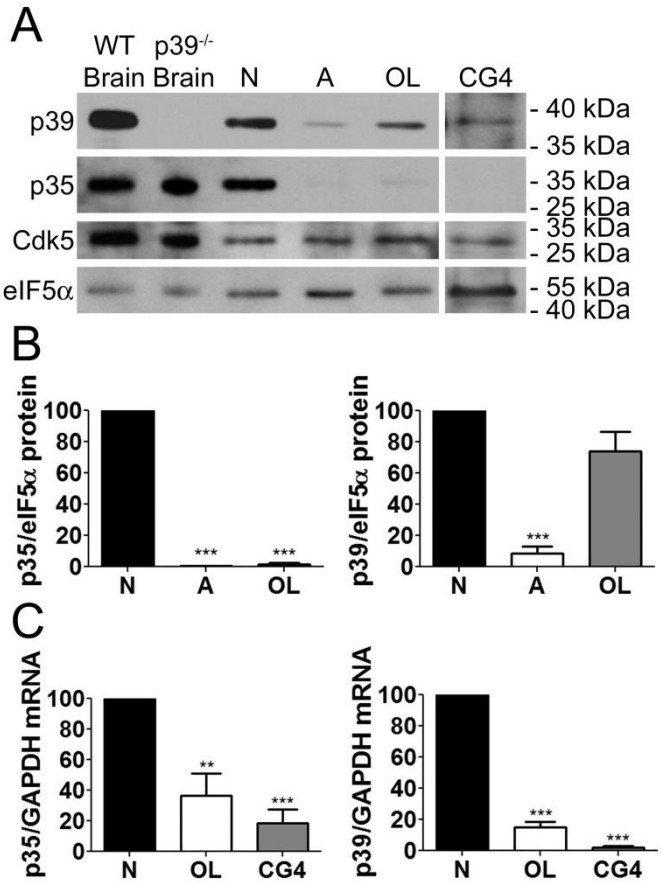
2.2 Results

2.2.1 Distinct expression profiles of p35 and p39 in neurons and OLs

Previous studies have demonstrated the essential function of Cdk5 in the development of neuronal and glial lineages. However, whether neurons and glia harbor similar or distinct expression profiles of p35 and p39 has not been defined. To address this question, we performed immunoblot analysis to detect Cdk5, p35, and p39 proteins using lysates derived from primary cultured rat neurons, astrocytes, and OLs (Figure 2-1A). The house keeping protein translation initiation factor 5α (eIF5α) was detected as a loading reference. On the same blot, lysates of $p39^{-/-}$ and WT mouse brains were loaded as specificity controls (Figure 2-1A). Densitometry of p35 and p39 in neurons from the same blot were used to assess the relative abundance of these proteins in OLs and astrocytes. Cdk5 was detected in all lysates examined. Both p35 and p39 were abundantly expressed in primary cultured neurons and the WT brain. In contrast, negligible amounts of p35 were detected in cultured OLs whereas p39 levels were 74±12% of that in neurons (Figure 2-1B). A similar pattern of Cdk5 activator expression was also observed in the OPC cell line CG4, which closely mimics molecular and cellular properties of primary OPCs in early differentiation (Louis et al. 1992). Among the examined neural cell types, astrocytes harbored the lowest levels of Cdk5 activators, with negligible amounts of p35 and p39 levels at 9±4% of that expressed in neurons (Figure 2-1A and B). The function of Cdk5 in normal astrocytes still remains elusive, although Cdk5 activities are clearly detectable in glioma cells (Filippova et al. 2012, Liu et al. 2008). These data suggest that in contrast to the more crucial role of p35 in neurons, p39 may function as the primary Cdk5 activator in OLs.

Figure 2-1: Differential Cdk5 activator expression in neurons and OLs. (**A**) Lysates from WT and $p39^{-/-}$ brain, primary cultured rat neurons (N), astrocytes (A), oligodendrocytes (OL), and CG4 cells were subjected to immunoblot analysis of p39, p35, Cdk5, and eIF5α. All cells underwent 5 days of differentiation in culture. p39 antibody specificity was confirmed by the absence of signal in $p39^{-/-}$ lysates. (**B**) Densitometer reading of p35 (left) and p39 (right) proteins are normalized to the housekeeping protein eIF5α as a loading reference. The normalized level of p35 and p39 protein in neurons on the same immunoblot was set as 100%, respectively, for estimation of relative abundance of these proteins in neurons and glia. (**C**) Total RNA was extracted from cultured neurons (N), oligodendrocytes (OL), and CG4 cells at DIV5 and qRT-PCR was performed for p35, p39, and GAPDH. The levels of p35 (left) and p39 (right) mRNAs relative to the GAPDH mRNA are graphically displayed. The relative mRNA level in neurons was set as 100% in each replicate experiment for normalization. For (**B**) and (**C**), three independent batches of cultures were used for immunoblots (n=3). The data are reported as mean ± SE ($p<0.0005$, one-way ANOVA; $^{**}p<0.01$, $^{***}p<0.001$, Tukey's post-test).

Figure 2-1

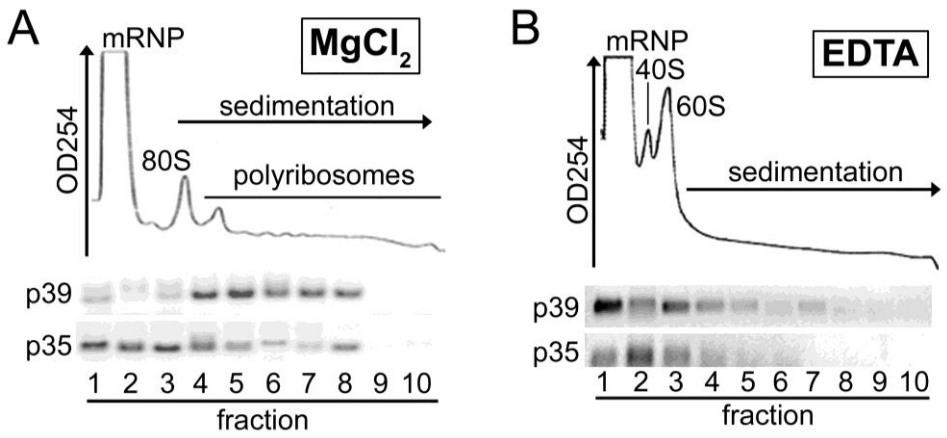

A
WT Brain
p39-/- Brain
N
A
OL
CG4
p39
p35
Cdk5
eIF5α
- 40 kDa
- 35 kDa
- 35 kDa
- 25 kDa
- 35 kDa
- 25 kDa
- 55 kDa
- 40 kDa
B
p35/eIF5α protein
p39/eIF5α protein
100
80
60
40
20
0
***
N
A
OL
C
p35/GAPDH mRNA
p39/GAPDH mRNA
**
***
N
OL
CG4

To explore molecular mechanisms that underlie the distinct protein levels of Cdk5 activators in neurons and OLs, we examined the expression levels of p35 and p39 mRNAs in neurons, OLs, and CG4 cells by qRT-PCR using specific primers. As shown in Figure 2-1C, the levels of p35 and p39 mRNAs in primary OPCs are 36±15% and 15±4% of the corresponding mRNA levels expressed in neurons, respectively. Similar levels of p35 and p39 mRNAs to those in OPCs were also detected in CG4 cells. Thus the mRNA levels could not explain the distinct expression profiles of p39 and p35 proteins in neurons and OLs. One possibility is that p39 and p35 mRNAs are differentially translated in OLs, leading to preferential p39 expression.

To test this hypothesis, we performed linear sucrose gradient fractionation, a common assay for determining endogenous mRNA translation efficiency based on their association with translating polyribosomes (Lau et al. 2010). CG4 cells that recapitulate the preferential expression of p39 protein in primary OLs (Figure 2-1A) were used to obtain sufficient amount of extracts for biochemical fractionation, which would be difficult to derive from primary cultured OLs. As shown in Figure 2-2A, 84.4% of p39 mRNA co-sediment with translating polyribosomes (fractions 4-10). The remaining p39 mRNA was detected in ribosome-free messenger ribonucleoprotein particles (mRNPs, fractions 1-3). In contrast, the majority of p35 mRNA was sequestered into mRNPs that are not engaged in translation. Moreover, when polyribosomes are dissociated by EDTA-treatment, p39 mRNA is shifted into mRNP fractions (Figure 2-2B), confirming the association of p39 mRNA with polyribosomes. These data suggest that active translation of p39, in contrast to translational suppression of p35, is an underlying mechanism for the preferential expression of p39 protein in OLs.

Figure 2-2: Active translation of p39 mRNA and translation suppression of p35 mRNA in CG4 cells. (**A** and **B**) Cytoplasmic RNA was isolated from CG4 cells and fractionated on a linear sucrose gradient in the presence of MgCl2 (**A**) or EDTA (**B**). Top: Plots of absorption at 254 nm (OD254) of linear sucrose gradients fractions. The direction of sedimentation, as well as absorption peaks for mRNP complexes, 40S and 60S ribosomal subunits, 80S monoribosome, and translating polyribosomes are indicated. Bottom: representative images of semi-quantitative RT-PCR products of p39 and p35 mRNA in each gradient fraction on agarose gel after electrophoresis.

Figure 2-2


A
mRNP
MgCl2
sedimentation
OD254
80S
polyribosomes
p39
p35
1 2 3 4 5 6 7 8 9 10
fraction
B
mRNP
40S
60S
EDTA
OD254
sedimentation
p39
p35
1 2 3 4 5 6 7 8 9 10
fraction

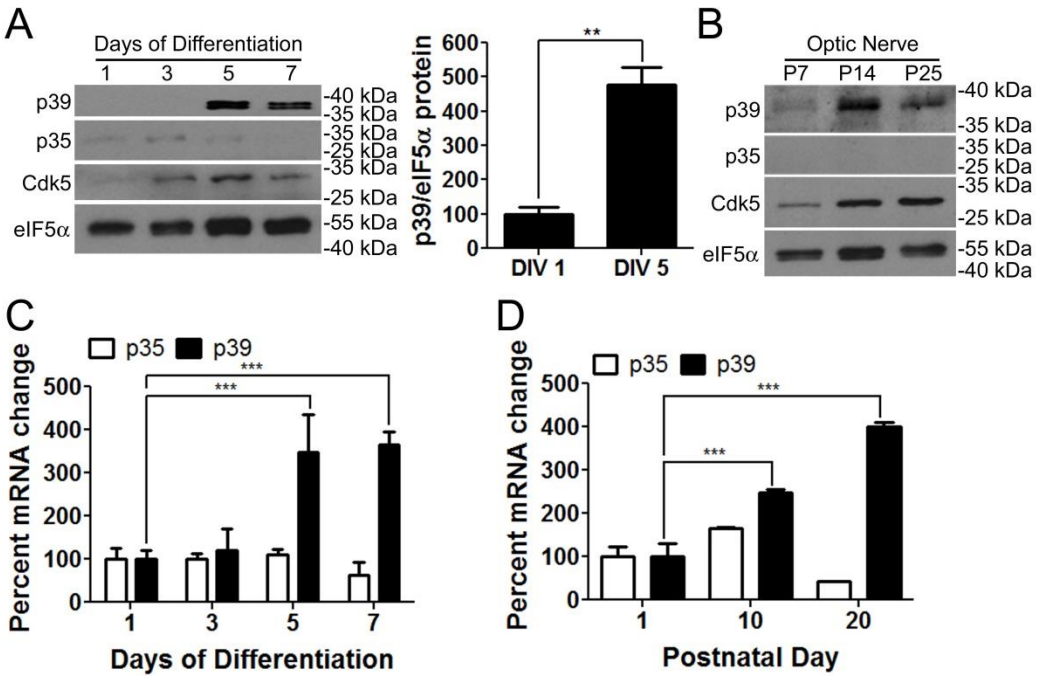
2.2.2 p39, but not p35, is upregulated during OL and myelin development

During OL differentiation, Cdk5 activity is markedly upregulated (Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998). To explore which activator is responsible for the enhanced Cdk5 activity, we analyzed the expression levels of Cdk5 and its activators during OL differentiation (Figure 2-3A, *left*). A notable increase of p39 protein was detected by immunoblot analysis accompanied by increased Cdk5 expression during OPC differentiation in culture. Densitometric analysis revealed a 5±0.5-fold increase of p39 protein in cells that had undergone 5 days of differentiation *in vitro* (DIV5) as compared to DIV1 (Figure 2-3A, *right*). To assess whether p39 protein is upregulated during OL and myelin development *in vivo*, optic nerves, highly enriched of OLs, were isolated from WT mice during the most vigorous myelinogenesis at postnatal days 7, 14, and 25. As shown in Figure 2-3B, a concomitant upregulation of Cdk5 and p39 proteins during myelin development was detected by immunoblot analysis in the optic nerves. Conversely, no appreciable changes of p35 protein levels were detected during OPC differentiation in culture (Figure 2-3A) or myelination of the optic nerve (Figure 2-3B).

To further investigate molecular mechanisms that underlie the selective upregulation of p39 during OL development, we performed qRT-PCR to quantify the levels of p39 and p35 mRNA during OL development in culture and *in vivo*. As shown in Figure 2-3C, p39 mRNA, but not p35 mRNA, was significantly upregulated by 3.5±0.9-fold in cultured OPCs after 5 days of induced differentiation. To determine whether p39 mRNA is upregulated in developing OLs *in vivo*, we FACS-isolated brain OLs from mice, which express EGFP driven by the OL-specific CNP promoter (Zhu et al. 2012), at postnatal days 1, 10, and 20 of myelinogenesis. A similar extent of p39 mRNA upregulation, 2.5±0.06-fold at P10 and 4±0.1-fold at P20, was detected in OLs isolated from the brain during *de novo* myelin development (Figure 2-3D). Thus, the developmentally programmed upregulation of p39 mRNA leads to increased production of p39 protein, which underlies the increase of Cdk5 activity during OL and myelin development reported by previously studies (Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998).

Figure 2-3: Selective upregulation of p39 during OL differentiation and myelin development. (**A**) Lysates from primary cultured rat oligodendrocytes at the indicated days of differentiation were subjected to immunoblot analysis of p39, p35, Cdk5, and eIF5α. A representative blot is shown (left). Band intensities were quantified by densitometry, and the levels of p39 relative to eIF5α from immunoblots in three batches of cultured oligodendrocytes at DIV1 and DIV5 (n=3) are shown (right). The densitometer reading in DIV1 cells are set as 100% to assess percent changes from independent experiments. The data are reported as mean ± SE (**$p<0.01$ by two-tailed *t*-test). (**B**) Lysates from optic nerves, highly enriched of myelinating oligodendrocytes, were isolated at the indicated postnatal days during myelin development and subjected to immunoblot analysis of p39, p35, Cdk5, and eIF5α. (**C** and **D**) Total RNA was extracted from primary cultured rat oligodendrocytes harvested at the indicated days of differentiation (**C**) and FACS-isolated oligodendrocytes from mouse brains during myelin development at the indicated postnatal days (**D**). Three independent samples were collected (n=3). qRT-PCR was performed for p35, p39, and GAPDH. The levels of p35 and p39 relative to GAPDH are shown. qRT-PCR reading in Day 1 samples are set as 100%. The data are reported as mean ± SE ($p<0.01$, two-way ANOVA; ***$p<0.001$, Bonferroni's post-test).

Figure 2-3


A
Days of Differentiation
1
3
5
7
p39
p35
Cdk5
eIF5α
-40 kDa
-35 kDa
-35 kDa
-25 kDa
-35 kDa
-25 kDa
-55 kDa
-40 kDa
p39/eIF5α protein
600
500
400
300
200
100
0
**
DIV 1
DIV 5
B
Optic Nerve
P7
P14
P25
p39
p35
Cdk5
eIF5α
-40 kDa
-35 kDa
-35 kDa
-25 kDa
-35 kDa
-25 kDa
-55 kDa
-40 kDa
C
p35
p39
Percent mRNA change
500
400
300
200
100
0
***
***
1
3
5
7
Days of Differentiation
D
p35
p39
Percent mRNA change
500
400
300
200
100
0
***
***
1
10
20
Postnatal Day

2.2.3 p39-dependent Cdk5 activation is essential for OL differentiation

To directly test whether p39 is primarily responsible for Cdk5 activity in OPCs, we specifically knocked down p39 mRNA in CG4 cells (69±4% reduction) using an siRNA that does not affect p35 (Figure 2-4A). To assess the role of p39 in Cdk5 activation, the Cdk5/activator complex was immunoprecipitated from p39 siRNA- and control siRNA-treated cells, resolved by SDS-PAGE, and transferred to PDVF membrane. Cdk5 activity was then determined by a well-established kinase assay using histone H1 as substrate, in which ^{32}P-H1 signal on autoradiography was normalized to the level of immunoprecipitated Cdk5 protein detected by immunoblot analysis of the same membrane. As shown in Figure 2-4B, p39-specific siRNA significantly reduced Cdk5 activity which was largely rescued by expression of exogenous p39 (p39-Flag).These data suggest that p39 plays a major role in Cdk5 activation in OLs.

Previous studies have established the essential role of Cdk5 in OPC development (Miyamoto et al. 2007). We next asked whether p39-mediated activation of Cdk5 advances OPC differentiation. CG4 cells can be induced for morphological differentiation, during which increasingly sophisticated processes develop over time, recapitulating primary OPCs. We treated CG4 cells with control- or p39-siRNA prior to the induction of differentiation. The complexity of process arborization, a well-established hallmark of OL differentiation, was analyzed based on primary, secondary, and tertiary processes (Figure 2-5A). At the start of differentiation, comparable percentages of cells harboring primary, secondary, or tertiary processes were observed in control- and p39-siRNA treated cultures. While control-siRNA treated cells progressively develop complex secondary and tertiary processes over two days of differentiation, p39-siRNA treatment resulted in a significantly increased percentage of cells that harbored only primary processes as compared to control siRNA-treated cells (Figure 2-5A). This was accompanied by a reciprocal reduction of cells that carry tertiary processes upon p39 siRNA-treatment, suggesting that p39 knockdown blocked initial differentiation.

Figure 2-4: p39 activates Cdk5 in OLs. (**A**) Total RNA was extracted from three independent experiments in which CG4 cells were transfected with p39-siRNA or a negative control-siRNA (n=3). qRT-PCR was performed for p35, p39, and GAPDH mRNA. The levels of p35 and p39 mRNA relative to GAPDH mRNA are shown. qRT-PCR reading in control-siRNA treated cells are set as 100%. The data are reported as mean ± SE ($p<0.001$, two-way ANOVA; ***$p<0.001$, Bonferroni's post-test). (**B**) Cdk5 was immunoprecipitated from lysates of CG4 cells cotransfected with negative control-siRNA and empty vector (pc), p39-siRNA and empty vector, or p39-siRNA and p39-flag (p39F). The immunoprecipitated Cdk5 or kinase reaction buffer alone was incubated with histone H1 and [γ-^{32}P] ATP in a kinase reaction. Reactions were resolved on SDS-PAGE and transferred to PVDF membranes. Top: The same membrane was used to detect H1 phosphorylation by autoradiography and immunoprecipitated Cdk5 by immunoblot analysis. Bottom: Band intensities were quantified by densitometry, and the levels of H1 phosphorylation relative to Cdk5 protein are shown. The normalized Cdk5 activity in control-siRNA treated cells is set as 100% for each experiment. The data are reported as mean ± SE (n=3; $p<0.05$, one-way ANOVA; *$p<0.05$, Tukey's post-test).

Figure 2-4

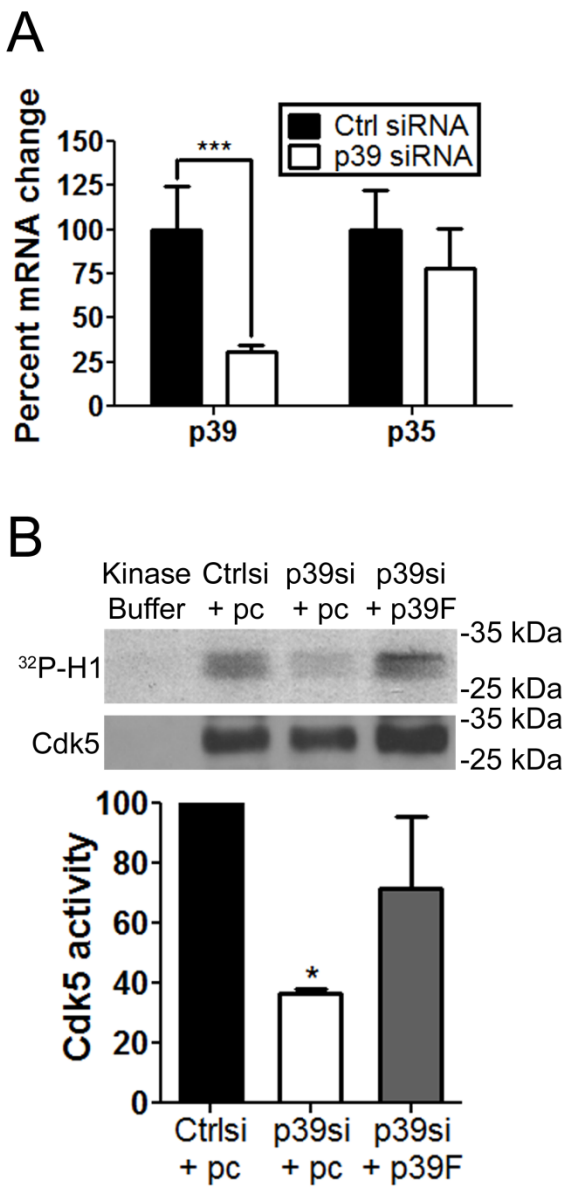
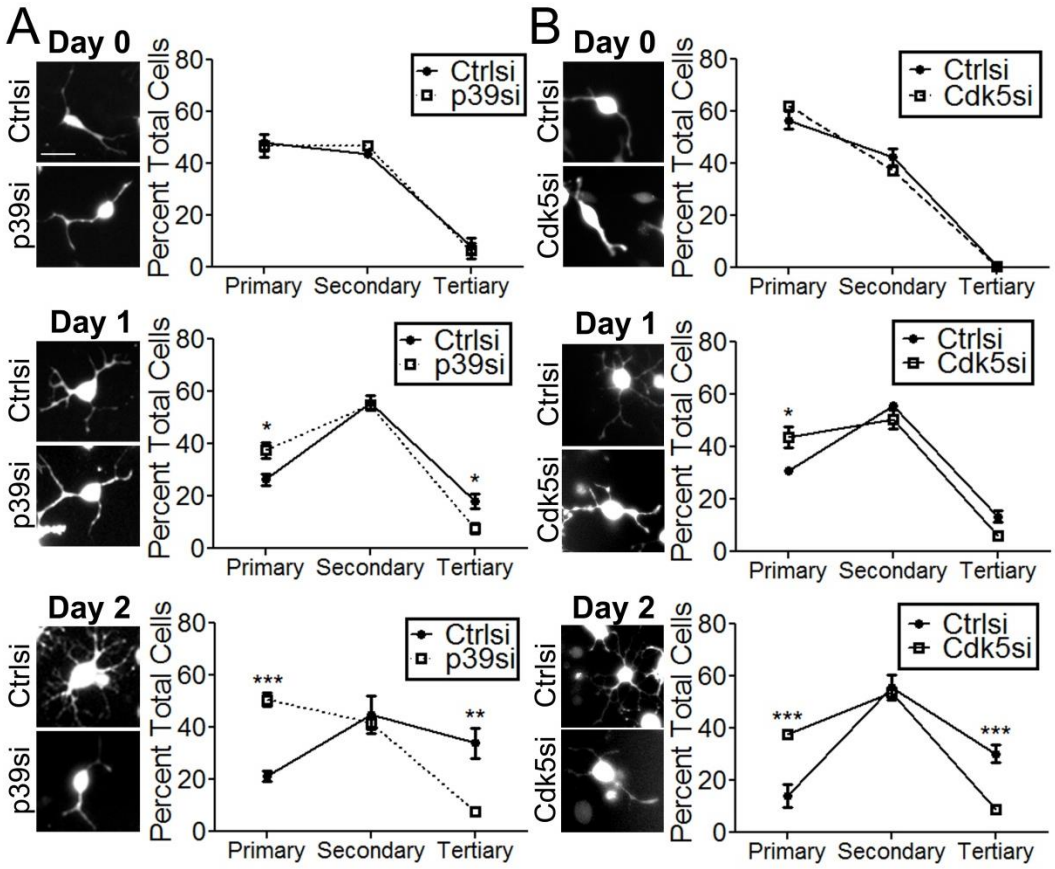

A
Percent mRNA change
150
125
100
75
50
25
0
***
Ctrl siRNA
p39 siRNA
p39
p35
B
Kinase Buffer
Ctrlsi + pc
p39si + pc
p39si + p39F
32P-H1
-35 kDa
-25 kDa
Cdk5
-35 kDa
-25 kDa
Cdk5 activity
100
80
60
40
20
0
*
Ctrlsi + pc
p39si + pc
p39si + p39F

Figure 2-5: p39-dependent Cdk5 activation is essential for OL differentiation. (**A**) p39-siRNA or control-siRNA were cotransfected with a GFP construct into CG4 cells in three independent experiments (n=3). At 0, 1, and 2 days of differentiation, images of GFP^+ cells (left) were captured from more than 50 randomly selected cells in each transfected culture and the percentage of total cells harboring process complexity up to primary, secondary, or tertiary processes were determined and plotted for each day of differentiation (right). (**B**) Cdk5-siRNA or control-siRNA were cotransfected with a GFP construct into CG4 cells. Analysis was performed as described for (**A**). For (**A**) and (**B**), the data are reported as mean ± SE (n=3; $p<0.01$, two-way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$, Bonferroni's post-test). Scale bar, 50 μm.

Figure 2-5


A
Day 0
Ctrlsi
p39si
Percent Total Cells
Primary Secondary Tertiary
Ctrlsi
p39si
Day 1
Day 2
B
Day 0
Ctrlsi
Cdk5si
Day 1
Day 2

A previous study reported that a siRNA that targets p35 also impaired OPC differentiation (Miyamoto et al. 2007). However, we were surprised to find that this siRNA caused a comparable reduction of both p35 and p39 mRNAs when transfected into CG4 cells (59±15% reduction of p35 and 77±11% reduction of p39 by qRT-PCR, $p<0.0005$, Two-way ANOVA; $p<0.01$for p35 and p39, Bonferroni's post-test; data not shown). Further examination revealed that this siRNA contains high levels of sequence complementarily against p39 mRNA as well. Thus the previously reported effects of this siRNA on OL development likely involved suppression of p39.

To further discern if the impaired morphogenesis of CG4 cells by the p39 siRNA is due to suppression of Cdk5 function, we transfected CG4 cells with an siRNA that significantly knocked down Cdk5 (65±6% reduction of Cdk5 mRNA by qRT-PCR, $p<0.01$, two-tailed *t*-test; data not shown). Indeed, we observed a similar profile of impairment in morphological differentiation as seen with p39-siRNA treatment (Figure 2-5B). Together, these data suggest that p39-dependent Cdk5 activation is essential for OPC morphogenesis in culture.

To determine whether p39 functions in the OL lineage *in vivo*, we examined OL development in $p39^{-/-}$ mice (Ko et al. 2001). Myelin basic protein (MBP) is an important hallmark for OL development and deficiency of Cdk5 in OPCs was previously reported to cause reduced MBP^{+} cells during OPC differentiation in culture (Miyamoto et al. 2007). Thus, we first analyzed the expression levels of MBP in the brains of WT and $p39^{-/-}$ at postnatal day 14 when p39 levels peak during normal OL development *in vivo* (Figure 2-3B). As shown in Figure 2-6A, the $p39^{-/-}$ brain harbors reduced expression of MBP that can be visualized by immunoblot analysis. In order to minimize the potential influence of p39 deficiency in neurons, which may in turn affect OLs through axo-myelin interactions, we performed immunofluorescence staining of MBP to examine OLs in the neonatal brain at postnatal day 6 before the onset of rigorous myelination. Reduced MBP immunofluorescence and process complexity, both indicative of impaired OL differentiation, were detected in $p39^{-/-}$ OLs in the corpus callosum that is composed of myelin-

enriched axon tracts connecting the left and right cerebral hemispheres (Figure 2-6B). In addition, the loss of p39 resulted in reduced numbers of MBP^{+} cells in the neonatal corpus callosum (Figure 2-6C).

However, by postnatal day 20, we observed a 2.5-fold increase of p35 protein in $p39^{-/-}$ optic nerves compared to that of the WT controls (Figure 2-7A). Considering the enriched OL soma on the optic nerve, the compensatory upregulation of p35 likely occurs in OLs. In the same samples, MBP expression in $p39^{-/-}$ mice is normal (Figure 2-7B). Moreover, by the age of one month, electron microscopy detected normal compact myelin in $p39^{-/-}$ corpus callosum (Figure 2-7C, *upper panel*). The g-ratio (ratio of axon diameter/axon+myelin diameter), a well-established method for quantifying myelin thickness, in the WT and $p39^{-/-}$ corpa callosa was nearly identical (Figure 2-7C, *bottom panel*). Thus, despite the delay of OL development in mice lacking p39, myelination is largely normal by the age of one month, likely due to compensatory mechanisms including p35 upregulation.

Figure 2-6: Loss of p39 delays oligodendroglia development in vivo. (**A**) Lysates from WT and $p39^{-/-}$ mouse brains at postnatal day 14 were subjected to immunoblot analysis of MBP and eIF5α. (**B**) Brain slices from WT and $p39^{-/-}$ corpa callosa at postnatal day 6 were stained for MBP. Representative images of MBP^{+} cells are shown. Scale bar, 10 μm. (**C**) Brain slices from WT and $p39^{-/-}$ mice at postnatal day 6 were stained for MBP. The total number of MBP^{+} cells in the corpus callosum from three adjacent slices at comparable anatomic position from each brain was quantified. Four pairs of WT and $p39^{-/-}$ mice were examined. The data are reported as mean ± SE (n=4; *$p<0.05$ by two-tailed *t*-test).

Figure 2-6

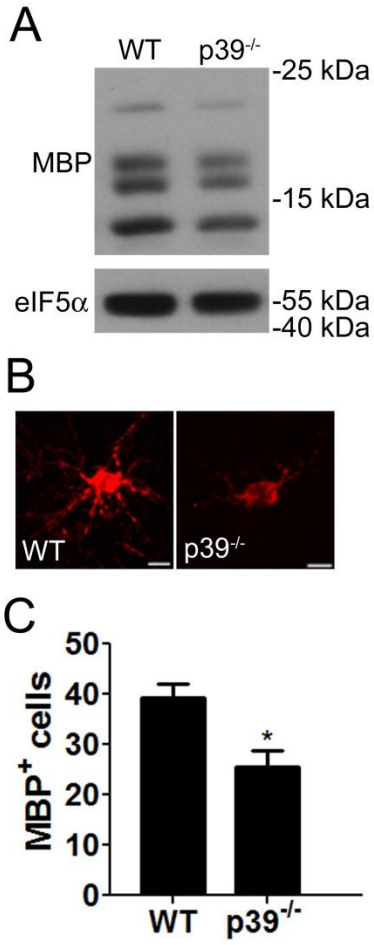
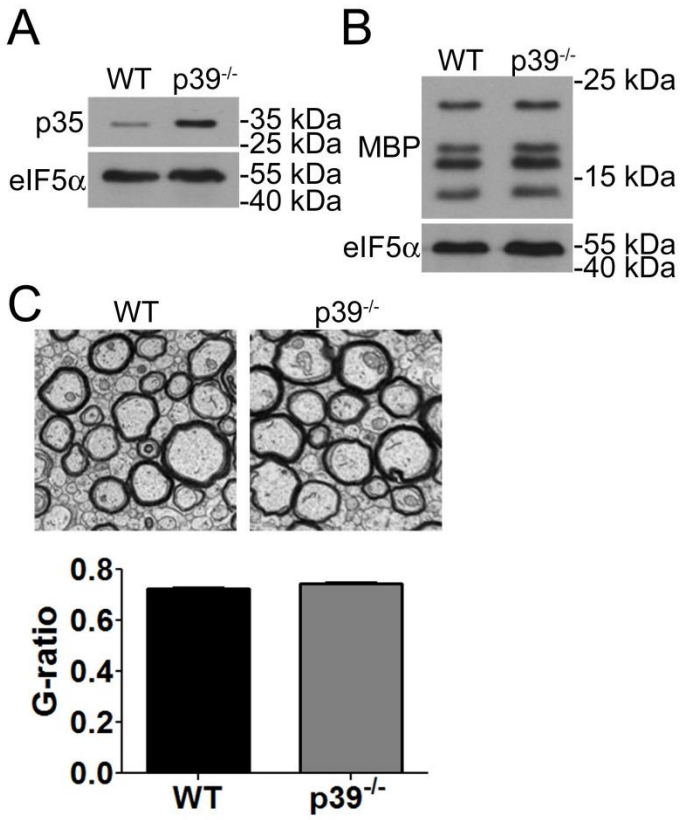

A
WT
p39-/-
-25 kDa
MBP
-15 kDa
eIF5α
-55 kDa
-40 kDa
B
WT
p39-/-
C
MBP+ cells
50
40
30
20
10
0
*
WT
p39-/-

Figure 2-7: p35 is upregulated in p39$^{-/-}$ optic nerves at the peak of myelination, and p39$^{-/-}$ young adults display normal MBP expression and myelin formation. (**A**) Optic nerves were isolated from multiple WT and p39$^{-/-}$ mice at postnatal day 20 and combined for each genotype to prepare protein lysates in order to detect the low levels of p35 in WT. Immunoblot analysis of p35 and eIF5α were carried out. Densitometer reading of p35 band intensity was normalized to that of eIF5α. (**B**) Lysates from the same preparations used in (A) were subjected to a parallel immunoblot analysis for MBP and eIF5α. (**C**) Top: Representative images of electron micrograph (EM) of cross sections of corpa callosa from 1 month old WT and p39$^{-/-}$ mice. Bottom: Average g-ratio of axons in the corpa callosa of 1 month old WT and p39$^{-/-}$ mice. The data are reported as mean ± SE.

Figure 2-7


A
WT
p39-/-
p35
-35 kDa
-25 kDa
eIF5α
-55 kDa
-40 kDa
B
WT
p39-/-
-25 kDa
MBP
-15 kDa
eIF5α
-55 kDa
-40 kDa
C
WT
p39-/-
G-ratio
0.8
0.6
0.4
0.2
0.0
WT
p39-/-

2.2.4 p39 is upregulated during remyelination and essential for repair of myelin lesions

Failure in repairing myelin lesions is a prevailing issue in demyelination diseases (Fancy et al. 2010). Thus, an important question is whether p39-dependent Cdk5 activation is functionally important for myelin repair besides *de novo* OL development. We first questioned whether p39 expression is dynamically changed during induced demyelination and/or myelin repair. To answer this question, we employed the cuprizone paradigm that induces demyelination primarily in the corpus callosum, which can be readily dissected for molecular and biochemical quantification of changes occurring during demyelination and the myelin repair process. Young adult mice that express EGFP specifically in cells of the OL lineage under the control of the PLP promoter were fed a cuprizone diet for 3 weeks to induce demyelination (3wk DM) and then returned to a normal diet for remyelination (3+3wk RM) (Crawford, Mangiardi, and Tiwari-Woodruff 2009). Demyelination is indicated by the reduced EGFP signal and reduced MBP immunofluorescence (Figure 2-8A, 3wk DM compared to Normal). In this model, both demyelination and impaired axonal conductance are largely recovered 3 weeks after returning animals to normal diet (3+3wk RM) (Crawford, Mangiardi, and Tiwari-Woodruff 2009). In fact, remyelination already initiates by the end of 3 weeks of cuprizone treatment, indicated by repopulation of the demyelinated corpus callosum with OPCs expressing Olig2 (Figure 2-8A, bottom panel). Enhanced p39 expression after 3 weeks of cuprizone treatment was evident in cells of the OL lineage expressing EGFP (Figure 2-8B*b* and *Bc* compared to *Ba*), including mature OLs marked by CC1 immunofluorescence (Figure 2-8B*d*). Moreover, p39 protein was increased by 3.1±0.3-fold in the dissected corpus callosum starting from the earliest phases of remyelination, and remained above basal levels even after remyelination was largely completed (Figure 2-8C). We next investigated the mechanisms that underlie the increase of p39 protein during myelin repair by measuring p39 mRNA levels by qRT-PCR. Similar to *de novo* OL differentiation, p39 mRNA expression was upregulated by 3.5±1.6-fold during myelin repair (Figure 2-8C, bottom panel). In contrast, p35 protein was unchanged during either the

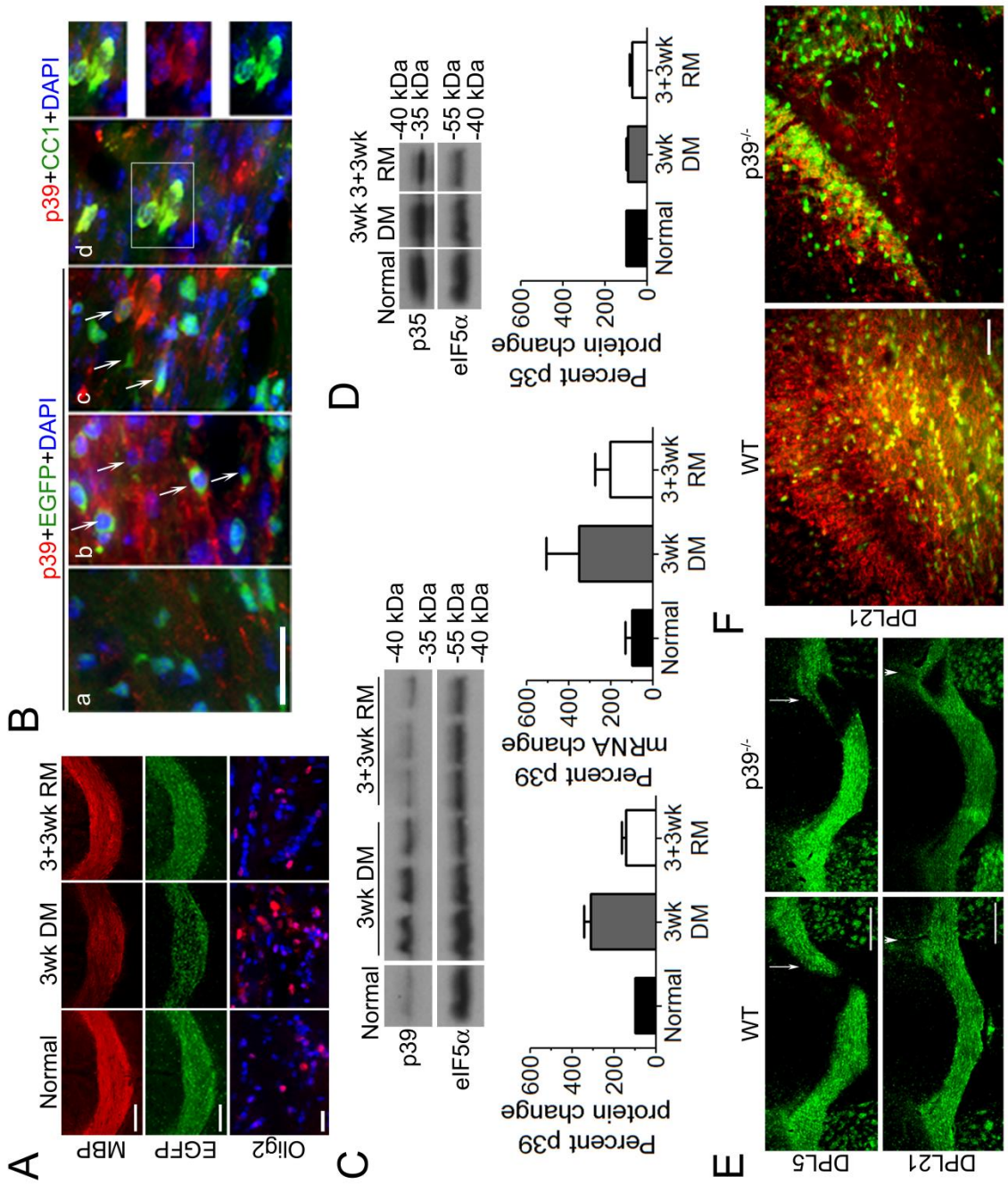
demyelination or the remyelination phase (Figure 2-8D). These data suggest an increased functional requirement for p39 during myelin repair.

To directly assess whether p39 is required for myelin repair, acute demyelination was induced by unilateral lysolecithin injection into the corpus callosum of WT and $p39^{-/-}$ mice that carry the PLP-EGFP transgene. Unlike the cuprizone model where demyelination occurs over weeks, lysolecithin injection produces a focal myelin lesion, clearly marked by the lack of EGFP-expressing OLs and myelin membrane, within 5 days (Aguirre et al. 2007). The re-appearance of $EGFP^{+}$ cells in the lesion area during myelin repair can be visualized. We chose to inject lysolecithin at 6 weeks of age because $p39^{-/-}$ mice have developed normal myelin by that age (Figure 2-7), which is also indicated by the similar intensity of EGFP-labeled myelin on the contralateral side comparable to that in the WT control (Figure 2-8E, top panels). Successful demyelination was achieved in both WT and $p39^{-/-}$ mice (Figure 2-8E, arrows in top panels). However, while WT mice almost fully repaired the demyelinated lesion 21 days post-injection, remyelination was severely impaired in $p39^{-/-}$ mice, as indicated by the obvious lesion that remained at the injection site (Figure 2-8E, arrowheads in bottom panels). Furthermore, MBP staining and $EGFP^{+}$ cells were reduced in the $p39^{-/-}$ lesion as compared to the WT lesion (Figure 2-8F). Together, these data provide the first evidence for selective upregulation of p39, but not p35, during remyelination and the functional requirement of p39-dependent Cdk5 activity for myelin repair.

Figure 2-8: p39 is selectively up-regulated during remyelination and required for myelin repair. (**A-C**) Mice expressing EGFP under the oligodendroglia-specific PLP promoter were subjected to the following diet paradigms: a normal diet for 6 weeks (Normal), a cuprizone diet for 3 weeks (3wk DM), or a cuprizone diet for 3 weeks followed by a normal diet for 3 additional weeks (3+3wk RM). (**A**) Top: Brain slices containing the corpus callosum were stained for MBP. Middle: EGFP signals in OLs and on axonal tracts. Bottom: Brain slices containing the corpus callosum were stained for the OL precursor marker Olig2. Scale bar, 100 μm for MBP and EFGP images, 20 μm for Olig2 images. (**B a-d**) Immunofluorescence of p39 (red) in corpa callosa of Normal (**a**), 3wk DM (**b**), 3+3wk RM (**c** and **d**) mice. DAPI staining (blue) marks nuclei. (**a-c**) OLs are $EGFP^{+}$ and increased p39 expression (red) is indicated by arrows. (**d**) Immunofluorescence of p39 (red) and the mature OL marker CC1 (green). Scale bar, 20 μm. (**C**) Top: Corpa callosa of Normal, 3wk DM, and 3+3wk RM mice were dissected, and lysates were subjected to immunoblot analysis of p39 and eIF5α. Bottom-left: Protein band intensities were quantified by densitometry, and the levels of p39 were normalized to that of eIF5α. The level of p39 protein in Normal was set as 100% and graphically displayed. Bottom-right: Total RNA was extracted from dissected corpa callosa of Normal, 3wk DM, and 3+3wk RM mice, and qRT-PCR was performed for p39 and GAPDH. The levels of p39 mRNA relative to GAPDH mRNA were calculated and the level of p39 mRNA in Normal was set as 100%. The data are reported as mean ± SE (n=3). (**D**) Top: Immunoblot analysis of p35 and eIF5α in dissected corpa callosa. Bottom: Protein band intensities were quantified by densitometry, and the levels of p35 relative to eIF5α are shown. Normal is set as 100%. The data are reported as mean ± SE (n=3). (**E**) WT and $p39^{-/-}$ mice expressing EGFP under the oligodendroglia-specific PLP promoter were unilaterally injected with lysolecithin to induce demyelination. Top: The demyelinated lesion was visualized by the lack of PLP-EGFP signals 5 days post lysolecithin injection (DPL5). Arrows indicate the site of injection. Bottom: Myelin repair was assessed 21 days post lysolecithin injection (DPL21). Arrowheads indicate the injection tracks, which are still visible. Scale bar, 500 μm. (**F**) Brain

slices containing corpa callosa from WT and $p39^{-/-}$ mice at DPL21 were stained for MBP. High magnification images of MBP staining and $EGFP^{+}$ cells are shown. Scale bar, 50 μm.

Figure 2-8


A
Normal
3wk DM
3+3wk RM
MBP
EGFP
Olig2
B
p39+EGFP+DAPI
p39+CC1+DAPI
a
b
c
d
C
Normal
3wk DM
3+3wk RM
p39
eIF5α
-40 kDa
-35 kDa
-55 kDa
-40 kDa
Percent p39 protein change
Percent p39 mRNA change
0
200
400
600
Normal
3wk DM
3+3wk RM
D
3wk 3+3wk
Normal DM
RM
p35
eIF5α
-40 kDa
-35 kDa
-55 kDa
-40 kDa
Percent p35 protein change
E
WT
p39-/-
DPL5
DPL21
F
WT
p39-/-
DPL21

2.3 Discussion

In this study, we demonstrated for the first time that neurons and glia employ distinct molecular mechanisms to control Cdk5 activity by differential regulation of Cdk5 activator expression. Moreover, using multiple experimental paradigms, our studies clearly established that in contrast to the predominant function of p35 in Cdk5 activation in neurons and many other peripheral cell types (Brinkkoetter et al. 2010, Lazaro et al. 1997, Liebl et al. 2010, Lilja et al. 2001, Philpott et al. 1997, Rosales and Lee 2006, Tripathi and Zelenka 2009, Ubeda, Kemp, and Habener 2004), p39 is the primary Cdk5 activator in OLs and plays essential roles to advance normal OL differentiation as well as repair of myelin lesion in the brain.

The critical roles of Cdk5 in controlling normal neuronal development and function have been well-established (Ohshima et al. 1996, Su and Tsai 2011). More recent studies have also established a parallel role of Cdk5 in OL development (He et al. 2010, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013). However, molecular mechanisms that regulate Cdk5 function in different neural cell lineages still remain vastly elusive. In particular, because the lack of overt detrimental phenotypes in adult $p39^{-/-}$ mice (Ko et al. 2001), the functional importance of p39 is not understood. We show for the first time that OLs primarily express p39 for Cdk5 activation, with scarce levels of p35. Moreover, the selective upregulation of p39, but not p35, during OL differentiation in culture and *in vivo* clearly demonstrated that p39 is responsible for the increased Cdk5 activity during OL differentiation (Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998). Given the fact that the most rigorous OL development occurs in the first few postnatal weeks (Miller 2002), OL-produced p39 must be an important contributor to the previously reported preferential increase of p39 during neonatal brain development (Humbert, Lanier, and Tsai 2000). Importantly, selective upregulation of p39 is also observed during myelin repair in adults, suggesting that similar mechanisms control p39 expression in neonatal and adult OPCs.

We have provided *in vitro* and *in vivo* evidence that p39-dependent Cdk5 activation is crucial for OPC differentiation, which expands the importance of p39-dependent Cdk5 function from the previous report that shRNA knockdown of p39 impairs OPC migration in culture (Miyamoto, Yamauchi, and Tanoue 2008). In fact, the reduced numbers of MBP^{+} cells in the corpus callosum of $p39^{-/-}$ mice likely originate from defects in both migration and differentiation, two concurrent events tightly coupled during *in vivo* myelinogenesis. Although the lack of p39 in neurons may also contribute to the delayed OL development *in vivo* in the $p39^{-/-}$ mice, impairment of morphological differentiation of CG4 cells by siRNA-mediated knockdown of p39 and Cdk5 clearly demonstrated the essential role of cell autonomous p39-dependent Cdk5 function in OPCs.

Unlike the conditional Cdk5 knockout mice that exhibit CNS hypomyelination (He et al. 2010, Yang et al. 2013), we found that $p39^{-/-}$ mice only exhibit a delay in early OL development without overt hypomyelination. One likely explanation is the compensatory increase of p35 in OLs due to the absence of p39, which may permit delayed myelination in $p39^{-/-}$ adult. In addition, given the fact that many Cdk5 target proteins can be phosphorylated by other kinases (Bibb et al. 2001, Bibb James A. et al. 1999, Grant, Sharma, and Pant 2001, Kansy et al. 2004, Wang, Grundke-Iqbal, and Iqbal 2007), compensatory crosstalk by other signaling mechanisms may also contribute to myelinogenesis in the absence of p39-dependent Cdk5 function. Nonetheless, the defects in early OPC development in $p39^{-/-}$ mice argue that the function of p39 could not be completely spared despite compensation by various possible mechanisms.

Importantly, in contrast to the largely intact *de novo* myelination in the absence of p39, myelin repair is severely impaired in the $p39^{-/-}$ mice after lysolecithin-induced acute demyelination. On one hand, the much more rapid lesion development may not leave sufficient time to induce compensatory changes. On the other hand, the more challenging environment of lysolecithin-induced lesion may exacerbate the functional defects of lacking p39-dependent Cdk5 signaling, which lead to failures of myelin repair. Notably, increased p39 expression can also be

observed in cells beyond the OL lineage during myelin repair. Thus, whether increased p39 expression from neurons and other types of glia cells together with OL-specific p39 upregulation are synergistically required for myelin repair is an interesting unanswered question that could be addressed once neural-lineage specific, inducible knockout of p39 can be achieved.

The low levels of p39 and p35 mRNAs in OLs as compared to that in neurons suggest the existence of neural lineage-specific mechanisms for differential mRNA biogenesis of these Cdk5 activators. We showed that p39 mRNA is active for translation in OLs. Thus, p39 protein levels are largely regulated by the abundance of p39 mRNA, likely involving unidentified OL-specific transcription factors as well as posttranscriptional mechanisms that control the stability of the p39 mRNA. Noticeably, upregulation of p39 mRNA is observed in *de novo* myelin development as well as myelin repair. Thus, molecular mechanisms that regulate p39 in neonatal OPCs are likely applicable to myelinating OLs during lesion repair in the adults and warrant rigorous investigation. In contrast, p35 mRNA is translationally repressed in OLs, which is an apparent mechanism that contributes to the scarce expression of p35 protein in OLs. The transacting factors that suppress p35 translation in OLs still remain unknown. Nonetheless, it is worth mentioning that a number of microRNAs are predicted to target the lengthy 3'UTR of p35 mRNA specifically, but not the p39 mRNA (Moncini et al. 2011). Which of these miRNAs are expressed in OLs and responsible for translational suppression of p35, is a challenging question to be addressed by future studies.

In conclusion, our studies have demonstrated differential regulation of Cdk5 activator expression in neurons and OLs. Further delineating molecular mechanisms that control p35 and p39 expression may allow differential and independent manipulation of Cdk5 function in neuronal and glial lineages during normal development, as well as in the pathogenesis of brain disorders involving cell type-specific Cdk5 dysregulation, such as neuronal degeneration in Alzheimer's disease and glioma tumorigenesis (An et al. 2009, Cheung and Ip 2012, Liu et al. 2008). In particular, identification of OL-specific mechanisms that control p39 expression may

ultimately help to develop novel strategies to advance CNS myelinogenesis in numerous disorders for which the pathogenic impacts of myelin impairment have become increasingly recognized, represented by multiple sclerosis and schizophrenia (Fields 2008).

Chapter 3: Phosphorylation of Cytoplasmic QKI by Cdk5 Induces Nuclear Translocation and Promotes Oligodendroglia Differentiation

3.1 Introduction

Development of oligodendroglia (OL), which myelinate neuronal axons for fast and efficient saltatory conduction and provide neurotrophic and metabolic support to maintain axon integrity, is essential for normal function of the central nervous system (CNS) (Bankston, Mandler, and Feng 2013). Myelin deficiency can cause debilitating neurological disorders, such as multiple sclerosis, and impairment of OL and myelin function have become increasingly associated with many neurodegenerative diseases, including Alzheimer's disease (Bankston, Mandler, and Feng 2013, Fields 2008).

Recent work has described the essential function of cyclin-dependent kinase 5 (Cdk5) in OL and myelin development (He et al. 2010, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013). Cdk5 is an unconventional member of the Cdk family as it does not advance cell cycle progression and mainly functions in postmitotic cells. Despite the accumulating reports of Cdk5 function in numerous cell types (Liebl et al. 2010, Lilja et al. 2001, Rosales and Lee 2006), the activity of the ubiquitously expressed Cdk5 is highest in the brain due to robust expression of the Cdk5 activators, p35 and p39 (Su and Tsai 2011, Tsai et al. 1993), which control the level and localization of Cdk5 activity (Ko et al. 2001, Takahashi et al. 2005). Classic studies of Cdk5 function in neuronal development have established many targets that mediate Cdk5 function by modulating the cytoskeleton upon Cdk5-dependent phosphorylation (Su and Tsai 2011).

In OLs, loss of Cdk5 activity impairs differentiation in culture (Bankston et al. 2013, Miyamoto et al. 2007). Moreover, OL-specific conditional knockout of Cdk5 in mice causes deficits in OL differentiation and myelin formation (Yang et al. 2013). While many of the cytoskeleton-associated Cdk5 targets in neurons also function in OLs, many OL- and myelin-specific proteins play key roles in OL and myelin development (Nave 1994, Zhao et al. 2006b). Our previous studies have demonstrated that unlike neurons that harbor abundant expression of both p35 and p39, OLs primarily employ p39 to activate Cdk5 with negligible p35 expression

(Bankston et al. 2013). Loss of p39 impairs early OL development in neonates and myelin repair in the adult brain (Bankston et al. 2013). However, while it is clear that p39-mediated Cdk5 activation plays important roles in OL development, the protein targets phosphorylated by Cdk5-p39 complex which in turn promote OL differentiation and CNS myelin development still remain vastly elusive.

One critical player that advances OL development is the selective RNA-binding protein QKI (Chen et al. 2007, Zhao et al. 2006b). Within the brain, QKI is a glia-specific member of the Signal Transduction Activation of RNA (STAR) protein family that links signaling cascades to RNA homeostasis (Vernet and Artzt 1997). Previous studies have demonstrated that QKI is essential for OL differentiation and myelin development (Chen et al. 2007, Friedrich 1974, Zhao et al. 2006b). QKI deficiency in OLs results in severe impairment of OL function and CNS hypomyelination in the quakingviable (qk^{v}) mutant mice (Friedrich 1974, Sidman, Dickie, and Appel 1964). Three protein isoforms of QKI exist, named QKI5, QKI6, and QKI7 after the length of their mRNA. The common N-terminal sequence in QKI encodes an extended hnRNPK homology (KH) RNA-binding domain, which is followed by isoform-specific C-termini derived from alternative splicing (Ebersole et al. 1996). QKI5 is the predominant isoform in early OL development and localizes to the nucleus at steady-state. In contrast, QKI6 and QKI7 are primarily cytoplasmic and are upregulated during OL development (Hardy 1998). The functional interplay between nuclear and cytoplasmic QKI isoforms is thought to govern the balance of OL proliferation and differentiation. Despite the distinct nuclear-cytoplasmic distribution, QKI isoforms can form heterodimers and shuttle in and out of the nuclei (Pilotte, Larocque, and Richard 2001, Wu et al. 1999). However, whether nuclear-cytoplasmic distribution of QKI can be regulated to impact QKI function in OL development is not understood.

In this study, we identified consensus target amino acid motifs for Cdk5 in QKI, and demonstrate that Cdk5 can phosphorylate recombinant QKI isoforms. We observed that T243 is an important site of Cdk5-dependent QKI phosphorylation. In fact, we discovered that QKI6

phosphorylated at T243 accumulated in the nucleus. Finally, differentiating OLs expressing phosphomimetic QKI6-T243E advanced beyond those expressing unphosphorylated QKI6-T243A. Together, these data uncovered a novel role for Cdk5-dependent QKI phosphorylation in nuclear translocation of QKI to advance OL differentiation.

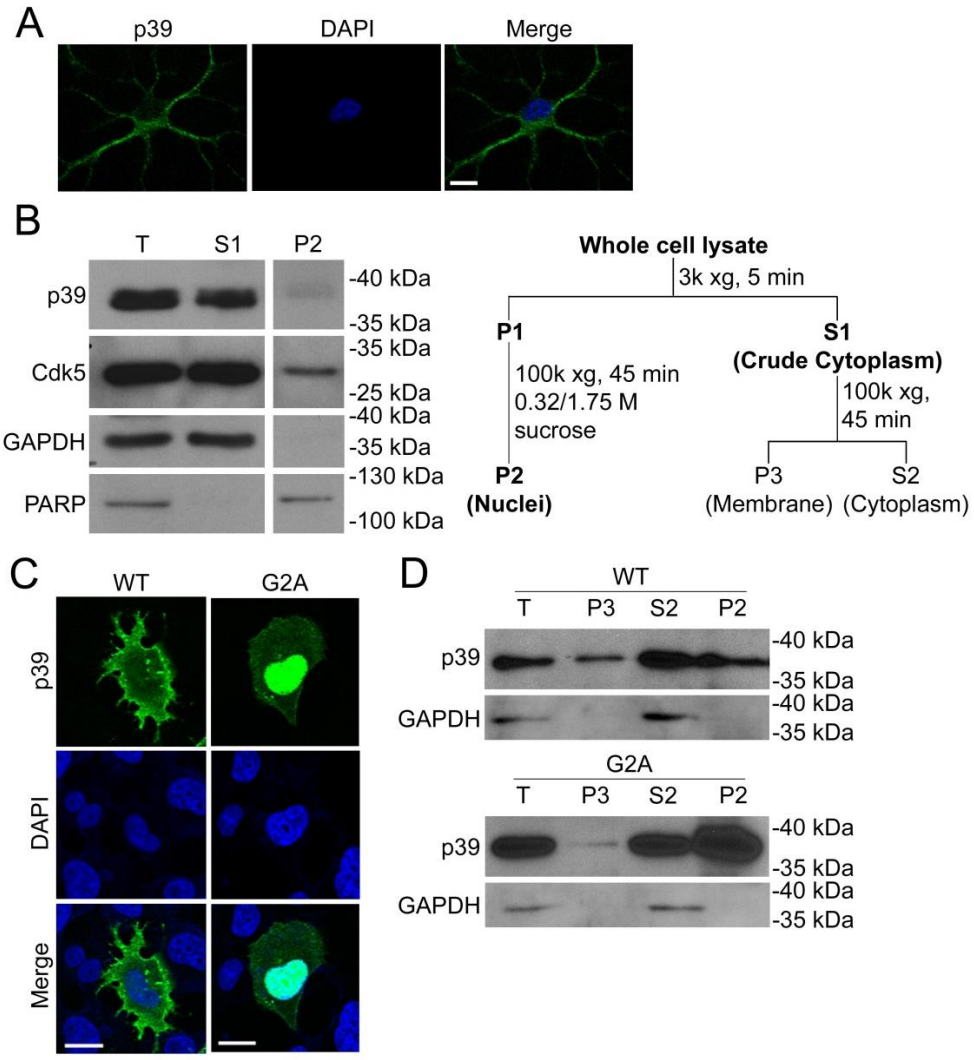
3.2 Results

3.2.1 Myristoylation retains p39 in the cytoplasm of OLs

Our previous work has demonstrated that p39 is the primary activator of Cdk5 in OLs and is essential for OL differentiation (Bankston et al. 2013), presumably through phosphorylation of OL protein targets. The localization of p39 in OLs determines the collection of targets through which Cdk5/p39 can act. Endogenous p39 displays a largely cytoplasmic localization in cell line and primary neurons, but cytoplasmic and nuclear Cdk5 activity has been reported in glioblastoma cells (Catania et al. 2001, Eggers et al. 2011, Liu et al. 2008). Immunofluorescent staining of endogenous p39 in primary cultured rat OLs after 5 days in culture (DIV5) revealed a primarily cytoplasmic localization that marked the soma and the extended processes (Figure 3-1A). The cytoplasmic localization of p39 was confirmed when mouse brain stem, an OL-rich region of the brain, was fractionated to isolate nuclear and cytoplasmic fractions following the procedure outlined in Figure 3-1B. Purified nuclei were obtained by ultracentrifugation of the crude nuclear pellet through a sucrose gradient. Immunoblot analysis of p39 and Cdk5 in these fractions was performed (Figure 3-1B). GAPDH and PARP were also detected as cytoplasmic and nuclear markers, respectively. The majority of p39 (~62%) and Cdk5 were detected in the cytoplasmic fraction (S1), with negligible p39 detected in the nuclear fraction (P2).

Figure 3-1: Myristoylation retains p39 in the cytoplasm of oligodendroglia. (A) Primary cultured rat oligodendroglia were immunostained for p39 (green) after 5 days in culture. (B) The brain stem of wildtype mice were isolated and fractionated according to the protocol at right to isolate nuclear (P2) and cytosolic (S1) fractions. Immunoblot was performed on total lysate (T), S1 fraction, and P2 fraction for p39, Cdk5, GAPDH (cytosolic marker), and PARP (nuclear marker). (C) HOG cells were transfected with either WT or G2A mutant p39-FLAG and immunostained using a FLAG antibody (scale bar= 20 μm). (D) HOG cells were transfected with either WT or G2A mutant p39-FLAG and fractionated according to the protocol in (B). Immunoblot was performed on total lysate, P3 fraction, S2 fraction, and P2 fraction (loaded as 1.5%, 19%, 2.8%, and 24% of Input, respectively) for p39 and GAPDH.

Figure 3-1


A
p39
DAPI
Merge
B
T
S1
P2
p39
Cdk5
GAPDH
PARP
-40 kDa
-35 kDa
-35 kDa
-25 kDa
-40 kDa
-35 kDa
-130 kDa
-100 kDa
Whole cell lysate
3k xg, 5 min
P1
100k xg, 45 min
0.32/1.75 M
sucrose
P2
(Nuclei)
S1
(Crude Cytoplasm)
100k xg,
45 min
P3
(Membrane)
S2
(Cytoplasm)
C
WT
G2A
p39
DAPI
Merge
D
WT
T
P3
S2
P2
p39
GAPDH
-40 kDa
-35 kDa
-40 kDa
-35 kDa
G2A
T
P3
S2
P2
p39
GAPDH
-40 kDa
-35 kDa
-40 kDa
-35 kDa

Previous studies have demonstrated that myristoylation of p39 on glycine 2 acts as an anchor that prevents nuclear translocation of p39 in other cell types, including neurons (Asada et al. 2008). To test whether this also occurs in OLs, we expressed WT or G2A mutant p39, deficient for myristoylation, that carry a C-terminal Flag-tag (p39-flag) in the human OL cell line HOG (Post and Dawson 1992). Immunofluorescent staining for flag-tagged p39 clearly indicated that in contrast to the lack of nuclear staining for WT p39, the G2A mutant p39 displayed dramatic nuclear accumulation while some G2A-p39 remains in the cytoplasm (Figure 3-1C). Subcellular fractionation of HOG cells expressing WT or G2A mutant p39-flag was also performed to isolate membrane, cytoplasmic, and nuclear fractions (Figure 3-1D). Immunoblot of these fractions revealed that loss of myristoylation reduced membrane association of p39 and increased nuclear accumulation of p39. The absence of GAPDH in the nuclear fraction confirms the lack of cytoplasmic contamination, suggesting that the nuclear accumulation of G2A p39-flag was due to increased nuclear translocation. These data reveal that myristoylation retains p39, and thus Cdk5 activity, in the cytoplasm of OLs, thus the major role of Cdk5 signaling in advancing OL development may rely on phosphorylation of cytoplasmic OL targets that remain largely undefined.

3.2.2 Threonine 243 is the major site of Cdk5-dependent QKI phosphorylation

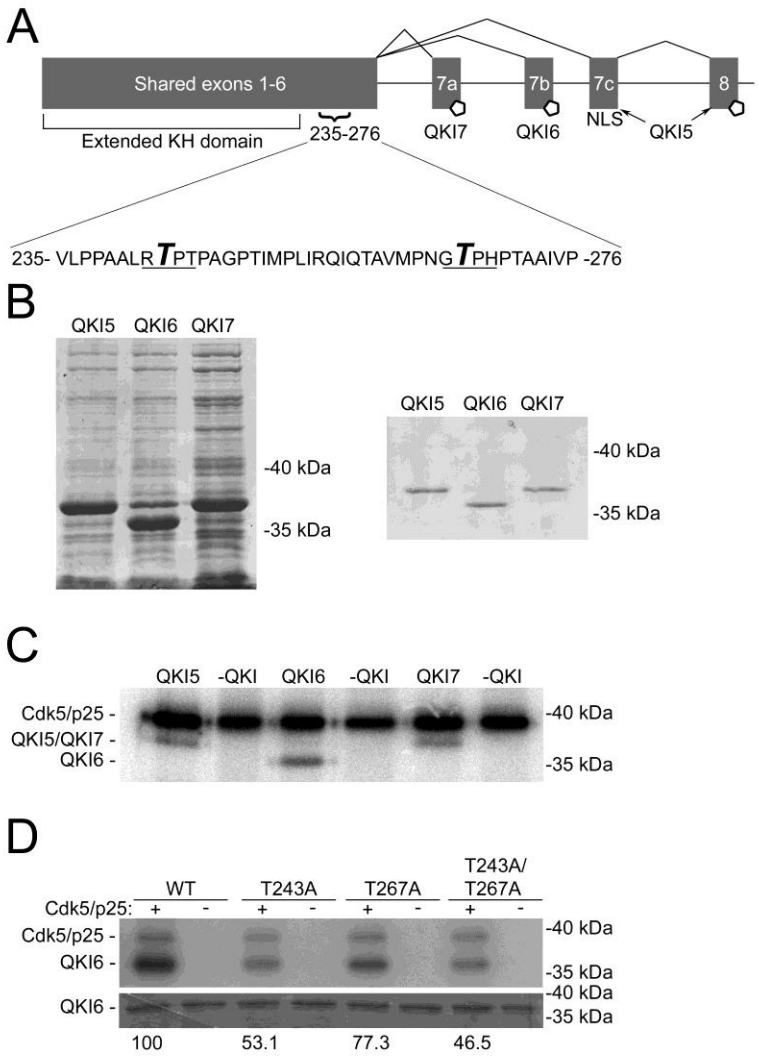
Few targets of Cdk5 in OLs have been identified that play central roles in OL development. One potential cytoplasmic target is the selective RNA-binding protein QKI, which is critical for OL differentiation and myelin development, is upregulated in the cytoplasm during OL development, and contains two sites matching the Cdk5 target consensus sequence of S/T-P with a neighboring basic residue (Figure 3-2A) (Su and Tsai 2011). Both potential sites, T243 (RTPT) and T267 (GTPH), are contained within the sequence shared by all QKI isoforms. To test whether Cdk5 may phosphorylate QKI, recombinantly expressed His_6-tagged QKI isoforms were purified from Rosetta2 E. coli (Figure 3-2B) and incubated in an *in vitro* kinase assay with active

Cdk5 complexes in the presence of [γ-^{32}P]-ATP (Figure 3-2C). The reactions were then resolved by SDS-PAGE, and QKI phosphorylation was assessed by autoradiography. All three major QKI isoforms were phosphorylated by Cdk5 *in vitro*.

Cytoplasmic QKI6 is the most abundant isoform in mature OLs and, like Cdk5 activity, is upregulated during OL differentiation (Hardy et al. 1996, Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998), making it the most likely QKI isoform to mediate Cdk5 function in OLs. To test whether the identified sites, T243 and T267, mediate Cdk5 phosphorylation of QKI, site-directed mutagenesis was performed to convert these threonines to alanines in QKI6. Recombinant His_6-tagged WT and mutant QKI6 were purified and incubated with pre-formed active Cdk5 complexes in an *in vitro* kinase assay in the presence of [γ-^{32}P]-ATP (Figure 3-2D). After the reactions were resolved by SDS-PAGE, the intensity of QKI phosphorylation measured by autoradiography was normalized to the intensity of coomassie-staining of each QKI isoform determined by densitometry from the same gel. Nearly half of ^{32}P-QKI6 signal was ablated by the T243A mutation, while T267A mutation reduced ^{32}P-QKI by 23%. In addition, double mutation of T243A and T267A caused no further reduction of QKI6 phosphorylation by Cdk5 as compared to that by T243A mutation. Thus, T243 is an important site mediating Cdk5-dependent phosphorylation of QKI, although additional Cdk5-target sites in QKI are still to be determined.

Figure 3-2: **Cdk5 phosphorylates QKI *in vitro*.** (A) Schematic representation of QKI isoform structure. Pentagons indicate stop codons for each isoform. All potential Cdk5 target sites are contained within the N-terminal shared sequence. A portion of this sequence containing two potential sites for Cdk5 phosphorylation is displayed below with the target residue in bold and the consensus site underlined. (B) His_6-tagged QKI isoforms were recombinantly expressed in Rosetta2 E. coli (left) and purified using Ni-NTA agarose beads (right). Lysates and purified proteins were resolved by SDS-PAGE, and the gels were coomassie-stained. (C) His_6-tagged QKI isoforms were recombinantly expressed in Rosetta2 E. coli and purified using Ni-NTA agarose beads. The purified QKI isoforms were then incubated with Cdk5/p25 complexes and [γ-^{32}P]-ATP in an *in vitro* kinase assay and resolved by SDS-PAGE. QKI phosphorylation was detected by autoradiography. (D) WT or mutant His_6-tagged QKI6 were recombinantly expressed in Rosetta2 E. coli and purified using Ni-NTA agarose beads. The purified QKI isoforms were then incubated with Cdk5/p25 complexes and [γ-^{32}P]-ATP in an *in vitro* kinase assay and resolved by SDS-PAGE. Brij-35 and additional cold ATP were included in the reactions to reduce the intensity of the Cdk5/p25 band. QKI phosphorylation was detected by autoradiography (top). The relative amount of QKI in each reaction is indicated by the coomassie-stained gel below. The intensity of QKI6 phosphorylation relative to the respective coomassie-stained band is indicated at bottom.

Figure 3-2


A
Shared exons 1-6
7a
7b
7c
8
Extended KH domain
235-276
QKI7
QKI6
NLS
QKI5
235- VLPPAALRTPTPAGPTIMPLIRQIQTAVMPNGTPHPTAAIVP -276
B
QKI5 QKI6 QKI7
-40 kDa
-35 kDa
QKI5 QKI6 QKI7
-40 kDa
-35 kDa
C
QKI5 -QKI QKI6 -QKI QKI7 -QKI
Cdk5/p25 -
QKI5/QKI7 -
QKI6 -
-40 kDa
-35 kDa
D
WT
T243A
T267A
T243A/
T267A
Cdk5/p25: + - + - + - + -
Cdk5/p25 -
QKI6 -
-40 kDa
-35 kDa
QKI6 -
-40 kDa
-35 kDa
100
53.1
77.3
46.5

3.2.3 T243 phosphorylation of cytoplasmic QKI induces nuclear translocation

Although our data suggests that Cdk5 may phosphorylate QKI at T243 *in vitro*, the functional role of this phosphorylation was unclear. To begin exploring the mechanism through which Cdk5 may regulate QKI function, we generated potential phosphodeficient (T243A) and phosphomimetic (T243E) mutants of the QKI isoforms. While HOG cells only express endogenous QKI5, high levels of expression can be achieved for all three QKI isoforms in HOG cells (Figure 3-3A). Importantly, WT and mutant QKI isoforms all expressed full length protein, as visualized by anti-Flag immunoblot (Figure 3-3B). HOG cells expressing WT, T243A, or T243E Flag-QKI5, -QKI6, or -QKI7 were immunostained using an anti-Flag antibody (Figure 3-4A), and the percentage of total cells displaying more than 20% nuclear QKI staining was quantified. Nearly all cells expressing WT QKI5, which is nuclear at steady-state, displayed positive nuclear immunostaining. In cultures expressing the normally cytoplasmic WT QKI6 and QKI7, few cells harbored any nuclear signal (Figure 3-4B). QKI6 T243A behaved similar to WT QKI6 with 20.3±1.5% of T243A expressing cells displaying positive nuclear staining compared to 25.2±2.1% of cells in WT QKI6 expressing cultures. A similar result was seen for QKI7 (17.9±0.7% for T243A and 27.8±5.7% for WT). In striking contrast, both QKI6 and QKI7 T243E mutants expressing cultures displayed positive nuclear staining in the majority of cells (79.6±3.4% for QKI6 T243E and 83.8±4.3% for QKI7 T243E). No change in localization was observed between WT, T243A, or T243E QKI5.

Figure 3-3: High expression levels of full-length WT and mutant QKI isoforms can be achieved in HOG cells. (A) HOG cells were transfected with empty vector or vectors encoding FLAG-tagged QKI5, QKI6, or QKI7. Lysates were immunoblotted with QKI5-, QKI6-, or QKI7-specific antibodies. eIF5α was detected as a loading control. (B) Lysates from HOG cells transfected with FLAG-tagged WT, T243A, or T243E QKI isoforms (indicated above each immunoblot) were immunoblotted using an anti-FLAG antibody. eIF5α was detected as a loading control.

Figure 3-3

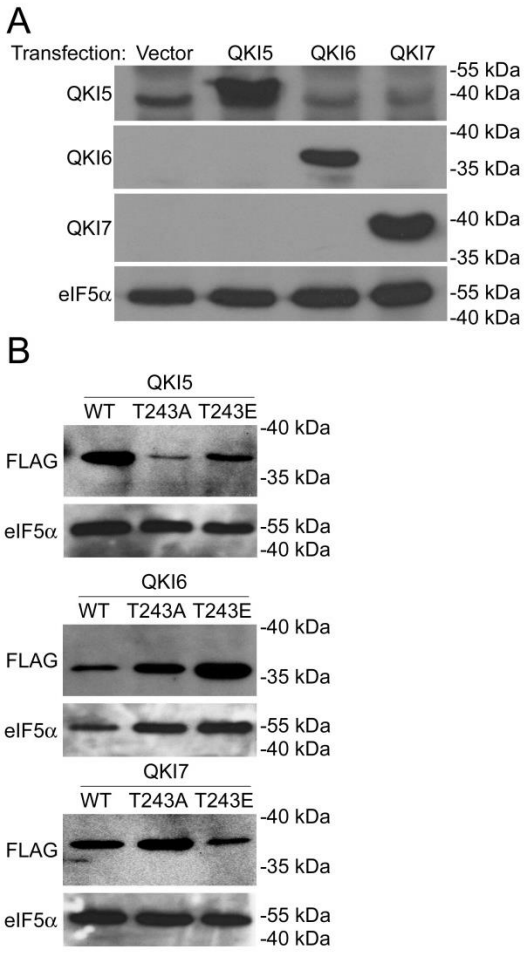
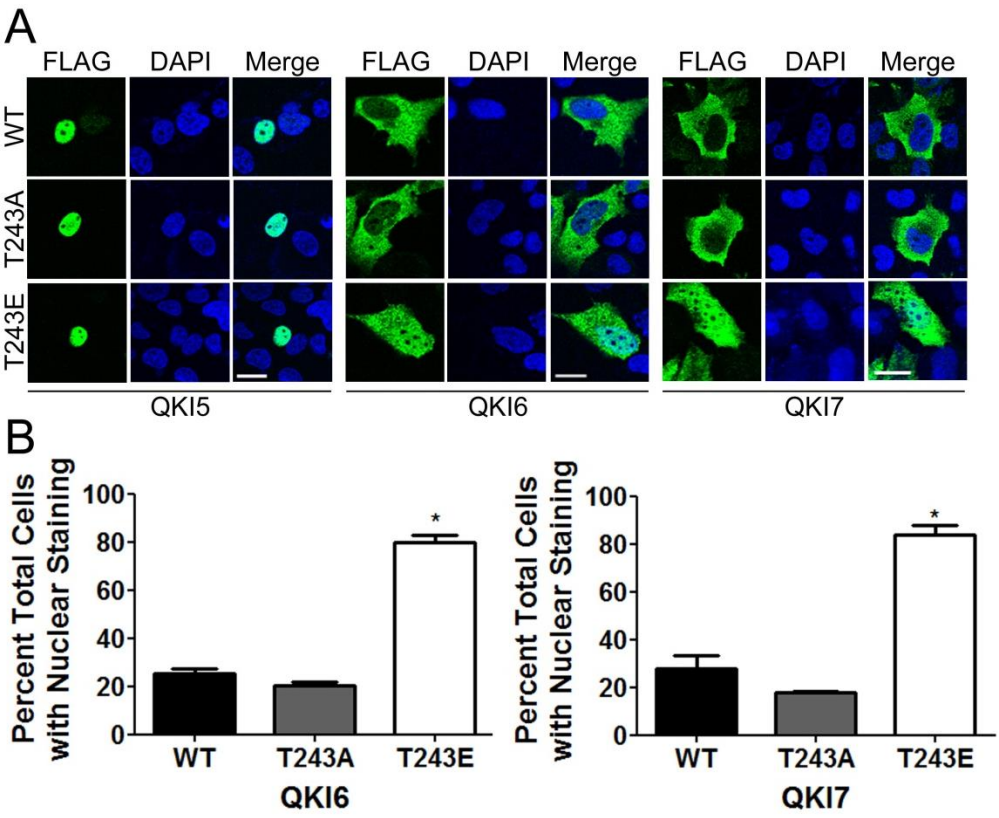

A
Transfection: Vector QKI5 QKI6 QKI7
QKI5
QKI6
QKI7
eIF5α
-55 kDa
-40 kDa
-40 kDa
-35 kDa
-40 kDa
-35 kDa
-55 kDa
-40 kDa
B
QKI5
WT T243A T243E
FLAG
eIF5α
QKI6
WT T243A T243E
FLAG
eIF5α
QKI7
WT T243A T243E
FLAG
eIF5α
-40 kDa
-35 kDa
-55 kDa
-40 kDa

Figure 3-4: QKI T243 phosphorylation induces nuclear translocation. (A) HOG cells were transfected with FLAG-tagged WT, T243A, or T243E QKI isoforms and immunostained using a FLAG antibody (scale bar= 20 μm). (B) HOG cells expressing FLAG-tagged WT, T243A, or T243E QKI isoforms were scored according to detection of nuclear staining and quantified as a percentage of total cells counted (n=3). The data are reported as mean ± SE ($p<0.0001$, one-way ANOVA; *$p<0.001$, Tukey's post-test).

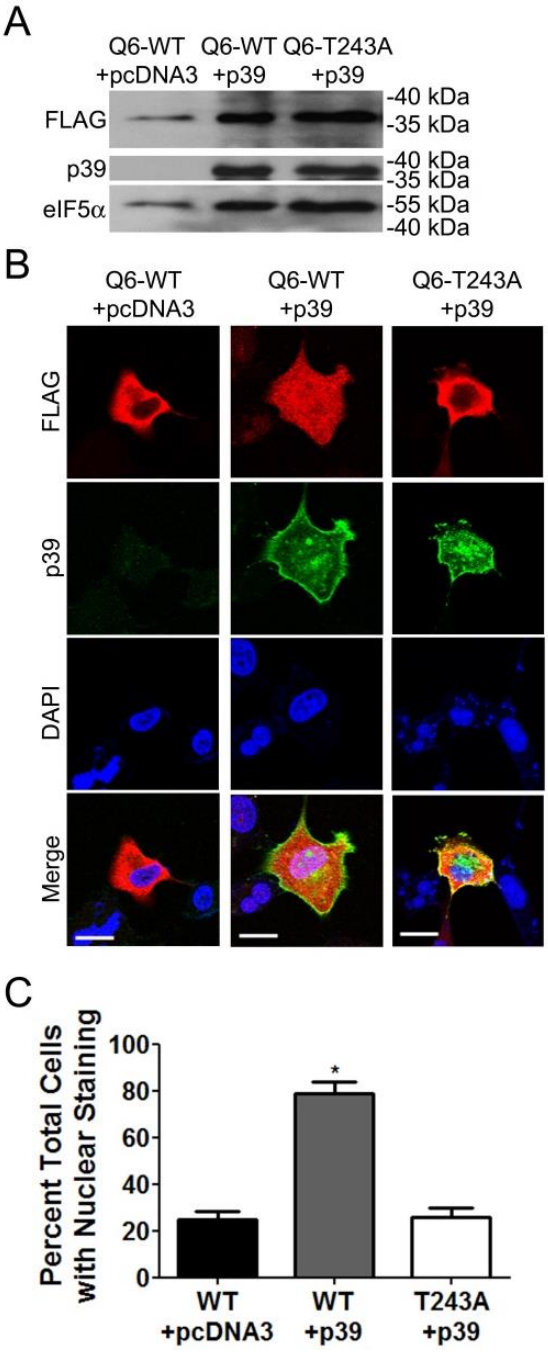
Figure 3-4


A
FLAG
DAPI
Merge
WT
T243A
T243E
QKI5
QKI6
QKI7
B
Percent Total Cells with Nuclear Staining
100
80
60
40
20
0
*
WT
T243A
T243E
QKI6
QKI7

Besides the increase of QKI6 during OL differentiation, p39 expression also increases and likely underlies the reported increase of Cdk5 activity (Bankston et al. 2013, Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998). To test whether increased p39-mediated Cdk5 activation induces nuclear translocation of WT QKI6, HOG cells were cotransfected with WT QKI6 and either empty vector or p39. In parallel transfections, T243A mutant QKI6 was cotransfected with p39. Similar levels of expression were achieved for WT and T243A mutant QKI6 and for p39 in these parallel transfections as detected by immunoblot with anti-Flag and anti-p39 antibodies (Figure 3-5A). Immunostaining with an anti-Flag antibody revealed that while WT QKI6 expressed alone displayed predominant cytoplasmic localization, WT QKI6 coexpressed with p39 displayed both nuclear and cytoplasmic staining (Figure 3-5B). This effect was not seen when T243A mutant QKI6 was coexpressed with p39. Similar to the T243E mutants of QKI6 and QKI7, a nearly 4-fold increase of the percent of cells with nuclear staining was seen when p39 was coexpressed with WT QKI6 (24.9±3.2% for QKI6 WT alone and 79.1±5.0% for QKI6 WT with p39) (Figure 3-5C). Nuclear translocation of QKI6 T243A was not induced by increased p39 expression (25.6±3.9% of total cells with positive nuclear staining). Only cells expressing similar levels of p39 and QKI6 were quantified. These data suggest that phosphorylation of T243 on cytoplasmic QKI isoforms by Cdk5 triggers nuclear translocation of QKI6 and QKI7.

Figure 3-5: Cdk5/p39 phosphorylation of cytoplasmic QKI induces nuclear translocation. (A) Lysates from HOG cells cotransfected with FLAG-tagged WT-QKI6 and empty vector (pcDNA3), FLAG-tagged WT-QKI6 and p39, or FLAG-tagged QKI6-T243A and p39 were immunoblotted using an anti-FLAG and anti-p39 antibodies. eIF5α was detected as a loading control. (B) HOG cells were cotransfected with FLAG-tagged WT or T243A QKI6 and empty vector or p39 and co-immunostained using FLAG and p39 antibodies (scale bar= 20 μm). (C) HOG cells expressing FLAG-tagged WT or T243A QKI6 with or without p39 were scored according to detection of nuclear FLAG staining in $p39^{+}$ and quantified as a percentage of total cells counted ($p<0.001$, one-way ANOVA; *$p<0.001$, Tukey's post-test, n=3).

Figure 3-5

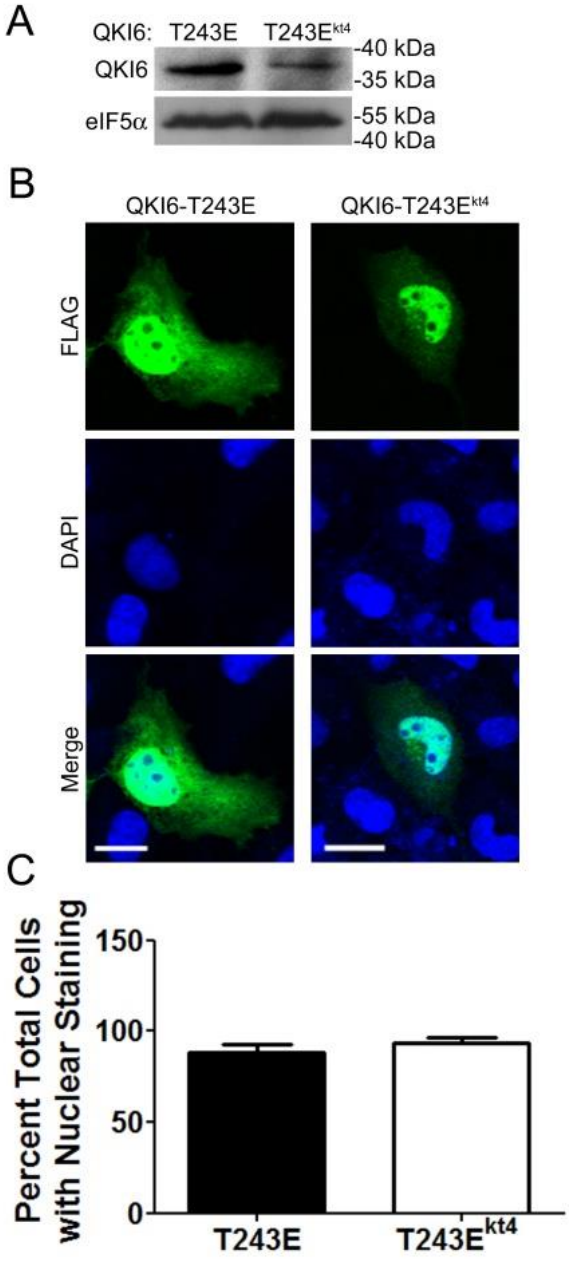

A
Q6-WT +pcDNA3
Q6-WT +p39
Q6-T243A +p39
FLAG
p39
eIF5α
-40 kDa
-35 kDa
-40 kDa
-35 kDa
-55 kDa
-40 kDa
B
Q6-WT +pcDNA3
Q6-WT +p39
Q6-T243A +p39
FLAG
p39
DAPI
Merge
C
Percent Total Cells with Nuclear Staining
100
80
60
40
20
0
*
WT +pcDNA3
WT +p39
T243A +p39

To test whether nuclear translocation of QKI6 or QKI7 was dependent on dimerization with endogenous nuclear QKI5 (Figure 3-3A), HOG cells were transfected with QKI6-T243E or QKI6-T243E^{kt4}, which carries the phosphomimetic mutation used in Figure 3-4 as well as an E48G mutation previously demonstrated to prevent QKI dimer formation. Immunoblot analysis demonstrated that QKI6-T243E^{kt4} still expressed a full length protein (Figure 3-6A). Upon immunofluorescent staining, we observed that QKI6-T243E^{kt4} accumulated in the nucleus, just as was seen for QKI6-T243E (Figure 3-6B). Quantification revealed that addition of the E48G mutation had no effect on the nuclear accumulation of QKI6-T243E (positive nuclear staining in 88.4±4.4% of cells for T243E and 93.2±3.4% of cells for T243E^{kt4}) (Figure 3-6C). Based on these observations, it appears that nuclear translocation of QKI6 upon phosphorylation by Cdk5 does not require dimerization with QKI5.

Figure 3-6: Nuclear translocation of QKI6 does not depend on dimerization.

(A) HOG cells were transfected with FLAG-tagged QKI6-T243E or –T243E^{kt4} and total lysates were immunoblotted for FLAG-QKI expression. eIF5α was detected as a loading control. (B) HOG cells were transfected with FLAG-tagged QKI6-T243E or -T243E^{kt4} and immunostained using FLAG antibody (scale bar= 20 μm). (C) HOG cells expressing FLAG-tagged QKI6-T243E or T243E^{kt4} were scored according to detection of nuclear FLAG staining and quantified as a percentage of total cells counted (n=3).

Figure 3-6

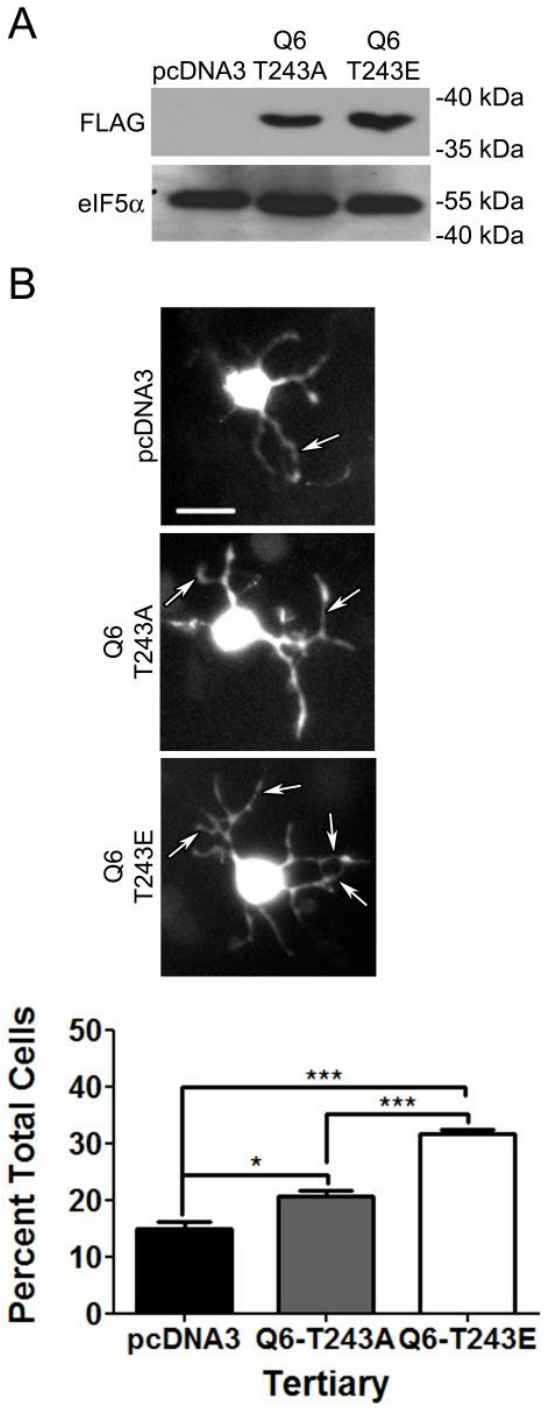

A
QKI6: T243E T243Ekt4
QKI6
eIF5α
-40 kDa
-35 kDa
-55 kDa
-40 kDa
B
QKI6-T243E
QKI6-T243Ekt4
FLAG
DAPI
Merge
C
Percent Total Cells with Nuclear Staining
150
100
50
0
T243E
T243Ekt4

3.2.4 T243 phosphorylation of cytoplasmic QKI promotes OL differentiation

We next asked whether and how phosphorylation by Cdk5 influences the ability of QKI6 to promote OL differentiation. The OL progenitor cell line CG4, which was previously used to demonstrate the essential functions of Cdk5 and QKI6 to promote OL differentiation, can be induced for morphological differentiation, during which they produce increasingly complex processes, recapitulating primary OLs (Bankston et al. 2013, Chen et al. 2007). CG4 cells were cotransfected with EGFP, to allow process visualization, and either empty vector (pcDNA3), QKI6-T243A, or QKI6-T243E. Cultures transfected with pcDNA3 provide a baseline for differentiation without addition of pro-differentiation factors. Immunoblot analysis confirmed expression of full length T243A and T243E Flag-QKI6 (Figure 3-7A). In three independent experiments, transfected CG4 were induced to differentiate, and the tertiary process arborization, a well-established hallmark of OL differentiation, was quantified (Figure 3-7B). Before induction of differentiation, a comparably small percentage of cells contained tertiary processes in all cultures. After two days of differentiation, cultures expressing QKI6-T243E contained significantly more cells with tertiary processes than cultures that expressed QKI6-T243A, suggesting that phosphorylation of QKI6 promotes OL differentiation. However, cultures transfected with QKI6-T243A did harbor more cells with tertiary processes than cultures transfected with pcDNA3, which is not surprising considering the established roles of QKI6 in promoting OL differentiation (Chen et al. 2007). Together, these data suggest that phosphorylation of QKI6 at T243 enhances the established function of QKI in promoting OL differentiation.

Figure 3-7: QKI6 phosphorylation promotes differentiation. (A) CG4 cells were transfected with FLAG-tagged WT-QKI6, QKI6-T243A, or QKI6-T243E and total lysates were immunoblotted for FLAG-QKI expression. eIF5α was detected as a loading control. (B) CG4 were cotransfected with EGFP and empty vector, QKI6-T243A, or QKI6-T243E and induced for differentiation in three separate experiments (n=3). Top, Images of GFP^+ cells were captured from more than 30 randomly selected cells in each transfected culture 2 days after induction of differentiation. Arrows indicate tertiary processes. Bottom, The percentage of total cells harboring tertiary processes was determined and plotted ($p<0.001$, one-way ANOVA; $*p<0.05$, $***p<0.001$, Tukey's post-test).

Figure 3-7


A
pcDNA3
Q6 T243A
Q6 T243E
FLAG
eIF5α
-40 kDa
-35 kDa
-55 kDa
-40 kDa
B
pcDNA3
Q6 T243A
Q6 T243E
Percent Total Cells
50
40
30
20
10
0
***
***
*
pcDNA3
Q6-T243A
Q6-T243E
Tertiary

3.3 Discussion

In this study, we demonstrate that Cdk5 phosphorylates the selective RNA-binding protein QKI, a key player essential for OL differentiation and CNS myelination. Moreover, we show that T243 in QKI is an important functional site for Cdk5-dependent phosphorylation, which enhances nuclear translocation of QKI from the cytoplasm and the ability of QKI to promote differentiation of OL progenitor cells. These data identify QKI as a novel downstream target that mediates the recently discovered function of Cdk5 in advancing OL differentiation (Bankston et al. 2013, Miyamoto et al. 2007).

Although the highest activity of Cdk5 is found in brain neurons (Su and Tsai 2011, Tsai et al. 1993), accumulating evidence indicate important functions of Cdk5 in various non-neuronal cell types (Rosales and Lee 2006). Nonetheless, the downstream targets phosphorylated by Cdk5 in different cell types are vastly undefined, except some regulators of cytoskeleton dynamics identified in neurons that are likely shared among other cell types (Baumann et al. 1993, Hahn et al. 2005, Kim Y. et al. 2006). In the CNS, QKI, which is expressed in all glial lineages but absent in neurons (Hardy 1998, Hardy et al. 1996), represents the first glial-specific target of Cdk5 identified in the CNS. The function of QKI in governing RNA homeostasis and translation is well established (Lakiza et al. 2005, Larocque et al. 2002, Saccomanno et al. 1999, Zhao et al. 2006a, Zhao et al. 2010). Therefore, our studies suggest an intriguing possibility to link Cdk5 signaling with the broad posttranscriptional pathways under the control of QKI.

Unlike neurons where Cdk5 function depends largely on its activator p35 (Chae et al. 1997, Ko et al. 2001), Cdk5 activity in OLs is primarily controlled by p39 whereas p35 expression is negligible (Bankston et al. 2013). Whether the preferential expression of p39 in OLs is an underlying mechanism for Cdk5 target selection still awaits future investigation. Despite the crucial role of p35-dependent nuclear Cdk5 activity for neuronal cell cycle re-entry and cell death upon DNA damage (Tian, Yang, and Mao 2009), we found that rodent and human OL lineage cells display predominant cytoplasmic localization of p39 at steady-state, for which

myristoylation of p39 at Glycine 2 appears to be an important mechanism. Thus, although recombinant Cdk5 can phosphorylate all QKI isoforms *in vitro*, QKI isoforms that display preferential cytoplasmic localization are more prominent targets of Cdk5 in OLs. The most obvious consequence of Cdk5-dependent phosphorylation of QKI is the induction of nuclear translocation of QKI6 and QKI7 from the cytoplasm. In contrast, the phosphomimetic T243E mutation did not alter nuclear-cytoplasmic distribution of QKI5, which is already predominantly localized in the nucleus at steady-state. How Cdk5-dependent phosphorylation induces nuclear translocation of QKI still remains unknown. Previous studies demonstrated that QKI can shuttle between the nucleus and the cytoplasm (Pilotte, Larocque, and Richard 2001, Wu et al. 1999). Notably, QKI isoforms range in size from 35 to 37 kDa, which are near the upper molecular weight limit for passive diffusion through the nuclear pore (Zuleger, Kerr, and Schirmer 2012). However, the distinct nuclear-cytoplasmic distribution of QKI isoforms argues the existence of either active transport and/or retention mechanisms in a QKI isoform-dependent manner, which must involve other unidentified proteins that interact with QKI isoforms in the nucleus and the cytoplasm. Cdk5-dependent phosphorylation of QKI likely modulates the interaction of cytoplasmic QKI isoforms with their partners, which in turn may enhance nuclear import, release cytoplasmic retention, or stabilize nuclear retention, thus resulting in nuclear accumulation of QKI.

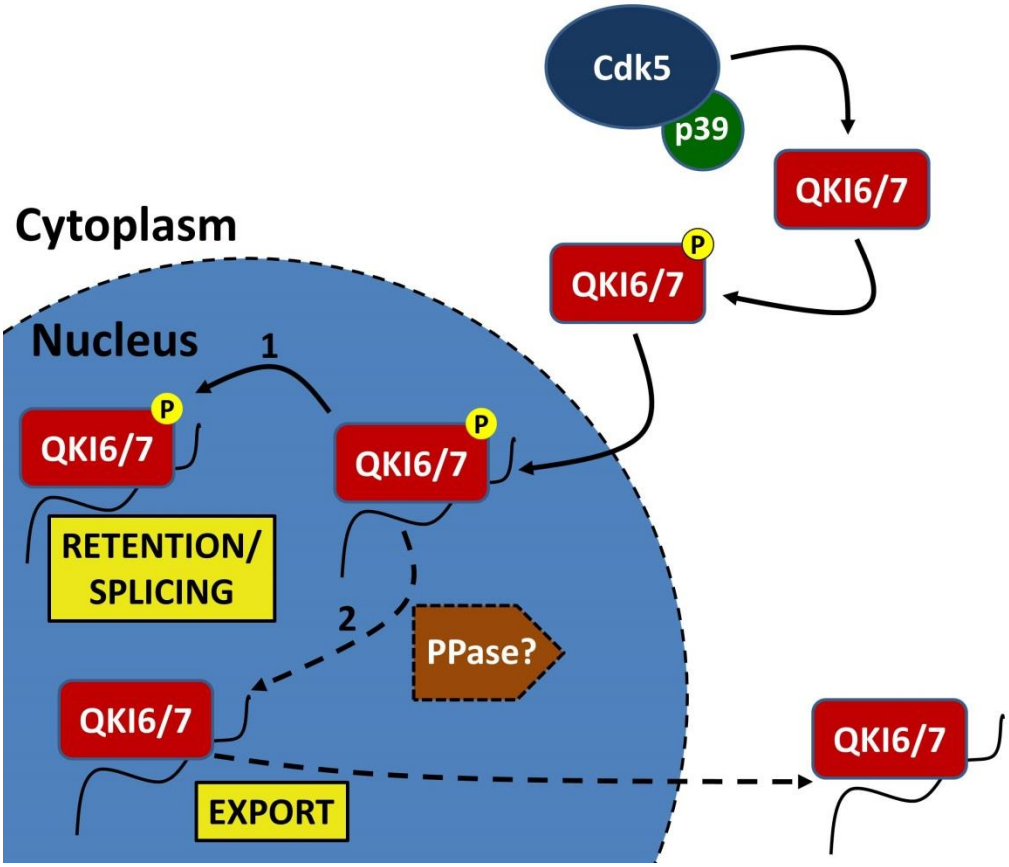
Despite their distinct nuclear-cytoplasmic profiles, all three QKI isoforms harbor activity to enhance early differentiation of OL progenitor cells (Chen et al. 2007). Prior to our findings reported here, QKI5 was thought to be primarily responsible for governing the processing and homeostasis of selective mRNA ligands in the nucleus whereas QKI6 and QKI7 control stability and translation of mRNA ligands in the cytoplasm. The marked nuclear translocation and increased activity of QKI6 in promoting OL differentiation upon Cdk5 phosphorylation at T243 uncovered an unexpected role for QKI. A working model postulates several possible mechanisms as described in Figure 3-8. Once in the nucleus, QKI6 and QKI7 may perform functions similar to

QKI5 in mRNA processing and/or cause nuclear retention of mRNAs that encode differentiation repressor proteins. Alternatively, because a substantial portion of T243-phosphorylated QKI6 is still detectable in the cytoplasm, phosphorylated QKI6 may bind mRNAs that encode differentiation activators in the nucleus and shuttle back to the cytoplasm for translation. These questions will be answered once the nuclear mRNA targets of QKI isoforms are identified by CLIP-seq. Another important issue that warrants rigorous investigation regards whether and how Cdk5-dependent phosphorylation alters the RNA binding activity of QKI, which may impact the ability of QKI in both the nucleus and the cytoplasm to promote OL differentiation.

Further delineating the mechanisms by which the Cdk5-QKI pathway promotes OL differentiation may provide novel strategies to advance OL development in diseases involving OL impairment. Uncovering mRNAs whose translation is either enhanced or inhibited by the Cdk5-QKI pathway could yield clues regarding factors whose expression must be elevated or suppressed to improve OL lineage development. Furthermore, treatments that either promote Cdk5-dependent QKI phosphorylation or merely nuclear translocation of QKI6 and QKI7 are interesting possibilities for advancing OL development that warrant further investigation.

Figure 3-8: Model of potential nuclear function of QKI6/7 to promote differentiation. Upon Cdk5-depdendent phosphorylation, QKI6/7 translocates to the nucleus where it promotes differentiation by two possible non-exclusive mechanisms: 1) Nuclear QKI6/7 binds mRNAs which encode inhibitors of differentiation and sequesters them in the nucleus. It is also possible that QKI6/7 may directly regulate splicing while in the nucleus; 2) QKI6/7 competes with QKI5 for binding to target mRNAs and transports these mRNAs to the cytoplasm through export mechanisms that possibly involve QKI6/7 dephosphorylation. mRNA-bound QKI6/7 in the cytoplasm would then localize and regulate the translation of the target mRNAs.

Figure 3-8

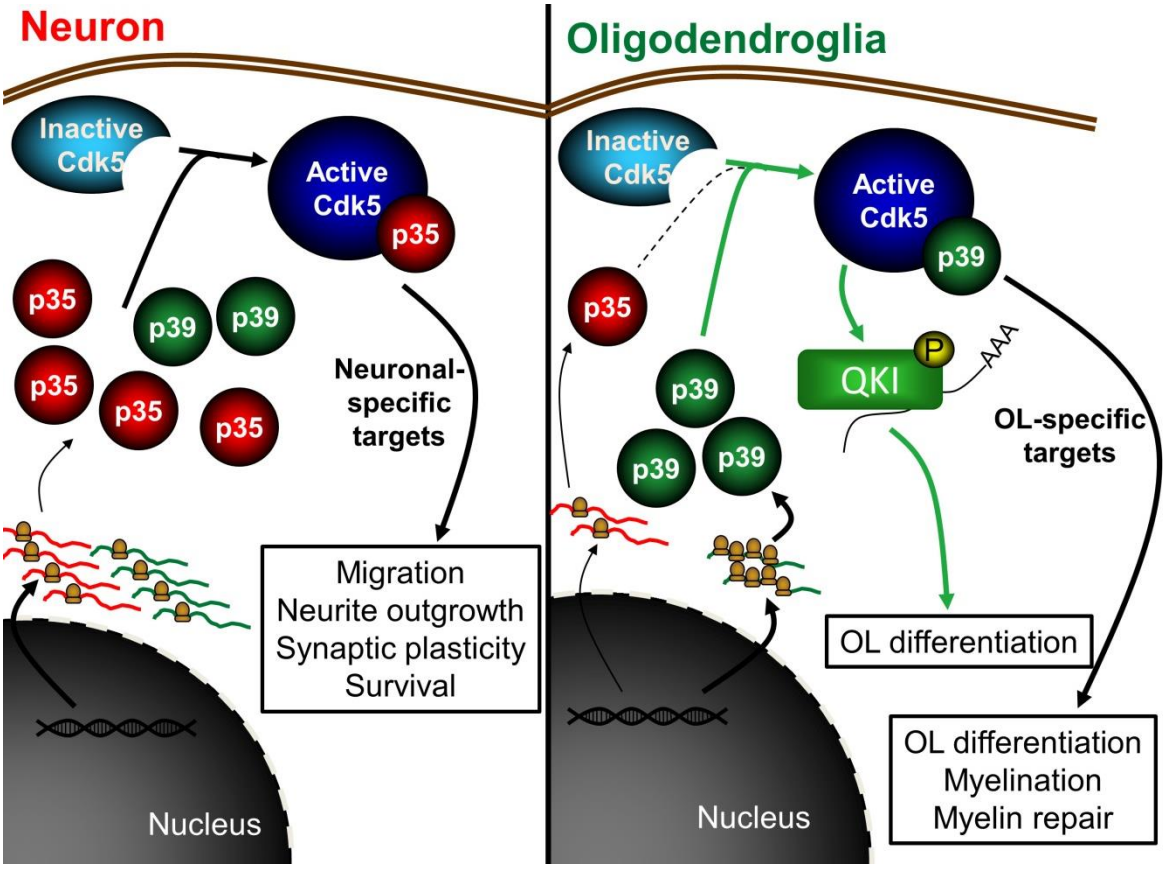

Cdk5
p39
QKI6/7
QKI6/7
P
Cytoplasm
Nucleus
1
QKI6/7
P
QKI6/7
P
RETENTION/
SPLICING
2
PPase?
QKI6/7
QKI6/7
EXPORT

Chapter 4: Conclusions and Future Directions

In this dissertation (summarized in Figure 4-1), we provide the first evidence that in contrast to most cell types where p35 is essential for Cdk5 activation (Rosales and Lee 2006, Su and Tsai 2011), p39 is the primary activator of Cdk5 in oligodendroglia (OL). Translational suppression of p35 mRNA and active translation of p39 appeared to underlie the preferential p39 expression in OLs. Suppressed translation was detected for both p35 and p39 mRNAs in neurons. We detected upregulation of p39 mRNA and protein during OL differentiation, myelin formation, and myelin repair. We further establish that p39 is essential for Cdk5 activation in OL, OL differentiation, and myelin repair. In addition, we present data suggesting that Cdk5/p39 promote OL differentiation through phosphorylation of glial-specific proteins critical for OL lineage development in addition to known Cdk5 targets such as cytoskeleton components shared by both neuronal and glial lineages. We have also demonstrated that Cdk5-dependent phosphorylation of the glial-specific protein, QKI, promotes OL differentiation through novel functions involving nuclear translocation of cytoplasmic QKI isoforms.

Figure 4-1: Conclusions. (Left) In neurons, p35 is essential for Cdk5 activation to promote migration, neurite outgrowth, synaptic plasticity, and cell survival (Rosales and Lee 2006, Su and Tsai 2011). (Right) In contrast, p39 is the primary activator of Cdk5 in oligodendroglia (OL). Active translation of p39, but not p35, likely underlies the preferential p39 expression in OLs. p39 is essential for Cdk5 activation in OL, OL differentiation, and myelin repair. In addition, Cdk5/p39 promote OL differentiation through phosphorylation of the glial-specific protein, QKI, and novel functions involving nuclear translocation of cytoplasmic QKI isoforms.

Figure 4-1


Neuron
Oligodendroglia
Inactive Cdk5
Active Cdk5
p35
p39
Neuronal-specific targets
Migration
Neurite outgrowth
Synaptic plasticity
Survival
Nucleus
QKI
P
AAA
OL-specific targets
OL differentiation
OL differentiation
Myelination
Myelin repair

4.1 Potential mechanisms regulating the preferential expression of p39 in OLs

While the critical functions of Cdk5 in neurons are well-established (Su and Tsai 2011), emerging evidence suggests that Cdk5 is also required for development of the OL lineage (Bankston et al. 2013, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013). Recent studies demonstrating that Cdk5 is essential for OL migration, differentiation, and myelin formation raise important questions regarding the regulation of Cdk5 activity in the OL lineage. In the first section of this thesis, we provided evidence that in contrast to neurons where p35 is the major activator of Cdk5, p39 is the primary activator of Cdk5 in OLs. The preferential expression of p39 in OLs was mediated by active translation of p39 mRNA and translational suppression of p35 mRNA. Considering the increasing reports of miRNA-dependent regulation of OL development (Lau et al. 2008, Li and Yao 2012), these data open the possibility of miRNAs, perhaps in co-operation with specific RNA-binding proteins, selectively targeting p35 mRNA during OL development. The 3' untranslated region (UTR) of p35 is 1.5 kb longer than that of p39, making p35 mRNA a more likely target for regulatory factors (Lew et al. 1994, Nilden, Backstrom, and Bark 1998). Future studies should focus on identifying the factors with the potential to selectively target p35 mRNA that are highly expressed in OLs relative to neurons.

Furthermore, we found that p39, but not p35, is upregulated during OL differentiation, concomitant with the reported increase of Cdk5 activity (Miyamoto et al. 2007, Tang, Strocchi, and Cambi 1998), as well as during myelin formation and repair. p39 is essential for Cdk5 activity in OL differentiation and myelin repair. The upregulation of p39 was observed for both p39 mRNA and protein, suggesting that increased transcription of p39 underlies the increase of Cdk5 activity during OL development. Furthermore, the increase of p39 mRNA seemed to underlie upregulation of p39 protein in both myelin development and repair, suggesting that similar mechanisms may regulate Cdk5 activation in myelinogenesis under developmental as well as pathological conditions. Further analysis of the p39 promoter sequence should reveal whether transcription factors that play important roles in OL development, such as Olig2 and YY1 (He

and Casaccia-Bonnefil 2008, Nicolay, Doucette, and Nazarali 2007), have potential binding sites in the p39 promoter region. ChIP using antibodies specific to these transcription factors followed by PCR with primers targeting p39 sequence would confirm whether binding of the p39 promoter by these transcription factors occurs. Experiments using overexpression or knockdown of these transcription factors in conjunction with detection of endogenous p39 or a reporter under the control of the p39 promoter would reveal the functional role of binding to the p39 promoter by these transcription factors. In summary, the mechanisms that mediate suppression of p35 and upregulation of p39 expression during OL development and myelin repair require future study, which could provide cell type-specific strategies against diseases involving failures of myelin development (dysmyelination) or loss of previously formed myelin (demyelination).

4.2 Why does myelin repair fail in p39-null mice?

Initial studies of Cdk5 and its activators in neurons suggested that p39 may play roles that are largely redundant to those of p35 due to the lack of overt phenotypes in p39-null mice at the adult stage (Chae et al. 1997, Ko et al. 2001, Ohshima et al. 1996). However, we have uncovered that a delay of myelin development exists in p39-null mice that is resolved in adulthood. The mechanisms that allow the progression of OL and myelin development in the absence of p39 remain unclear. The detection of compensatory p35 upregulation in the highly OL-enriched optic nerve of p39-null mice suggests that increased p35-dependent Cdk5 activation in OLs may mask the loss of p39. The p39-null mice also provide an opportunity to identify factors that suppress p35 expression in OLs as they may be downregulated in p39-null OLs, allowing p35 upregulation to occur. Nevertheless, repair of myelin after toxic insult was severely impaired in p39-null mice. How could loss of p39 have minimal effects during development but cause such severe impairment in myelin repair? One possible scenario might be the lack of sufficient p35 upregulation to compensate for the functional requirement of increased p39 expression during myelin repair. The much shorter time span of myelin repair may overwhelm the mechanisms

necessary for upregulation of p35 in p39-null mice, leading to insufficient Cdk5 activity to drive myelin repair. A lack of p35 compensation during myelin repair of p39-null mice could also result from differences between OLs in the developing brain and adult brain. The factors necessary for the expression of p35 in p39-null mice could be developmentally downregulated or the accessibility of the p35 locus itself in OLs could change as p39-null mice reach adulthood.

On the other hand, if p35 is upregulated in p39-null mice after a demyelination event, then the failure of myelin repair in these mice suggests that functions unique to p39 may be required for myelin repair. An alternative possibility is that p35 may be toxic to OLs exposed to the harsh environment of a demyelinated lesion. OLs are vulnerable to glutamate excitotoxicity, especially during demyelination in animal models and MS patients, which involves vast Ca^{2+}-influx (Matute et al. 2002). Activated immune cells, such as those involved in the pathology of multiple sclerosis, as well as damaged neurons release large quantities of glutamate (Collard et al. 2002, Frigo et al. 2012, Rimaniol et al. 2001). An influx of Ca^{2+} causes, among many other changes, increased activation of calpains, which can cleave p35 and p39 to form the more stable p25 and p29, resulting in hyperactivation of Cdk5 (Kusakawa et al. 2000, Patzke and Tsai 2002, Su and Tsai 2011). Considering that p25 generation is associated with many neurodegenerative disorders and appears to be neurotoxic whereas little evidence exists for detrimental effects of p29 (Cheung and Ip 2012), OLs may preferentially express p39 as the "safer" activator of Cdk5. When p35 is aberrantly expressed in p39-null OLs, the permissive developmental environment may present little risk for use of p35 by OLs. However, upon challenging the p39-null OLs by induction of myelin repair, the stressful lesion condition is predicted to convert p35 to p25, which may be harmful and impair OL maturation and thus myelin repair. Whether p35 is indeed converted to p25 in p39-null OLs during myelin repair or whether calpain inhibitors could improve myelin repair in p39-null OLs are important questions that certainly warrant further investigation.

It is important to recognize that the use of conventional p39-null mice in our studies leaves questions regarding whether loss of p39 in neurons plays a role in the delayed myelin development and severely impaired myelin repair observed in these mice. Future studies using OL-specific knockout of p39 will eliminate the influence of any neuronal defects on myelin development and repair, allowing focus on the OL-specific function of p39. Furthermore, these conditional p39-null mice will aid in the study of myelin repair by providing a system for inducible knockout of p39 after the completion of myelin development but before induction of demyelination, separating the roles of p39 in myelin development from those in myelin repair. Studying myelin repair in conditional p39-null mice could also minimize the influence of p35 upregulation.

4.3 Novel nuclear function of cytoplasmic QKI isoforms upon phosphorylation-dependent nuclear translocation

Despite recent studies that clearly demonstrate the critical role of Cdk5 in OL development (Bankston et al. 2013, Miyamoto et al. 2007, Miyamoto, Yamauchi, and Tanoue 2008, Yang et al. 2013), few targets of Cdk5 in OLs have been identified. Furthermore, no targets of Cdk5 that play central roles in OL development have been reported. In the second section of this thesis, we establish that p39-mediated Cdk5 activation phosphorylates QKI at threonine 243 (T243) to promote early OL differentiation. This is the first glial-specific target of Cdk5 and suggesting that Cdk5 may regulate OL development by targeting proteins unique to the OL lineage rather than only those shared by neurons known to control cytoskeleton dynamics. Previous studies have demonstrated that QKI is essential for OL differentiation and myelin development (Chen et al. 2007, Friedrich 1974, Zhao et al. 2006b). QKI deficiency in OLs results in severe impairment of OL function and CNS hypomyelination in the quakingviable (qk^{v}) mutant mice (Friedrich 1974, Sidman, Dickie, and Appel 1964). The mechanisms by which Cdk5-dependent QKI phosphorylation promotes early OL differentiation remain unclear. Our

finding that Cdk5-dependent phosphorylation induces nuclear translocation of the cytoplasmic QKI isoforms, QKI6 and QKI7, suggests that novel nuclear functions of the cytoplasmic QKI isoforms are involved in OL differentiation. Each QKI isoform was previously reported to advance early OL differentiation, although QKI6 and QKI7 appeared to be more potent factors for OL differentiation than QKI5 (Chen et al. 2007). Before this study, a model of QKI isoform function in OL development postulated that although QKI isoforms can shuttle between the nucleus and the cytoplasm, QKI5 is mainly responsible for regulating the nuclear behavior of target mRNAs, e.g. pre-mRNA splicing (Wu et al. 2002) and nuclear retention of mRNA targets (Larocque et al. 2002). In contrast, QKI6 and QKI7 were thought to control stability and translation of direct QKI target mRNAs in the cytoplasm, represented by mRNAs encoding myelin structural proteins and canonical splicing factors (Lakiza et al. 2005, Li et al. 2000, Saccomanno et al. 1999, Zhao et al. 2006a, Zhao et al. 2010). Our findings add a new layer to this model in which Cdk5-dependent phosphorylation causes a significant amount of QKI6 and QKI7 nuclear translocation and potential shuttling between the nucleus and cytoplasm. In fact, the increase in the nuclear pool of QKI6 and QKI7 advances differentiation, possibly through novel functions in the nucleus.

The exact mechanism by which QKI6 promotes OL differentiation while in the nucleus is unknown. One possibility is that QKI6 binds mRNAs that encode inhibitors of differentiation and retains them in the nucleus. Binding of the mRNA encoding actin-interacting protein 1 (AIP-1), a mediator of actin disassembly, by QKI6 causes the developmental downregulation of AIP-1 during OL differentiation and myelin formation (Doukhanine et al. 2010). It is possible that AIP-1 downregulation is mediated by nuclear retention of its mRNA by phosphorylated QKI6. Nuclear retention of these inhibitor mRNAs would prevent their translation, thus attenuating the inhibitory effect of the encoded protein and allowing OL differentiation to progress. A second model postulates that QKI isoforms may bind mRNAs in the nucleus and facilitate their export to the cytoplasm. While nuclear QKI5 is capable of exiting the nucleus and could thus mediate

export of QKI target mRNAs (Wu et al. 1999), nuclear QKI6 and QKI7 could expedite the mRNA export process by returning to the cytoplasm while bound to the target mRNAs and promoting cytoplasmic retention of the bound mRNAs. Furthermore, the developmental increase of both QKI6 and Cdk5 activity would increasingly favor cytoplasmic retention of QKI target mRNAs that are critical for OL development, such as MBP and MAP1B, as they are being upregulated. This would allow export of these mRNAs under translational suppression by QKI6, which is reported to suppress mRNA translation (Lakiza et al. 2005, Saccomanno et al. 1999), for proper localization and local translation in myelin. Alternatively, nuclear translocation of QKI6 and QKI7 could relieve translational repression of target mRNAs in the cytoplasm. A third model hypothesizes regulation of alternative splicing by QKI6 and QKI7 when in the nucleus by direct binding to pre-mRNAs. Many key regulators of OL development have multiple isoforms generated by QKI-dependent alternative splicing, such as MBP, PLP, and MAG (Zhao et al. 2006b). Recent studies have elucidated the role of cytoplasmic QKI isoforms in regulating the alternative splicing of these pre-mRNAs through regulation of splicing factor expression (Zhao et al. 2010). However, functions for QKI5 in regulating alternative splicing through direct pre-mRNA binding have also been reported (Wu et al. 2002). Moreover, QKI-dependent regulation of global alternative splicing events by binding sequences proximal to exon-intron boundaries has been uncovered in myotubes (Hall et al. 2013). In light of our data demonstrating nuclear translocation of QKI6 and QKI7 from the cytoplasm, it is possible that these QKI isoforms may regulate alternative splicing not only by controlling translation of splicing factors within the cytoplasm, but also through direct pre-mRNA binding in the nucleus. Consistent with this idea, recent work has provided evidence that QKI6 colocalizes with spliceosomal proteins in the nuclei of vascular smooth muscles cells (VSMCs) and regulates alternative splicing of the myocardin pre-mRNA through direct binding proximal to the 3’ splice site of alternative exon 2a (van der Veer et al. 2013). These non-exclusive models present interesting possibilities regarding how QKI isoforms regulate OL development. The mechanisms that regulate these nuclear events are

not fully understood. Specifically, the factors that regulate the import and export of cytoplasmic QKI to and from the nucleus are unknown.

4.4 Potential mechanisms mediating the nuclear localization of phosphorylated QKI6/7

Although it is clear from our study that Cdk5-depdendent phosphorylation of cytoplasmic QKI induces nuclear translocation, the mechanisms that mediate the entry of cytoplasmic QKI into the nucleus in response to this phosphorylation require further study. Threonine phosphorylation of the QKI homolog held out wings (HOW) by the MAPK, Erk, enhances HOW dimer formation (Nir et al. 2012). The cells used in our experiments, HOG cells, express endogenous QKI5. Thus, we sought to determine whether phosphorylated QKI6 may enter the nucleus through increased QKI5 binding. However, a mutation that prevents QKI dimer formation did not affect nuclear translocation of phosphomimetic QKI6, suggesting that nuclear translocation of cytoplasmic QKI likely occurs through factors other than QKI5.

One hypothesis postulates that phosphorylation of QKI6 and QKI7 may promote their interaction with nuclear anchor proteins, similar to phosphorylation of Erk. Upon phosphorylation by the MAPKK, MEK, a proline-directed serine/threonine kinase like Cdk5, Erk translocates to the nucleus (Lidke et al. 2010). Recent work has uncovered that the nuclear translocation of Erk is mediated by the Dual-specificity phosphatase (DUSP) family of nuclear anchors (Mandl, Slack, and Keyse 2005). Members of the DUSP family that are upregulated and required for OL differentiation have recently been identified, including DUSP15 (Schmidt et al. 2012). Although the DUSP family was thought to act exclusively on MAPKs, emerging reports indicate that members of the STAT family, histone H3, and focal adhesion kinase (FAK) are bound and dephosphorylated by DUSPs (Jardin and Sticht 2012, Jeong et al. 2013, Li et al. 2010). Therefore, it is possible that the requirement of DUSP proteins for OL differentiation may involve mediating the nuclear translocation of cytoplasmic QKI upon Cdk5-dependent phosphorylation. Previous work from our lab suggests that the methyltransferase PRMT5 binds QKI6 in OLs (Chen Y,

unpublished data). Upregulated during OL development, PRMT5 is required for OL differentiation through mechanisms that include epigenetic regulation in the nucleus (Huang et al. 2011). In addition, QKI5 is methylated *in vivo*, and QKI interacts with coactivator-associated arginine methyltransferase 1 (CARM1) (Côté et al. 2003, Weimann et al. 2013). R242 was identified as the site of QKI methylation (Weimann et al. 2013). Considering that QKI methylation at R242 and Cdk5-dependent QKI phosphorylation at T243 occur at neighboring residues, whether crosstalk between these post-translational modifications influences their function is an interesting question for future studies. Phosphorylation and methylation of neighboring residues within a protein can modulate the association of that protein with binding partners (Fischle et al. 2005, Varier et al. 2010). Additionally, methylation of SAM68 alters its nucleo-cytoplasmic distribution (Côté et al. 2003). Whether Cdk5-dependent phosphorylation regulates QKI6 binding to PRMT5, CARM1, or proteins that recognize QKI6 methylation in order to facilitate QKI6 nuclear translocation is an interesting possibility to be explored by future studies. Conversely, binding of QKI6 to proteins in the cytoplasm may prevent its nuclear translocation. Tau phosphorylation by proline-directed serine/threonine kinase at sites proximal to PXXP motifs disrupts the interaction of Tau with SH3 domain-containing proteins (Reynolds et al. 2008). T243 of QKI is within ten amino acids of a putative PXXP that may mediate binding of QKI by SH3 domain proteins. Whether T243 phosphorylation induces nuclear translocation of cytoplasmic QKI by disrupting binding of QKI to SH3 domain-containing proteins in the cytoplasm requires further investigation.

Perhaps it is more important to determine whether QKI6 and QKI7 remain in the nucleus or they are exported back to the cytoplasm. Reversal of Cdk5-dependent QKI phosphorylation by nuclear phosphatases may be required for nuclear export of QKI6 and QKI7. The phosphomimetic QKI6-T243E used in our study adds a negative charge to QKI6 that in contrast to kinase-dependent phosphorylation is not reversible. It is possible that QKI6-T243E may accumulate in the nucleus to a greater extent than phosphorylated WT-QKI6. Use of QKI6-

T243E may aid in the study of the nuclear functions of QKI6 in OL differentiation. On the other hand, induction of WT-QKI6 nuclear translocation by increasing Cdk5 activation would provide a system for defining the mechanisms mediating QKI6 nucleo-cytoplasmic shuttling through Cdk5 or phosphatase inhibition. It is important to note that QKI is small enough to transit the nuclear pore by passive diffusion (Zuleger, Kerr, and Schirmer 2012). Therefore, QKI6 and QKI7 may normally be retained in the cytoplasm through binding to other cytoplasmic proteins. Cdk5 would then modulate the balance between cytoplasmic and nuclear retention of QKI. QKI phosphorylation by Cdk5 may attenuate binding to cytoplasmic anchors, releasing QKI from cytoplasmic retention and allowing QKI diffusion to the nucleus. Cdk5-dependent QKI phosphorylation may also enhance QKI binding to nuclear anchors. However, the fact that QKI normally functions as a dimer could prevent the passive diffusion of QKI between the cytoplasm and nucleus.

4.5 Other potential roles for Cdk5-dependent QKI phosphorylation in promoting OL differentiation

In cells with nuclear accumulation of QKI6 and QKI7, cytoplasmic QKI staining is still observed. Therefore, the nuclear functions of QKI6 and QKI7 likely occur in addition to the well-established roles of QKI in the cytoplasm. However, the effect of Cdk5-dependent phosphorylation on the function of QKI in the cytoplasm must be determined to fully understand the role of the Cdk5/QKI pathway in OL development. QKI regulates OL development by controlling the stability, localization, and translation of QKI target mRNAs that encode key proteins for OL differentiation and myelin development in the cytoplasm (Bockbrader and Feng 2008). Previous studies from our lab demonstrated that phosphorylation of QKI by the Src family protein tyrosine kinase, Fyn, reduces QKI RNA-binding (Zhang et al. 2003). A similar consequence is seen for HuR phosphorylation by Cdk5 (Filippova et al. 2012). However, phosphorylation of RBPs does not always reduce their RNA-binding activity. For instance,

phosphorylation of polypyrimidine tract binding protein associated factor (PSF) by anaplastic lymphoma kinase (ALK) enhances the binding of PSF to target RNAs (Galietta et al. 2007). Thus, Cdk5-dependent phosphorylation could actually increase QKI RNA-binding. In neurons, phosphorylation by Cdk5 inhibits Src-dependent phosphorylation of PSD-95 (Zhang et al. 2008). In addition, Cdk5 inhibition enhances Src-dependent phosphorylation of NR2B. Furthermore, Cdk5-dependent phosphorylation of apoptosis-associated tyrosine kinase 1 (AATYK1) suppressed phosphorylation by Src (Tsutsumi et al. 2010). Hence, Cdk5-dependent phosphorylation appears to counteract or negatively affect Src-dependent phosphorylation within the same target protein. Whether and how the RNA-binding activity of QKI is altered by Cdk5-dependent phosphorylation, and how that may impact Fyn-dependent phosphorylation of QKI is an intriguing question for future studies. Experiments first determining whether Cdk5-dependent phosphorylation enhances or inhibits RNA-binding are essential. These experiments, involving crosslinking immunoprecipitation (CLIP) of QKI/target mRNA complexes, can also determine whether the observed effect on RNA-binding is restricted to the nucleus or cytoplasm. Should we find that nuclear phosphatases mediate the export of cytoplasmic QKI from the nucleus, the now unphosphorylated QKI may bind and export target mRNAs, which would then be released in the cytoplasm upon Cdk5-dependent phosphorylation of QKI, which returns to the nucleus. If Cdk5-dependent QKI phosphorylation increases RNA-binding, we would next determine whether this occurs through inhibition of Fyn-dependent QKI phosphorylation.

4.6 Conclusions, future directions, and implications for human health

In this dissertation, we have revealed that neurons and OLs utilize distinct activators for Cdk5 activation. We have further provided clear evidence for the critical role played by p39 in advancing OL and myelin development, and perhaps most critical in myelin repair, likely through activation of Cdk5. Activated Cdk5 in turn phosphorylates QKI, a central protein in the OL differentiation and myelinogenesis and the first glial-specific target of Cdk5. Cdk5-dependent

phosphorylation induced nuclear translocation of QKI6 and QKI7, implying novel nuclear function of the cytoplasmic QKI isoforms in the nucleus exist. Our data establishing that p39 is required for OL differentiation and Cdk5-dependent QKI phosphorylation promotes OL differentiation suggest that the Cdk5/QKI pathway may perform critical roles in advancing development of the OL lineage.

The OL-specific Cdk5/p39-QKI pathway identified in this dissertation may aid in the development of novel strategies to guard against or repair OL impairment in neurological disorders. Careful study of the propensity for p25 or p29 generation by OLs in stress conditions as well as the possible cytotoxicity of p25 and p29 in OLs may guide future efforts to enhance myelin repair through increased Cdk5 activity. This is especially important considering the elevated oxidative damage and glutamate present in MS lesions (Frigo et al. 2012). Furthermore, defining OL-specific factors that underlie the upregulation of p39 could provide targets for enhancing Cdk5 activity in diseases involving both dysmyelination and demyelination. Additionally, emerging evidence implicating OLs and Cdk5 in many of the same neurodegenerative diseases demands further exploration of the importance of Cdk5 dysfunction in OLs for the pathogenesis of these diseases. Our identification of a glial-specific Cdk5 target has provided an alternative to modulation of Cdk5 activity in OLs. Enhanced OL development and function may be achieved by finding ways to promote the functional effects of Cdk5-dependent target phosphorylation by manipulating the target itself (e.g., nuclear translocation of QKI6 or enhancing QKI6 nuclear functions). However, determination of the mechanisms that regulate QKI6 nucleo-cytoplasmic shuttling as well as the nuclear functions of QKI must first be accomplished. While many targets of Cdk5 in neurons are also found in OLs, it is likely that the full collection of Cdk5 targets remains largely undefined. Proteomic profiles after Cdk5 activity changes are needed to provide additional candidates for modulation of Cdk5 downstream function in OL and myelin development. The findings presented in this dissertation provide new strategies for enhancing OL and myelin development as well as myelin repair in neurological disorders in

which dysmyelination or demyelination have caused or contributed to the pathogenesis of these diseases. Furthermore, the evidence linking Cdk5 and QKI function, which are established as key factors in OL development, suggests that OL-specific functions of Cdk5 evolved which are not merely adapting neuronal Cdk5 functions to the OL lineage. Identifying additional OL-specific targets of Cdk5 could greatly expand our understanding of OL development and offer many possibilities for enhancing OL and myelin development as well as myelin repair in neurological diseases using treatments that modulate Cdk5-dependent function specifically in OLs.

Chapter 5: Materials and Methods

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5.1 Chapter 2

Animal Care All animal procedures adhere to NIH regulations under the approval of IACUC by Emory University, University of Kentucky, and UCLA. The $p39^{-/-}$ mice were generated in a previous study (Ko et al. 2001). Mice expressing EGFP under the oligodendrocyte-specific 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) and proteolipid protein (PLP) promoters were also previously described (Mallon et al. 2002, Zhu et al. 2012). Sprague-Dawley rats were purchased from The Jackson Laboratory.

FACS-isolation of Oligodendrocytes FACS-isolation of $EGFP^+$ OLs from PLP-EGFP mice at postnatal days 1, 10, and 20 was performed as previously described (Zhu et al. 2012). Briefly, finely minced brain tissues (6-10 brains) were digested with papain in Hank's balanced salt solution (HBSS) (15 U/mL) at 37ºC for 15 min to produce cell suspensions. The cell suspensions were then dissociated by sequential passages through gauge 18, 21, and 23 needles. Dissociated cell suspensions were sorted on a MoFlo (Beckman Coulter) using a 100 micro nozzle tip at 30 psi. The FACS sorting scatter profile was Forward Scatter for relative size versus Side Scatter for granularity. Apoptotic cells appeared as smaller and more granular and were excluded from further sorting process. The $EGFP^+$ cells were sorted based on fluorescence intensity and stored at -80ºC before being subjected to RNA and protein quantification.

Cuprizone-induced Demyelination The cuprizone demyelination-remyelination paradigm and tissue dissection were performed as previously reported (Crawford, Mangiardi, and Tiwari-Woodruff 2009). Briefly, male PLP-EGFP mice at 8 weeks of age were fed with 0.2% cuprizone (Sigma) mixed into milled chow (Harlan Tekland). Mice were fed the cuprizone diet for 3 weeks to induce demyelination (3wk DM). To analyze remyelination, a parallel group of mice were returned to normal chow for an additional 3 weeks following cuprizone treatment (3+3wk RM).

Control mice demonstrating normal myelination (Normal) were maintained on normal pellet chow.

Lysolecithin-induced Demyelination Focal demyelination was induced by unilateral lysolecithin injection as described (Aguirre et al. 2007). Briefly, 2 μl 1% lysolecithin (Sigma) in 0.9% NaCl was injected into the corpus callosum of WT and $p39^{-/-}$ PLP-EGFP mice at 6 weeks of age. Stereotactic coordinates of 1.1 mm anterior and 1 mm lateral to the bregma, and 1.8 mm deep from the skull surface were used for injection. Control mice received saline injection. To minimize reflux along the needle track, the needle was not removed until 5 min after injection. Brains were harvested at 5 and 21 days post lysolecithin injection (DPL5 and 21) to evaluate demyelination and remyelination, respectively. The demyelinated lesion was marked by the loss of EGFP signal.

Cell Culture and Treatment Primary cortical neurons were raised from rat brains at embryonic day 18 as described (Meberg and Miller 2003). Dissociated neurons were plated in poly-D-lysine coated 35 mm2 dishes (300,000 cells/dish) in B27/Neurobasal medium supplemented with 1X GlutaMax.

Primary rat OPCs were isolated by shaking off from a confluent glia monolayer prepared from Sprague-Dawley rats at postnatal day 2 following a well-established procedure (Osterhout et al. 1999). OPCs are purified by differential plating and maintained in a defined serum-free medium supplemented with PDGF (10 ng/mL) and bFGF (20 ng/mL) overnight before being induced for differentiation in defined medium lacking growth factors. The remaining astrocytes were trypsinized and sub-cultures were allowed to grow for 3 days before harvest.

CG4 cells were propagated and differentiated as previously described (Chen et al. 2007, Zhao et al. 2006a). Briefly, proliferating CG4 cells were cultured in Dulbecco's modified Eagle's medium containing 1% heat-inactivated fetal bovine serum, insulin (5 μg/mL), transferrin (50

μg/mL), PBPS [(putrescine (100 mM), biotin (10 ng/mL), progesterone (20 nM), and selenium (30 nM)]. Platelet-derived growth factorAA (PDGFAA, Sigma) and basic fibroblast growth factor (bFGF, Promega) were added to the proliferation medium (final 10 ng/mL each). To induce differentiation, proliferation medium was replaced with differentiation medium, which contains Dulbecco's modified Eagle's medium, insulin, PBPS, transferrin, tri-iodothyronine (50 nM), and 0.5% fetal bovine serum.

For p39, p35, or Cdk5 knockdown, CG4 cells were transfected with control-, p39-, p35-, Cdk5-siRNA (Invitrogen) using Lipofectamine 2000. The p39-siRNA (Cat# RSS366369) was predesigned. siRNA duplexes targeting the nucleotide sequence 5'-AAGCUGUACUCCACGUCCAUC-3' of rat Cdk5 mRNA were synthesized by Invitrogen. Previously described siRNA duplexes targeting the nucleotide sequence 5'-GAUGCUGCAGAUCAAUGCU-3' of rat p35 mRNA were synthesized by Thermo Scientific.

Morphological Analysis CG4 cells were cotransfected with an EGFP plasmid together with either control-, p39-, or Cdk5-siRNA. At 0, 24 and 48 hours of differentiation, live images of randomly selected GFP^+ cells were captured using the Olympus IX-51 inverted fluorescent microscope, followed by quantification of primary, secondary, and tertiary processes as previously described (Chen et al. 2007). Cells were categorized into the following groups: 1) only harboring primary processes, 2) the most complex processes are secondary processes, and 3) the most complex processes are tertiary processes. The percentages of cells in each category from three independent transfections were calculated and statistically compared. More than 50 randomly selected cells were analyzed from each transfected culture.

Cdk5 Kinase Assay CG4 cells were co-transfected with p39-specific siRNA or negative control siRNA (Invitrogen). To exclude potential off-target effects, a p39-Flag construct was used to rescue p39 knockdown. Twenty-four hours after transfection, cells were lysed in a buffer

containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 5 mM sodium fluoride, 0.5% NP-40, and protease inhibitors. After preabsorption with Protein A beads, lysates containing equal amount of total protein were incubated with 2 μg anti-Cdk5 antibody (Santa Cruz, C-8) for 3 hours before capture by Protein A beads. After washing with lysis buffer and kinase buffer (20 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 100 μM EDTA, 100 μM EGTA, and protease inhibitors), beads were resuspended in 25 μL kinase buffer plus 25 μM cold ATP, 10 μCi [γ-^{32}P]-ATP, and 100 ng/μL histone H1. After incubation at 30ºC for 30 minutes, proteins from each reaction were resolved by SDS-PAGE and transferred to PVDF membranes. From the same membrane, phosphorylation of H1 was detected by autoradiography and immunoprecipitated Cdk5 was detected by immunoblot analysis and quantified based on densitometry. For each experiment, the relative level of phosphorylated H1 to Cdk5 protein was quantified as a percentage of levels in immunoprecipitates from control-siRNA transfected cells. The average relative level of phosphorylated H1 to Cdk5 protein was determined from three independent experiments.

Linear Sucrose Gradient Fractionation Cytoplasmic extracts from CG4 cells lysed with gradient buffer (20 mM Tris pH 7.5, 100 mM KCl, 5 mM $MgCl_2$) containing 1% Triton X-100 were loaded onto linear sucrose gradients (15–45%, w/v), centrifuged at 39,000 rpm in a SW41 rotor for 60 minutes at 4ºC, and fractionated as previously described (He et al. 2010). To disassociate polyribosomes, parallel cytoplasmic extracts from CG4 cells were prepared in the presence of 20 mM EDTA and no $MgCl_2$, followed by fractionation on a gradient containing 1 mM EDTA, but lacking $MgCl_2$.

mRNA Quantification For tissue and cells, total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions and mRNAs were quantified by qRT-PCR. After linear sucrose gradient fractionation (Lau et al. 2010), RNA was isolated by phenol/chloroform,

followed by semi-quantitative RT-PCR. The following primers were used: p39-specific primers, 5'-AACCTGGTGTTCGTGTACCTGCT-3' (forward) and 5'-AGATCTCGTTGCCCATGTAGGAGT-3' (reverse); p35-specific primers, 5'-AACAGCAAGAACGCCAAGGACAAG-3' (forward) and 5'-ATGTTGCTCTGGTAGCTGCTGTTG-3' (reverse); Cdk5-specific primers, 5'-ATGATGACGATGAGGGTGTGCCAA-3' (forward) and 5'-GGTCACCATTGCAGCTGTCGAAAT-3' (reverse); GAPDH primers, 5'-GGTGAAGGTCGGTGTGAAC-3' (forward) and 5'-CCTTGACTGTGCCGTTGAA-3' (reverse).

Immunoblot Analysis Whole cell lysates were prepared by sonication in 1X Laemmli buffer for SDS-PAGE. Resolved proteins were transferred to PVDF membranes, which were blocked in 10% nonfat dry milk in phosphate-buffered saline with 0.1% Tween-20. The membranes were then probed with primary antibodies. Anti-p39 (1:1000) was kindly provided by Dr. Nancy Ip (Hong Kong University of Science and Technology), or purchased from Santa Cruz (F-4); anti-Cdk5 (1:1000) and anti-p35 (1:1000) were from Cell Signaling; anti-eIF5α (1:10000) was from Santa Cruz; anti-MBP (1:9000) was from Millipore.

Immunohistochemistry Mice were deeply anesthetized with isoflurane and intracardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed and postfixed in 4% paraformaldehyde. Cryostat brain sections were permeabilized with PBS containing 0.1% Triton X-100 for 20 minutes followed by blocking with PBS containing 1% BSA or 10% normal goat serum (NGS) for 1 hour. Sections were incubated with the following primary antibodies according to the manufacturer's suggestions: anti-p39 (Santa Cruz), anti-MBP and anti-Olig2 (Millipore), and anti-CC1 (Abcam). After washes, sections were incubated with secondary antibodies and nuclear stain DAPI (2 ng/mL; Molecular Probes) in PBS containing 2%

NGS at room temperature for 1 hour, washed 3 times with PBS and mounted with either Vectashield mounting medium (Vector Laboratories) or Fluoromount-G (Southern Biotech).

MBP+ Cell Quantification Immunofluorescent staining for myelin basic protein (MBP), a well-accepted marker for OL differentiation, was performed on three adjacent brain sections from four pairs of WT and $p39^{-/-}$ mice at postnatal day 6. The total numbers of MBP^{+} cells in the corpus callosum from all three adjacent sections for each mouse were counted in a blinded manner and graphically displayed.

Statistical analysis All bar graphs in the manuscript are presented as *means*± *SEM*. Student's *t*-test was used for two sample comparisons. For multiple sample comparisons, one-way ANOVA was carried out followed by Tukey's post-test and two-way ANOVA analysis was performed followed by Bonferroni's post-test as indicated in the corresponding figure legends.

5.2 Chapter 3

Animal Care All experiments with mice (*Mus musculus*) were carried out in compliance with NIH regulations with approval of the IACUC at Emory University. Sprague-Dawley rats (*Rattus norvegicus*) were purchased from The Jackson Laboratory.

Subcellular Fractionation Brain stem from WT and $p39^{-/-}$ mice were homogenized in a buffer containing PIPES (5 mM, pH 6.4), NaCl (10 mM), $MgCl_2$ (3 mM), $CaCl_2$ (3 mM), sucrose (0.32 M), and protease inhibitors, followed by lysis using 0.5% Triton X-100. The lysate was subjected to 3000 x g centrifugation for 5 minutes to yield crude cytoplasmic supernatant and nuclear pellet fractions. The pellet was resuspended in homogenization buffer (0.32 M sucrose) and centrifuged through 1.75 M sucrose at 100,000 x g for 45 minutes to produce cytoplasmic-free nuclei. All fractions were lysed in 1X Laemmli buffer by sonication.

Cell Culture Primary rat OPC cultures were generated by shaking from a confluent glial monolayer derived from Sprague-Dawley rats at postnatal day 2 following a well-established procedure (Osterhout et al. 1999). Purified OPC cultures were established by differential plating and maintained in a defined serum-free medium supplemented with platelet-derived growth factor (PDGF, 10 ng/mL) and basic fibroblast growth factor (bFGF, 20 ng/mL) overnight before differentiation was induced using defined medium that lacks growth factors.

CG4 cells were propagated and differentiated as previously described (Chen et al. 2007). Briefly, CG4 cells were proliferated in DMEM containing 1% heat-inactivated fetal bovine serum, insulin (5 μg/mL), transferrin (50 μg/mL), PBPS [putrescine (100 mM), biotin (10 ng/mL), progesterone (20 nM), and selenium (30 nM)], and supplemented with 10 ng/mL PDGFAA (Sigma) and bFGF (Promega). Differentiation was induced using differentiation medium consisting of DMEM, insulin, PBPS, transferrin, tri-iodothyronine (50 nM), and 0.5% fetal bovine serum.

HOG cells were proliferated in DMEM containing 10% fetal bovine serum. HOG and CG4 cells were transfected using Lipofectamine 2000 (Invitrogen).

Morphological Analysis CG4 cells were cotransfected with an EGFP plasmid and pcDNA3, QKI6-T243A, or QKI6-T243E. Live images of randomly selected GFP^+ cells were captured at 0, 24, and 48 hours of differentiation using the Olympus IX-51 inverted fluorescent microscope. Tertiary processes were quantified as previously described (Bankston et al. 2013). From three independent experiments, the percentage of cells with tertiary processes were quantified and statistically compared. More than 30 randomly selected cells were analyzed from each transfected culture.

Plasmid Construction and Mutagenesis To generate a p39 construct with a C-terminal flag tag, p39 was PCR amplified from a plasmid containing the mouse p39 cDNA (provided by Dr. Mao, Emory University) using primers containing the flag coding sequence 5'-CTCCCGCGGATGGGCACGGTGCTGTCGCTTTCCC-3' (forward) and 5'-CTCGGATCCGTCGACTCATCATCACTTATCGTCGTCATCCTTGTAATCGCGGTCCAAGTTCATAGTCC-3' (reverse).

All mutagenesis was performed using the QuikChange Lightning Mutagenesis Kit (Agilent). The following primers were used: p39-flag G2A 5'-CCCGCGGATGGCCACGGTGCTGT-3' (forward) and 5'-ACAGCACCGTGGCCATCCGCGGG-3' (reverse); flag-QKI T243A 5'-CAGCTGCTCTGCGTGCACCTACGCCAGCTG-3' (forward) and 5'-CAGCTGGCGTAGGTGCACGCAGAGCAGCTG-3' (reverse); flag-QKI T243E 5'-CCCACCAGCTGCTCTGCGTGAACCTACGCCA-3' (forward) and 5'-TGGCGTAGGTTCACGCAGAGCAGCTGGTGGG-3' (reverse); flag-QKI T267A 5'-

GTCATGCCAAACGGAGCTCCTCACCCAACTG-3' (forward) and 5'-CAGTACGGTTTGCCTCGAGGAGTGGGTTGAC-3' (reverse).

To generate the QKI6-T243E^{kt4} construct, the sequence encoding the E48G mutation from the QKI6^{kt4} plasmid was subcloned into the QKI6-T243E plasmid. Successful mutagenesis and subcloning was confirmed by sequencing the plasmids.

Recombinant QKI Protein Production Rosetta 2 competent cells were transformed with pET28a-QKI encoding His_6-tagged wildtype QKI isoforms or mutant QKI6 removing potential Cdk5 phosphorylation sites. 10 mL of LB medium containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol were inoculated with a single colony of each transformant and grown to saturation at 37ºC. Auto-induction media containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol was inoculated (1:100 dilution) using these cultures. Protein production was induced by growing the cultures to saturation with vigorous shaking at 37ºC for approximately 18 hours.

Cell pellets were resuspended in 20 mM Tris-HCl (pH 8.0) buffer with 500 mM NaCl, 5 mM imidazole, and protease inhibitors, lysed by sonication, and centrifuged at 18000 xg for 15 minutes. The supernatant was incubated with Ni-NTA beads (Qiagen) at 4ºC for 1 hour. His_6-tagged protein-bound beads were washed using 20 mM Tris-HCl (pH 8.0) buffer with 500 mM NaCl, 60 mM imidazole, and protease inhibitors, and proteins were eluted in 20 mM Tris-HCl (pH 8.0) buffer with 500 mM NaCl, 250 mM imidazole, and protease inhibitors. Eluted proteins were analyzed by SDS-PAGE.

In vitro Cdk5 Kinase Assay 100 ng of purified His_6-tagged wildtype QKI isoforms or mutant QKI6 (T243A, T267A, or T243A/T267A) were incubated with 2 ng of active Cdk5/p25 complexes (Millipore) in (10 mM MOPS (pH 7.0), 10 mM magnesium acetate, 0.5% glycerol, 0.01% 2-mercaptoethanol, 100 μg/mL BSA, 25 μM cold ATP, 1 μCi [γ-^{32}P]-ATP; 1 mM EDTA

and 0.001% Brij-35 were added for the reactions with WT and mutant QKI6) in an *in vitro* kinase assay at 30ºC for 30 minutes. Reactions were terminated by boiling in 1X Laemmli buffer and resolved by SDS-PAGE. The gels were coomassie stained, dried, and QKI phosphorylation was detected by autoradiography.

Immunoblot Analysis Whole cell lysates were prepared by sonication in 1X Laemmli buffer for SDS-PAGE. Proteins were then transferred to PVDF membranes, which were blocked in 10% nonfat, dry milk in phosphate-buffered saline with 0.01% Tween-20. The membranes were then probed with primary antibodies. Anti-p39 (1:1000, F-4), anti-GAPDH (1:500, V-18), and anti-eIF5α (1:10000) were purchased from Santa Cruz; anti-Cdk5 (1:1000) was from Cell Signaling; anti-PARP (1:1000) was from Transduction Labs; anti-FLAG (1:1000, M2) was from Sigma.

Immunohistochemistry Cells were plated on dishes containing coverslips and fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS). Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with PBS containing 1% BSA or 1% BSA and 5% normal goat serum (NGS). Cells were incubated with the following primary antibodies: anti-p39, kindly provided by Dr. Nancy Ip (Hong Kong University of Science and Technology) or purchased from Santa Cruz, or anti-FLAG (Sigma) at 4ºC overnight. After washes, cells were incubated with secondary antibodies in PBS containing 1% BSA and 0.01% Tween-20 at room temperature for 1 hour. Cells were then washed with PBS three times and mounted with Vectasheild mounting medium containing DAPI nuclear stain (Vector Laboratories).

Statistical Analysis All bar graphs display *means* ± *SEM*. For multiple comparisons, one-way ANOVA was performed followed by Tukey's post-test and two-way ANOVA followed by Bonferroni's post-test was carried out as indicated in the figure legends.

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