The genetic link between *qkI* and *p53* in the central nervous system

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Abstract

A definitive role for the QKI RNA-binding protein has been demonstrated in multiple biological processes, including myelinogenesis, vascular remodeling and cell fate determination. *qkI* has also been implicated as a candidate tumor suppressor gene as the *qkI* locus maps to a region of genetic instability in Glioblastoma Multiforme (GBM), an aggressive brain tumor of astrocytic lineage. Mice homozygous for the qk^v mutation have not been reported to develop GBM, suggesting that additional genetic mutations may be required to induce tumorigenesis. In order to increase the potential tumorigenetic effect of the qk^v mutation as well as to investigate whether *p53* and *qkI* are involved genetically in myelin formation, we bred qk^v/qk^v mice onto a *p53-/-* background. qk^v/qk^v*; p53-/-* mice demonstrated a reduced survival rate compared to *p53-/-* and qk^v/qk^v littermate controls and developed the cerebellar tumor medulloblastoma at a low frequency. qk^v/qk^v*; p53-/-* mice also displayed neurological defects including hydrocephaly and Purkinje neuron degeneration, however myelination was not further impaired compared to qk^v/qk^v mice. In support of *qkI* as a potential tumor suppressor, we show that QKI negatively regulates of oncogenic signaling factors including *gli1* and ERK1/2. These findings indicate a new role for *qkI* in tumor development.

Sommaire

La protéine liant l'ARN, QKI, est impliquée au niveau de nombreux processus biologiques, dont la myélogenèse, le remodelage vasculaire, et la détermination du sort cellulaire. Le gène codant pour QKI, *qkI*, est considéré comme un possible suppresseur tumorale puisque situé dans une région fréquemment altérée chez les patients atteint de glioblastome multiforme, un cancer du cerveau très aggressif. Par contre, les souris homozygotes pour une mutation de *qkI* (qk^{v}) ne développent pas de glioblastome, ce qui suggère que des altérations génétiques additionnelles pourraient être requise au développement de glioblastomes. Afin de mieux comprendre le potentiel antitumorale de QKI, des souris $qk^{v/+}$ ont été croisées avec des souris $p53^{-/-}$. Ces nouvelles souris qk^{v}/qk^{v}; $p53^{-/-}$ ont une longévité réduite comparativement à la portée contrôle $p53^{-/-}$ et développent à faible fréquence des tumeurs cérébelleuses embryonaires, médulloblastomes. Les souris qk^{v}/qk^{v}; $p53^{-/-}$ possèdent aussi des défauts neurologiques incluant l'hydrocéphalie et la dégénération des neurones de Purkinje. Supportant l'hypothèse que *qkI* est un suppresseur tumorale, nous démontrons que QKI inhibe les facteurs oncogéniques gli1 et ERK1/2. Les découvertes regroupées ici démontrent un nouveau rôle pour *qkI* dans le développement tumorale

Preface

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Table of contents

List of Figures and Tables

List of Abbreviations

APC	Adenomatous polyposis coli
BCS	Bovine calf serum
BSA	Bovine serum albumin
Bp	Base-pair
CDK	Cyclin-dependent kinase
CFS	Common fragile site
CNP	2,3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CSF	Cerebral spinal fluid
CSKII	Casein complex II
DNA	Deoxyribonucleic acid
E-	Embryonic day
ECL	Enhanced chemiluminescense
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Type III EGFR variant deletion mutant
EGL	External granular layer
EGR2	Early growth response gene-2
ENU	N-ethyl-N-nitrosourea
ERK	Extracellular signal-related kinase
GBM	Glioblastoma Multiforme
GCNP	Granule neuron precursor cells
GSG	GRP33/Sam68/GLD-1
GSK 3-β	Glycogen synthase kinase 3-β
HEK	Human embryonic kidney
HNF-3β	Hepatocyte Nuclear Factor-3beta

hnRNP	Heterogeneous nuclear ribonucleoprotein
HOW	Held out wings
IGL	Internal granular layer
Kb	Kilo-base
KH	K homology
LOH	Loss of heterozygosity
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
Mb	Mega-base
MBEN	Medulloblastoma with extensive nodularity
MBP	Myelin basic protein
Mdm2	Murine double-minute 2
Miple	Midkine and pleiotrophin heparin-binding growth factor
mRNA	Messenger Ribonucleic acid
NBCCS	Nevoid basal cell carcinoma syndrome
NCID	Notch intracellular domain
Nf1	Neurofibromatosis type I
NLS	Nuclear localization signal
OPC	Oligodendrocyte precursor cell
P-	Post natal day
PACRG	Parkin co-regulated gene
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pea15	Phosphoprotein enriched in astrocytes 15
PI3K	Phosphatidyllinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-biphosphate
PLP	Proteolipid protein

PNET	Primitive neuroectodermal tumors
PNS	Peripheral nervous system
PRMT1	Protein Arginine N-Methyltransferase 1
Ptch1	Patched1
PTEN	Phosphatase and tensin homology deleted on chromosome 10
Ptn	Pleiotrophin
QKI	Quaking
qk^v	Quaking viable
QRE	Quaking response element
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SBE	Star binding element
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SH3	Src homology 3
SHH	Sonic Hedgehog
Smo	Smoothened
Src-PTK	Src family protein tyrosine kinase
STAR	Signal transduction and activator of RNA
SUFU	Suppressor of fused
SVZ	Sub-ventricular zone
TGE	*tra-2 GLI* element
UTR	Un-translated region
WHO	World health organization
WNT	Wingless

Introduction

1. Quaking

1.1 Quaking: General Characteristics

The study of neurological diseases has been greatly advanced with the use of mouse models. In particular, spontaneously occurring mutant mice have played an important role in identifying key proteins involved in demyelinating diseases such as multiple sclerosis. One such mouse, the autosomal recessive qk^v mutant, was first described in 1964 by Sidman and colleagues. The qk^v/qk^v mouse exhibited severe hypomyelination of the central and peripheral nervous system and severe hindlimb shaking (Sidman et al. 1964). The mutation responsible for the qk^v/qk^v phenotype was later identified as a one megabase deletion that includes the promoter/enhancer region of the *qkI* gene, resulting in altered expression of *qkI* alternatively spliced gene products (Lorenzetti et al. 2004).

QKI belongs to the evolutionarily conserved signal transduction and activator of RNA (STAR) or GRP33/Sam68/GLD-1 (GSG) family of RNA binding proteins, which includes other members such as Sam68 and GLD-1 (Lukong and Richard 2003). STAR proteins contain the highly conserved KH RNA binding domain and are involved in the regulation of RNA metabolism, including stabilization, splicing, and translation (Lukong et al. 2008). QKI has been shown to be implicated in myelinogenesis by binding and stabilizing myelin basic protein (MBP) mRNA as well as promoting maturation of oligodendrocyte precursor cells (OPCs) by stabilizing $p27^{kip1}$ mRNA (Larocque et al. 2002; Larocque et al. 2005). The QKI response element or QRE has been defined as the bipartite consensus sequence NACUAAY-N(1-20)-UAAY (Galarneau and Richard 2005). Many gene products involved

in cell growth, maintenance, and cell fate determination have been found to harbor a QRE in their mRNAs, and have since been confirmed to be regulated by QKI (Galarneau and Richard 2005). QKI proteins are highly conserved among species and have been implicated in multiple diverse biological pathways, highlighting the pivotal role of RNA binding proteins in cellular function.

1.2 Genomic organization

The genomic organization of *qkI* was first described in mice by Kuniya Abe's group in 1999, having been previously identified as the candidate gene responsible for the qk^{v} mutation (Ebersole et al. 1996). The *qkI* locus has been shown to be well conserved among vertebrates, including *Xenopus* (Zorn et al. 1997), zebrafish (Tanaka et al. 1997), chicken (Mezquita et al. 1998), and mammals (Kondo et al. 1999). The *qkI* gene was mapped to the long arm of chromosome 17 in mice, and on 6q25-26 in humans. Mammalian *qkI* spans approximately 65 kb in length and is located downstream of the *parkin co-regulated* gene and the *parkin* gene (Kondo et al. 1999; Lockhart et al. 2004).

The *qkI* gene undergoes a complex pattern of alternate splicing, producing six different mRNA transcripts (Kondo et al., 1999). The three major isoforms of -5, -6, and -7 kb were termed quaking 5 (*qkI-5*), quaking 6 (*qkI-6*), and quaking 7 (*qkI-7*), respectively (Ebersole et al., 1996). The three other isoforms, *qkI-5B, qkI-7B*, and *qkI-G* were only identified later by Kuniya Abe's group in 1999. The *qkI* gene contains nine distinct exons, the first six being common to all 6 transcripts except *qkI-G*. The STAR module is encoded by exons 2, 3, 4 and 5. The KH domain is encoded by 3 different exons: the distal portion of 3, all of exon 5, and the proximal region of exon 6. The QUA1 or NK domain is encoded by exon 2 and the

proximal portion of exon 3, while only the distal region of exon 6 encodes the QUA2 or CK domain. Unlike all other transcripts, the *qkI-G* isoform is terminated before exon 5 and thus lacks a functional RNA binding domain (Kondo et al. 1999).

The three major isoforms differ only by the alternative splicing at the 3' end of the *qkI* gene, yielding products that differ only in their carboxyl tails (Chenard and Richard 2008). Exon 7 is a differentially spliced in a complex manner to produce *qkI-5*, *-6* and *-7*. To generate *qkI-7*, the first 45 bases of exon 7 (termed 7a) encode the 3' end of the coding region and the remainder of exon 7 encodes the 3'UTR. For *qkI-6*, exon 6 is spliced into a 24 bp portion of exon 7 (termed exon 7b). Exon 7b begins approximately 1.2 kb downstream of 7a and encodes the 3' end of the coding sequence of *qkI-6*. Exon 7c begins 2 kb downstream of exon 7b and is only 76 bp long. The *qkI-5* transcript is produced from the splicing of exon 7c into exon 8, and the majority of exon 9 encodes its 3'UTR. Of the three major isoforms, *qkI-5* is the only transcript that includes exons 8 and 9 (Kondo et al. 1999).

qkI transcripts have a common 5'UTR, however each of the three major isoforms contain a unique and extensive 3'UTR. The 3'UTR is highly conserved among homologues, indicating an evolutionarily conserved mechanism of post-transcriptional regulation (Zorn et al., 1997). Although *qkI-6* and *qkI-7* share a similar 3'UTR generated from internal alternative splicing of exon 7, the *qkI-5* UTR is encoded completely by exon 9 (Kondo et al., 1999). Given that the major transcripts are differentially expressed during development, regulation at the RNA level is most likely a means of gene expression control. Indeed, QKI-6 and QKI-7 have a similar pattern of expression in later stages of development, whereas *qkI-5* has been shown to have an earlier onset in stages of embryogenesis (Ebersole et al. 1996).

1.3 QKI protein features

QKI proteins belong to the evolutionarily conserved GSG or STAR family of RNA binding proteins. The STAR family of proteins includes other members such as the *Caenorhabditis elegans* Gld-1, *Drosophila melanogaster* HOW, Sam68, and the human fragile X protein FMR1 (Vernet and Artzt 1997; Volk et al. 2008). STAR proteins are defined as containing a single heterogeneous nuclear ribonucleoprotein (hnRNP) K homology (KH) domain, or maxi-KH domain, flanked by conserved N- and C- terminal sequences (Lukong et al. 2008). The STAR family of RNA binding proteins belongs to a larger super-family of KH-RNA binding proteins that contain anywhere from 2 -15 copies of the KH domain (Lewis et al. 2000). The highly conserved KH RNA-binding domain has been well characterized and is one of the most common RNA binding domains found in nature (Siomi et al. 1993). The KH domain consensus sequence was determined to be approximately 70 amino acid sequence containing an invariant Gly-X-X-Gly (GXXG) motif and a variable loop (Valverde et al. 2008). The three dimensional structure of the KH module was first characterized in the human endoplasmic reticulum associated protein Vigilin in 1996 (Musco et al. 1996). The KH domain exists as a stable compact globular structure that forms a $\beta_1\alpha_1\ \alpha_2\ \beta_2\ \beta_3$ fold (Type I) or an $\alpha_1\beta_1\ \beta_2\ \alpha_2\ \alpha_3\ \beta_3$ fold (Type II) (Grishin 2001). Type I folds are typically found in eukaryotes and are composed of three-stranded anti-parallel β -sheets packed against two to three α –helices (Musco et al. 1996). The GXXG motif forms a conserved loop structure between α_1 and α_2. RNA adopts a linear conformation and fits into the hydrophobic groove formed by α_1 ,α_2 ,β_2, the GXXG motif and the variable loop, forming direct contact with the protein (Valverde et al., 2008)

The STAR domain contains approximately 200 amino acids and is composed of 3 modules: the KH domain, a ~75 amino acid NK or QUA1 domain at its N-terminus, and a ~25 amino acid CK or QUA2 domain at its C-terminal region (Vernet et al., 1997). The importance of the NK domain is also highlighted by the embryonic lethal mouse mutant qk^{kt4}, which is caused by an A to G transition that alters glutamic acid 48 to glycine (J. Wu et al. 1999). The residues within the *qkI* NK domain were later characterized to be essential for self-association and the E48G substitution was purported to abrogate predicted coiled-coiled interactions (Chen and Richard 1998). The embryonic lethal mouse mutant qk^{2} was determined to be caused by a T to A transversion in the CK domain, altering valine 157 to glutamic acid (Cox et al. 1999). The V157E substitution has since been shown to completely abolish RNA binding activity in QKI (Larocque et al., 2002).

STAR proteins bind a consensus RNA sequence known as the STAR-binding element (SBE). The SBE was originally identified in GLD-1, and is defined as the hexanucleotide sequence (U>G>C/A)A(C>A)U(C/A>U)A (Ryder et al. 2004). The SBE has been identified in many different STAR mRNA targets; in addition, homologs within the STAR family have been shown to be interchangeable in mediating mRNA metabolism specifically through SBE binding (Lakiza et al. 2005; Ryder et al. 2004). QKI proteins bind a more specific variation of the SBE called the Quaking response element (QRE), a bipartite consensus motif defined by the sequence NACUAAY-N1-20-UAAY (Galarneau et al., 2005). The QRE has been found in known QKI mRNA targets such as myelin basic protein (MBP), early growth response gene-2 (*EGR2*) and the cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$. An additional 1,433 putative mRNA targets were identified with the use of bioinformatics. A subset of mRNA targets identified using the QRE was validated to interact with QKI *in vivo*,

implicating QKI in multiple pathways such as cell differentiation, development, and maintenance (Galarneau et al., 2005).

At the C-terminus, the major QKI isoforms contain elements suggesting a role in signal transduction, such as multiple proline-rich motifs, arginine-glycine repeats, and tyrosine residues (Chenard and Richard 2008). Similar to Sam68, QKI contains multiple PXXP motifs, suggesting that QKI may bind Src homology 3 (SH3) containing proteins (Ebersole et al. 1996). QKI has been shown to be tyrosine-phosphorylated by the Src family protein tyrosine kinase (Src-PTK) $p59^{fyn}$, and this was found to negatively affect the interaction between QKI and MBP mRNA (Zhang et al. 2003). Although QKI was determined to be a substrate of Protein Arginine N-Methyltransferase 1 (PRMT1) *in vivo* (Cote et al. 2003), the significance of its capacity to be arginine-methylated has yet to be elucidated.

1.4 QKI expression

QKI isoforms are differentially expressed both temporally and spatially. *qkI* mRNA is first expressed at E7.5 (Embryonic day 7.5) in the neuroepithelium head folds (Ebersole et al., 1996). *qkI-5* is the most abundant isoform during the perinatal stages, however its expression declines after the first two weeks of age (Hardy et al. 1996; Kondo et al. 1999). *qkI-6/7* mRNA is also expressed at the perinatal stages, however expression levels peak during the period of myelination at post-natal day 14 (P14) (Hardy et al., 1996). As opposed to *qkI-5*, *qkI-6/7* continue to be expressed at high levels during adulthood (Hardy et al., 1996). As adults, *qkI* is expressed both at the protein and RNA level in the brain, lung, heart,

testis and uterus, and to a lesser extent, the liver, spleen, kidney and muscle (Kondo et al., 1999).

During embryogenesis, *qkI* mRNA was detected in the brain, heart, lung, and testis (Ebersole et al., 1996). In early stages of embyrogenesis, neural progenitor cells of the sub-ventricular zone (SVZ) express high levels of QKI-5 (Hardy et al., 1996). Cortical neurons were observed to be devoid of any QKI expression, whereas glial cells including myelin producing oligodendrocytes were demonstrated high levels of QKI expression (Hardy et al., 1996).

QKI-5 possesses a different cellular localization compared with QKI-6 and -7. The longer carboxy tail of QKI-5 contains a nuclear localization signal (NLS), thus its localization is primarily nuclear whereas QKI-6 and -7 are cytoplasmic (J. Wu et al. 1999). The QKI-5 NLS is defined by the 7 amino acid sequence RXHPYQ/GR that bears no resemblance to the classical NLS sequence (Wu et al., 1999). This novel NLS was termed NLS-STAR as it was also identified in other STAR family members such as Sam68 and ETLE/T-STAR (Wu et al., 1999). It is hypothesized that QKI mediates some level of RNA regulation through heterodimerization with the other QKI isoforms, allowing for differential localization within the cell depending on its association (Chenard and Richard, 2008).

1.5 QKI animal models

The importance of QKI has been highlighted by a diverse set of animal models. As mentioned earlier, the spontaneously occurring qk^v/qk^v mutation was the first to be described. The qk^v/qk^v mutation is a one megabase deletion and its proximal breakpoint is located in the promoter region of the *qkI* gene. The distal breakpoint is located between exons 5 and 6 of

the *parkin* gene (Lockhart et al. 2004). The *parkin co-regulated* gene (*PACRG*) is located between the *qkI* and *parkin* loci, thus the qk^{v}/qk^{v} mutation affects the expression of 3 different genes. The qk^{v}/qk^{v} mutation results in loss of *parkin* and *PACRG* function, whereas *qkI* expression becomes abnormally expressed (Lockhart et al., 2004; Hardy et al., 1996). In qk^{v}/qk^{v} mutants, *qkI-6/7* expression is preferentially lost in myelin producing cells of the central and peripheral nervous systems, while for the most part expression remains unaffected in all other cell types (Hardy et al., 1996). Several independent studies have confirmed that the difference in *qkI* isoform expression is responsible for the dysmyelinating phenotype observed in qk^{v}/qk^{v} mice. *parkin -/-* mice do not demonstrate any defect in myelination (Goldberg et al. 2003; Itier et al. 2003). In addition, the over-expression of QKI-6 in qk^{v}/qk^{v} mutants has been shown to rescue the myelination defect (Zhao et al. 2006). Finally, QKI has been shown to regulate important mRNA targets involved in myelination and oligodendrocyte differentiation (Larocque et al. 2002; Larocque et al. 2005; J. I. Wu et al. 2002; Zhang et al. 2003).

Other ENU mutations have been useful in identifying the biological roles of QKI. The ENU-induced *qkI* alleles qk^{k2}, qk^{kt1}, $qk^{kt3/4}$, qk^{1-1} resulted in embryonic lethality in homozygotes, indicating an essential role for QKI during embryonic development (Cox et al. 1999; Ebersole et al. 1996; Justice and Bode 1988; Shedlovsky et al. 1988). Indeed, *qkI* null mutants demonstrated multiple defects such as open neural tubes, pericardial effusion, and inefficient vascular remodeling of the yolk sac vitelline vessels causing embryonic lethality at E9.5 and E10.5 (Z. Li et al. 2003).

1.6 Biological roles of QKI

1.6.1 mRNA metabolism

The qk^v/qk^v mouse has been observed to display defective expression of the major myelin-associated proteins including MBP, myelin-associated glycoprotein (MAG), proteolipid protein (PLP), and 2,3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (DeWille and Farmer 1992; Fujita et al. 1990; Hardy 1998a; Zhang and Feng 2001). MBP was the first myelin-associated mRNA to be validated as a QKI target (Larocque et al. 2002). qk^v/qk^v oligodendrocytes showed nuclear retention of MBP mRNA, resulting in improper localization to the cellular processes (Barbarese 1991). Nuclear retention of MBP mRNA was mimicked *in vivo* by QKI-5 over-expression in oligodendrocytes (Larocque et al., 2002). These findings suggest that proper expression of isoforms -6 and -7 allows for MBP mRNA export to the oligodendrocyte processes (Larocque et al., 2002).

QKI has been shown to regulate alternative splicing events for the myelin-associated protein, MAG. MAG is a transmembrane-associated myelin protein that co-exists as two isoforms: S-MAG and L-MAG (Braun et al. 1990). The different isoforms are generated via alternate splicing by exclusion of exon 12 in L-MAG (Bo et al. 1995). qk^v/qk^v mice were observed to display higher ratios of S-MAG to L-MAG, indicating preferential inclusion of exon 12 (Fujita et al. 1990). It was not until 2002 that the role of QKI in MAG mRNA metabolism was elucidated. Over-expression of QKI-5 was found to suppress inclusion of exon 12 in MAG mRNA, resulting in preferential expression of L-MAG (Wu et al., 2002). These findings provide a possible explanation for the altered ratios of MAG isoforms observed in qk^v/qk^v mice.

In addition to myelin-related targets, QKI has been demonstrated to regulate other mRNAs that possess a *tra-2 GLI* element (TGE) (Lakiza et al., 2005). The TGE was named for two regulatory 3'UTR sequences identified in *tra-2* mRNA, the *Drosophila* homolog of GLI (Goodrich et al. 1996). The QKI homolog GLD-1 in *C.elegans* was found to be required for repression of *tra-2* mRNA through TGE binding, resulting in hermaphrodite spermatogenesis (Clifford et al. 2000). QKI-6 was later determined to also act as a translational repressor of mRNA targets possessing TGEs including *tra-1*, *tra-2*, and *Gli1* (Lakiza et al. 2005; Saccomanno et al. 1999).

1.6.2 Cell fate determination

Another feature of qk^{v}/qk^{v} mice is the abundance of immature oligodendrocytes, suggesting a defect in oligodendrocyte maturation due to lack of QKI-6 and -7 expression (Larocque et al., 2002). Indeed, QKI-6/7 over-expression were shown to promote cell cycle arrest in neural progenitor cells, favoring glial cell fate speciation (Larocque et al., 2005). QKI-6/7 were determined to bind and protect the mRNA of the CDK inhibitor $p27^{kip1}$, which is known to be elevated in differentiating oligodendrocytes (Larocque et al. 2005; Tokumoto YM 2001). The accumulation of $p27^{kip1}$ leads to cell cycle exit in OPCs, allowing for terminal differentiation into oligodendrocytes (Friessen et al. 1997). Over-expression of QKI-6/7 *in vivo* was also shown to direct migration of OPCs into areas of high myelination such as the corpus callosum (Larocque et al., 2005). More recently, QKI-6/7 was shown to increase $p27^{kip1}$ levels in differentiating Schwann cells, indicating that QKI acts as a positive regulator of myelination in the PNS as well as the CNS (Larocque et al. 2009).

1.6.3 Apoptosis

Over-expression of QKI-7 was observed to induce apoptosis in NIH 3T3 mouse fibroblasts, HeLa cells, and primary rat oligodendrocytes (Chen and Richard 1998). Interestingly, QKI-7(E48G) was found to be a more potent inducer of apoptosis, suggesting that self-association could inhibit cell death. Indeed, heterodimerization of isoforms QKI-5 and -7 leads to reduced levels of apoptosis in NIH 3T3 cells due to nuclear translocation of QKI-7. The unique 14 amino acid C-terminus of QKI-7 was determined to be sufficient for the induction of apoptosis (Pilotte et al., 2001). Taken together, these findings suggest a unique regulatory mechanism of apoptotic inducers through nuclear translocation.

1.6.4 Vascular remodeling

As mentioned earlier, the importance of *qkI in vivo* has been highlighted by the lethality of ENU mutants *in utero*. The cause of embryonic lethality observed in ENU-induced qk^{k2} homozygotes was determined to be caused by blood vessel defects, indicating an essential role for *qkI* in vascular remodeling (Noveroske et al. 2002). Similar to qk^{k2} mutants, vascular architecture was defective in *qkI*-/- embryos, confirming the importance of *qkI* in blood vessel development (Z. Li et al. 2003). Vascular defects were also observed in homozygotes for the qk^{l-1} mutation (Cox et al. 1999). The qk^{l-1} lesion was determined to be due to the loss of a *qkI*-5 splice site, resulting in the loss of *qkI*-5 expression (Cox et al., 1999). Since *qkI*-5 is the most abundant isoform in developing embryos, these findings highlight the importance of *qkI*-5 expression during early embryogenesis (Ebersole et al., 1996).

1.6.7 Cancer

The long arm of chromosome 6 is known to harbor a common fragile site (CFS) that is frequently lost or mutated in a variety of cancers (Cesari et al. 2003; Smith et al. 2006). The breakpoint includes several genes, one of which has been identified as the *qkI* gene (Ichimura et al. 2006; Yin et al. 2009). Chromosomal aberrations at 6q26-27 are common in glioblastoma multiforme, an aggressive brain tumor of astrocytic lineage (Mulholland et al. 2006). In addition to its mapping to a region of genomic instability, *qkI* mRNA transcripts have been shown to be down-regulated specifically in glioblastoma tumors (Z. Z. Li et al. 2002). More recently, QKI was discovered to be down-regulated in colorectal cancers, in part due to promoter hypermethylation (Yang et al. 2009). QKI over-expression in the colon epithelium resulted in increased levels of $p27^{kip1}$ as well as an increase in membrane bound β-catenin, a downstream effector of the WNT pathway (Yang et al., 2009). Increased levels of membranous β-catenin are an indicator of gastric cell differentiation, and are also a marker for a favorable prognosis in gastric carcinomas (Ramesh et al. 1999). Taken together, these findings establish *qkI* as a candidate tumor suppressor gene.

2. Primary tumors of the central nervous system

2.1 Glioblastoma

Malignant gliomas are the most common occurring brain tumors in adults. Despite clinical advances over the last few decades, prognosis remains poor as gliomas are resistant to traditional treatments including surgical resection, chemotherapy, and radiation therapy (Purow and Schiff 2009). As the name suggests, histologically and immunohistochemically, gliomas display characteristics of differentiated glial cells. Gliomas are classified according to the glial cell lineage they most resemble, namely astrocytic, oligodendroglial, and ependymal cells, or a combination of astrocytic and oligodendroglial cells termed oligoastrocytoma (Maher et al. 2001). Diffuse astrocytic glioma (or astrocytoma) is a more commonly occurring form of glioma in adults, and is graded according to histological criteria of malignancy such as mitotic activity, necrosis, microvascular proliferation, cellularity and nuclear atypia (Weiss et al. 2002). Pilocytic astrocytoma (WHO grade I) is considered benign and rarely progresses to more invasive forms of glioma. Astrocytoma (WHO grade II), although classified as a low grade tumor, usually progresses into highly malignant Anaplastic Astrocytomas (WHO grade III) and Glioblastoma multiforme (WHO grade IV) within 5 to 10 years of diagnosis (Maher et al., 2001). Glioblastoma multiforme (GBM) is the most aggressive form of astrocytoma with a median survival rate of only 9-12 months after diagnosis (Sanai et al. 2005). GBM can arise without any previous diagnosis (termed primary GBM), or less commonly, from the malignant progression of low grade astrocytomas (termed secondary GBM) (Kleihues and Ohgaki 1999). Given the invasiveness of GBM and its failure to respond to more traditional methods of cancer treatment,

understanding the molecular mechanisms contributing to GBM malignancy is of utmost importance in developing new and effective therapies.

2.2 Altered signaling pathways in GBM

2.2.1 The ARF/MDM2/p53 pathway

Tumor suppressor protein *TP53*, located on chromosome 17p, is one of the most commonly mutated genes in cancer, including GBM (Louis 1994). p53 protects against genomic instability through its ability to induce cell cycle arrest at the G1 phase, allowing for DNA damage repair. In the event that the cell has undergone irreparable DNA damage, p53 is capable of initiating apoptosis. In normal conditions, p53 is rapidly degraded; however in response to cellular stress or damage such as ionizing radiation, hypoxia, carcinogens and certain chemical drugs, p53 levels accumulate due to protein stabilization (Zupanska and Kaminska 2002). Activation of p53 through serine-phosphorylation, dephosphorylation at other residues, as well as acetylation allows for conformational changes leading to exposure of the p53 NLS (Appella and Anderson 2001). Upon translocation to the nucleus, p53 acts as a transcriptional activator of genes involved in DNA repair and apoptosis. Thus, loss of p53 function allows for unchecked cell growth, escape from apoptosis and genomic instability.

Mutations abrogating p53 function and allelic loss of chromosome 17p were among the first genetic lesions identified in GBM (Nigro et al. 1989). *p53* lesions are present in all grades of astrocytoma at an average of 30% (Nozaki et al. 1999), suggesting that the inactivation of *p53* is an early event in gliomagenesis. Patients with Li-Fraumeni syndrome, a hereditary condition characterized by a germline mutation in one allele of *p53*, are pre-disposed to early onset cancer, including astrocytoma (Malkin 1993). Genetic alterations in

p53 were found in more than 65% of secondary GBM, but were detected in less than 10% of primary GBM cases (Watanabe et al. 1996). The same study indicated that more than 90% of secondary GBM cases harbored mutations in *p53* before progressing into more malignant grades (Watanabe et al. 1996). These findings suggest that loss of *p53* is important in tumor initiation in secondary GBM, but not in tumor progression.

The half-life of p53 is tightly controlled in normal cells. Another frequently mutated gene in GBM, *Mdm2* (Murine double-minute 2), acts to negatively regulate p53 protein stability by targeting it for ubiquitin-mediated proteosome degradation. Thus, Mdm2 over-expression or amplification provides another mechanism to bypass p53 regulation in cancer cells. In contrast with *p53*, *Mdm2* lesions are found in 10% of malignant astrocytomas, but are rarely observed in lower grades (Costanzi-Strauss et al. 1998). In addition, Mdm2 over-expression is more commonly observed in primary GBM than in secondary GBM (Kleihues and Ohgaki 2000). These findings suggest that, although primary GBM less frequently displays mutations affecting p53 function, the p53 pathway is still inactivated due to Mdm2 over-expression.

2.2.2 The PTEN/PI3K/AKT pathway

The tumor suppressor PTEN (phosphatase and tensin homology deleted on chromosome 10) regulates cell cycle progression through its suppression of phosphatidyllinositol-3-kinase (PI3K)/AKT pathway activation (Endersby and Baker 2008). In response to growth factors, the lipid kinase PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2), converting it to phosphatidylinositol-3,4,5-biphosphate (PIP3) (Engelman et al. 2006). The membrane-bound PIP3 recruits the transcription factor AKT to the plasma membrane,

allowing for PI3K-dependent phosphorylation (Knobbe et al. 2002). AKT activation leads to phosphorylation of multiple targets promoting cell cycle progression and cell survival. PTEN negatively regulates PI3K/AKT signaling by de-phosphorylating PIP3, thus acting in opposition to PI3K (Maehama and Dixon 1998). Due to its inhibitory role, PTEN is an important tumor suppressor that is frequently mutated in a variety of cancers, including glioma. The most frequent genetic alteration observed in GBM overall is loss of heterozygosity (LOH) of chromosome 10q, which includes the *PTEN* locus (Nozaki et al. 1999). Approximately 70% of both primary and secondary GBM display 10q LOH (Ohgaki et al. 2004). Specific mutations affecting PTEN function occur in 30% of primary GBM, but are rarely observed in secondary GBM (Tohma et al. 1998), suggesting that the loss of *PTEN* occurs mainly in the formation of GBM *de novo*. Interestingly, specific mutations of *p53* and *PTEN* appear to be mutually exclusive (Tohma et al. 1998), supporting the observation that loss of *p53* contributes primarily to the progression of low grade astrocytoma to secondary GBM, whereas *PTEN* mutations mainly confer tumorigenic advantage to the development of primary GBM.

2.2.3 The p16/CDK4/Rb pathway

The *INK4a* (or *CDKN2A*) locus produces two gene products through alternative reading frames in the second exon: the cycle cell regulators p16 and $p19^{ARF}$. $p19^{ARF}$ inhibits Mdm2 function, and therefore could be a possible tumor suppressor; however $p19^{ARF}$-specific mutations are rarely observed in cancer (Kleihues and Ohgaki 2000). Consistent with these findings, mutant mice deficient for $p19^{ARF}$ display glioma only at low rates (Kamijo et al. 1999). p16 negatively regulates the cyclin-dependent kinases CDK4 and CDK6, resulting in their inability to inactivate Rb by phosporylation. The inhibitory role of

p16 acts as a tight control over the cell cycle G_1/S transition, therefore the effectors of this pathway are frequently altered in a variety of cancers (Merlo 2003). Indeed, the homozygous deletion of *p16* is observed in approximately 40% of GBM overall (Myers et al. 1997). Primary and secondary GBM demonstrate similar rates of mutations affecting Rb expression, however deletion of the *INK4A* locus is more frequently observed in primary GBM compared to secondary GBM (36% vs. 4%, respectively) (Biernat et al. 1997). On the other hand, *CDK4* amplification is more commonly observed in secondary GBM compared to primary GBM (23% vs. 4%, respectively). In the absence of *p53* mutations, loss of either the *Rb* or *PTEN* tumor suppressors appears to be essential for progression to secondary GBM (Nozaki et al. 1999). These findings highlight distinct subsets of mutations required for GBM tumorigenesis.

2.2.4 EGFR

The receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR) is involved in signaling pathways regulating migration, proliferation, and cell development. *EGFR* gene amplification is observed in over 30% of primary GBM, but is a rare event in secondary GBM and low grade astrocytomas (Collins and James 1993). Interestingly, the relationship between loss of *p53* and *EGFR* over-expression appears to be mutually exclusive (Watanabe et al. 1996), suggesting that *EGFR* amplification does not confer additional tumorgenetic advantage in the absence of *p53*. These findings were consistent with *in vitro* studies demonstrating that *p53-/-* primary cortical astrocytes were not transformed in the presence of epidermal growth factor (EGF) (Bogler et al. 1995).

In addition to mutations affecting *EGFR* expression, mutations affecting EGFR structure have also been identified in GBM. The most common *EGFR* mutation is the deletion of the extracellular domain encoded by exons 2-7, resulting in a truncated, constitutively active form of EGFR that enhances proliferation and resistance to apoptosis (Wong et al. 1992). The expression of this oncogenic form of EGFR, known as type III EGFR variant deletion mutant (EGFRvIII), correlates with poor prognosis in GBM patients (Feldkamp et al. 1999). However, EGFRvIII was shown to be incapable of inducing high-grade tumors in the absence of other oncogenic mutations such as the loss of the *INK4a/ARF* locus (Ding et al. 2003).

Deregulation of EGFR signaling leads to unrestrained activation of multiple pathways, including the ERK1/2 mitogen-activated protein kinase (MAPK) pathway. The adaptor protein Grb2 binds to phosphorylated EGFR, resulting in recruitment of son of sevenless (SOS), and activation of downstream effectors Ras, Raf, MEK and finally ERK1/2. Activated ERK1/2 translocate to the nucleus, where they initiate the transcription of genes involved in cell growth and proliferation (Huang et al. 2009). Fibroblast cells expressing the EGFRvIII variant demonstrate four times higher activation of MEK compared to cell lines with *wt EGFR* (Montgomery et al. 1995). Activated Ras is frequently observed in primary GBM as well as most GBM cell lines. The activation of Ras is known to be an important mediator of tumorigenesis in general, although specific mutations affecting *Ras* are rare in GBM (Guha et al. 1997).

2.2 Medulloblastoma

Medulloblastoma is the most commonly occurring malignant brain tumor in children. Approximately 30% of medulloblastoma cases in children are incurable, and current clinical therapies have the potential for damaging effects later in life. Medulloblastomas belong to a larger group of CNS tumors termed primitive neuroectodermal tumors (PNETs), which are characterized as embryonal tumors arising from the neuroepithelium of the subependymal ventricles (Gilbertson and Ellison 2008). Over the past 20 years, WHO classification has evolved to include several distinct subtypes in addition to classic medulloblastoma: desmoplastic/nodular medulloblastoma, anaplastic medulloblastoma, large cell medulloblastoma, and medulloblastoma with extensive nodularity (MBEN). Classic medulloblastoma is the most commonly occurring form and is characterized histologically by small, compact cells with a high nucleus-to-cytoplasm ratio. Immunohistochemically, medulloblastomas express markers of both neuronal and glial cell lineage characteristic of embryonal neural precursor cells (Gulino et al. 2008).

Medulloblastoma occurs exclusively in the cerebellum. The molecular pathways deregulated in medulloblastoma are closely related to pathways that govern the normal development of the cerebellum (Marino 2005). The formation of the normal cerebellar architecture draws from two distinct pools of progenitor cells. Glial cells, GABAergic neurons including Purkinje neurons, and Golgi interneurons are formed from progenitor cells originating from the ventricular zone of the fourth ventricle (Morales and Hatten 2006). Progenitor cells within the rostral rhombic lip of the fourth ventricle generate the gluamatergic neurons of the deep cerebellar nuclei and the granule neuron precursor cells (GCNPs). GCNPs migrate to the cerebellum forming two distinct layers: the external

granular layer (EGL) and the internal granular layer (IGL) (Gilbertson and Ellison 2008). Signaling pathways regulating GCNP proliferation have been shown to be implicated in medulloblastoma tumorigenesis, and therefore will be the focus of this discussion.

2.4 Altered signaling pathways in medulloblastoma

2.4.1 SHH

GCNPS of the EGL can be induced to proliferate in response to the Purkinje cell-secreted mitogen Sonic Hedgehog (SHH). SHH is a Purkinje cell-secreted mitogen that binds the transmembrane receptor, Patched1 (Ptch1). Without SHH ligand stimulation, Ptch1 suppresses the activity of another transmembrane protein, Smoothened (Smo). Upon binding SHH, Ptch1 disassociates from Smo, relieving it of its inhibition. Smo signaling leads to the activation of the Gli transcription factors, resulting in expression of genes involved in proliferation and cell growth (Guessous et al. 2008). The link between SHH signaling and medulloblastoma was first established when the genetic lesion responsible for the Gorlin syndrome was elucidated in 1996. The Gorlin syndrome, also known as nevoid basal cell carcinoma syndrome (NBCCS), is caused by a germline mutation in the *ptch1* gene and is characterized by developmental abnormalities as well as a predisposition to a variety of tumors, including medulloblastoma (Hahn et al. 1996). *Ptch1* mutations are observed in up to 20% of sporadic medulloblastomas (Zurawel et al. 2000); however a subset of these has been shown to retain the *wt ptch1* allele (Gilbertson and Ellison 2008), suggesting that *ptch1* is haploinsufficient in tumorigenesis. In agreement with these findings, mice with a heterozygous deletion of *ptch1* develop spontaneous medulloblastomas with a penetrance of approximately 15% (Goodrich et al. 1996).

Other components of the SHH pathway have been found to be altered in human medulloblastoma. Suppressor of fused (SUFU), a negative regulator of SHH signaling, has been found to be mutated in 2% of sporadic medulloblastomas, and patients with a germline mutation in *SUFU* display a predisposition to medulloblastoma development (Taylor et al. 2002). In addition, mice with a heterozygous deletion of *SUFU* on a *p53-/-* background have been shown to develop tumors including medulloblastoma and rhabdomyosarcoma (Lee et al. 2007). Recently, a potential tumor suppressor has been identified on chromosome 17p13.2, the most frequently deleted locus in medulloblastoma (Di Marcotullio et al. 2004). The locus encodes for REN^{KCTD11}, a gene expressed in differentiating GCNPs and non-proliferating granule cells. REN has been shown to promote apoptosis and cell growth arrest by up-regulating $p27^{kip1}$ expression and inhibiting Gli1 transactivation (Argenti et al. 2005). Finally, over-expression of REN was found to suppress medulloblastoma growth *in vivo* (Di Marcotullio et al. 2004).

2.4.2 WNT

The Wingless (WNT) signaling pathway plays an important role in the proliferation of neural progenitor cells in the CNS (Ciani and Salinas 2005). WNT transduces its signal by binding its cell-surface receptor, the seven-pass transmembrane protein Frizzled. Frizzled activation upon WNT binding results in the destabilization of a complex composed of multiple proteins including Axin, glycogen synthase kinase 3-β (GSK 3-β), casein complex II (CSKII) and adenomatous polyposis coli (APC). The destabilization of the Axin-APC complex results in its inability to target β-catenin for proteosomal degradation. The accumulation of nuclear β-catenin leads to activation of target genes promoting cell cycle progression and cell-to-cell attachment (Jozwiak et al. 2007). The WNT signaling pathway

was implicated in medulloblastoma formation when it was observed that patients with type 2 Turcot syndrome, a germline mutation in *APC*, were predisposed to medulloblastoma development (Hamilton et al. 1995). Activating mutations in β-catenin have also been observed in up to 9% of sporadic medulloblastoma, although *APC* mutations are less frequent. In addition, other WNT signaling effectors have been found to be altered in sporadic medulloblastoma, including GSK-3β and Axin (2% and 4%, respectively). Overall, genetic lesions affecting the WNT pathway account for approximately 20% of all medulloblastoma cases (Jozwiak et al. 2007). Interestingly, medulloblastomas harboring mutations in the WNT signaling pathway tend to arise in an older subset of children, and is an indicator of favorable prognosis and survival (Ellison et al. 2005). In parallel, transgenic mice expressing a constitutively active form of β-catenin in neural cells were not observed to develop any CNS tumors (Kratz et al. 2002). These findings highlight the need to clarify the role of the WNT signaling pathway in medulloblastoma tumorigenecity.

2.4.3 Notch

The highly conserved Notch family of receptors mediates cell-to-cell signaling, cell fate determination and survival (Ehebauer et al. 2006). Upon binding the transmembrane protein Jagged on the cell surface of an adjacent cell, the Notch intracellular domain is proteolytically cleaved to produce a liberated protein. The cleaved Notch intracellular domain (NCID) translocates to the nucleus, where it induces the transcription of target genes including *NF-κB2, p21*, and *Hes* family of transcription factors (Jozwiak et al. 2007). Notch-1 and Notch-2 appear to have contrasting effects on medulloblastoma development. Notch-1 was found to inhibit growth of embryonal tumor cell lines such as medulloblastoma *in vivo*, whereas Notch-2 was found to promote tumor growth (Fan et al. 2004). Consistent with

these findings, amplification of *Notch-2* has been observed in up to 15% of PNETs, whereas Notch-1 expression is barely detectable. These findings may in part be explained by the physiological expression of the different Notch proteins: Notch-1 is primarily expressed in differentiated cells, whereas Notch-2 is expressed in proliferating progenitor cells (Guessous et al. 2008).

The expression of Hes1, a target gene of Notch signaling, is associated with a shorter survival time in medulloblastoma patients (Fan et al. 2004). Recently, the same group has demonstrated an important negative regulatory role for Notch in Hes1 expression. Down-regulation of Hes1 levels due to inhibition of Notch signaling lead to cell cycle exit, differentiation and apoptosis in medulloblastoma cell lines (Fan et al. 2006). Interestingly, suppression of Notch2 signaling led to a fivefold reduction in the CD133-positive stem cell fraction, indicating a specific role for Notch in maintaining the so-called cancer stem cell population in medulloblastoma.

Hypothesis and Objectives

A definitive role for the QKI RNA-binding protein has been demonstrated in multiple biological processes, including myelinogenesis, vascular remodeling and cell fate determination. The *qkI* locus maps to a region of genetic instability in GBM tumors, identifying it as a possible tumor suppressor; however its exact role in GBM formation has yet to be elucidated. qk^v/qk^v mice have not been reported to develop brain tumors, suggesting that additional genetic lesions may be required to induce tumorigenesis. *p53* is one of the most frequently mutated genes in cancer, including GBM; therefore, we hypothesized that the qk^v/qk^v mutation in addition to the loss of *p53* may confer enough genetic instability to induce gliomagenesis in mice. Terminal differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes has been shown to be dependent on p53 *in vitro* (Billon et al. 2004), suggesting that the loss of *p53* in addition to *qkI* in OPCs may lead to a more severe myelination defect. Our objective was to characterize the double mutant qk^v/qk^v*; p53-/-* mouse, focusing on any possible alterations in the CNS myelination phenotype or modified tumorigenesis. Secondly, we sought to identify the signaling pathways that QKI may potentially regulate in the CNS tumors GBM and medulloblastoma.

Materials and Methods

Animals:

Animals were sacrificed in accordance with a protocol approved by the Animal Care Committee at McGill University. Animals were housed in ventilated cages with a 12/12 light/dark hr cycle. *p53*$^{-/-}$ mice (Catalog# 002101) and *qk*^{v}/*qk*^{v} mice (Catalog# 000506) were obtained from JAX laboratories. Both mice strains were maintained on a C57BL/6 background. Mice homozygous for the *p53* mutation were bred to heterozygous *qk*^{v} mice and subsequent trans-heterozygous crosses were used to generate *p53*$^{-/-}$ males heterozygous for the *qk*^{v} mutation. These males were subsequently bred to trans-heterozygous females to generate double mutant mice. Mice were screened for the *p53 wild-type* allele via genomic PCR using oligonucleotides (5'-ATA GGT CGG CGG TTC AT-3') and (5'-CCC GAG TAT CTG GAA GAC AG-3'). The mutant *p53* allele was identified using the following oligonucleotides: (5'-CTT GGG TGG AGA GGC TAT TCG-3') and (5'-GTC GGG CAT GCG CGC CTT GAG-3'). The *qk*^{v} mutation was amplified using primers directed against the breakpoint (5'-TCT AAA GAG CAT TTT CGA AGT-3') and (5'-TTG CTA ACT GAA TAT TAC T-3').

Immunocytochemistry:

Mice were anaesthetized with isoflourane and perfused with ice-cold PBS followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose overnight at 4 °C and embedded in OCT compound (Tissue-Tek) over dry ice in acetone. Tissues were cryostat sectioned at 10 microns and collected on +/+ slides (Fisher). Tissue sections were blocked in 10% goat serum in Tris-buffered saline + 0.5 % Triton X-100 for 1 hr followed by incubation

with primary antibodies overnight at room temperature. Slides were incubated with Alexa-flour 488 or 546 IgG at a dilution of 1:400 for 4 hr.

Immunoblotting:

Samples were lysed in Laemmli sample buffer (2% sodium dodecyl sulphate SDS, 5mM $NaPO_4$ pH7, 0.1mM DTT, 5% β-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue) and separated on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) by electrophoresis. Samples were transferred onto a nitrocellulose membrane (Bio-Rad) and blocked in blocking buffer (1% Bovine serum albumin (BSA), 1% skim milk powder, 0.1% azide, 25mM Tris-HCl pH 7.4, 150mM NaCl). Membranes were probed with anti-ERK1/2 (Cell Signaling Technology), anti-phospho-ERK1/2 (Cell Signaling Technology), or anti-Gli1 antibodies overnight at 4 °C. Membranes were incubated with affinity purified anti-Sam68, affinity purified anti-QKI-5,-6 and -7, or anti-myc antibodies for 3 hours at room temperature. Membranes were incubated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit or horseradish peroxidase-conjugated goat anti-mouse for 3 hours at room temperature (Sigma) and visualized using enhanced chemiluminescense (ECL) on autoradiography film (Kodak).

Tissue cell culture:

Cells were incubated in 5% CO2 at 37 ºC in DMEM (Hyclone) supplemented with 10 % bovine calf serum (BCS) (Hyclone). Knockdown was performed using siRNA directed against QKI-5,-6 and -7 (5'-GGACUUACAGCCAAACAACTT-3') (Dharmacon) at a concentration of 3 nM. siRNA directed against GFP was used as a negative control (5'-AAUUGCCACAACAGGGUCGUG-3') (Dharmacon). U373 GBM cells were transfected

using the reverse method with Lipofectamine RNAiMAX (Invitrogen), according to manufacturer's instructions. After 24 hours transfection, cell media was replaced with DMEM supplemented with 0.1% BCS for serum starvation or DMEM supplemented with 10% BCS as a control. After 16h, cells were serum-stimulated with DMEM supplemented with 10% BCS for 1 hour.

Antibodies:

Monoclonal anti-MBP antibodies were obtained from Sternberger Monoclonals (SMI99). Monoclonal anti-Calbindin-D-28K antibodies were purchased from Sigma (C-9848). Antibodies against ERK1/2, phospho-ERK1/2 and Gli1 were obtained from Cell Signaling Technology. Anti-myc antibody was obtained from Santa Cruz Biotechnology. Antibodies against Sam68, QKI-5,-6, and -7 were generated as described previously (Chen and Richard 1998; Larocque et al. 2005).

Luciferase:

Plasmids expressing HA-tagged Gli1 and Gli3 and the 8xGli-BS-Luc luciferase reporter construct with 8x Gli binding elements were generous gifts from Dr. Steven Cheng (Department of Developmental Genetics, Nanjing Medical University, 140 Hanzhong Road, Xianzhi Lou 1908, Nanjing, Jiangsu, PR China), as described previously (Cheng et al, 2002). Myc-tagged QKI-5,-6,-7 as well as myc-QKI-6(V157E) were synthesized as described previously (Larocque et al. 2002). DNA was transfected into Human embryonic kidney (HEK) 293 cells, harvested after 48 hr and luciferase activities were measured using a dual luciferase assay kit (Promega). Total amounts of DNA transfected into each well of a 12 well plate were as follows: 500 ng 8xGli-BS-Luc, 25 ng pTK-RL (Promega), 100ng Gli1 or

Gli3, and 500 ng QKI-5,-6, -7, or QKI-6(V157E). Empty vector myc-pcDNA was added as required to maintain constant DNA levels. Results were normalized to Renilla luciferase activities.

Results

qk^v/qk^v; $p53^{-/-}$ mice display increased neurological defects

Resulting qk^v/qk^v; $p53^{-/-}$ progeny demonstrated distinct phenotypes from both parent strains. To assess myelination defects, post-natal day 30 (P30) coronal sections of the corpus callosum were stained with an antibody specific to MBP. As expected, qk^v/qk^v mice showed reduced myelination of the corpus callosum compared to *wt, p53-/-* and *p53-/-* ;$qk^v/+$ littermates (Figure 1a). However, qk^v/qk^v; $p53^{-/-}$ mice did not show an increased severity of hypomyelination compared to qk^v/qk^v mice (Figure 1a), suggesting that *p53* does not cooperate with *qkI* in the regulation of myelin formation *in vivo*. The loss of both *wt p53* alleles did not affect the onset of hindlimb shaking in mice pups, since both qk^v/qk^v; $p53^{-/-}$ mice, qk^v/qk^v; $p53^{+/-}$ mice and qk^v/qk^v mice displayed tremors by P14. However, the onset of stress induced tonic-clonic seizures in qk^v/qk^v; $p53^{-/-}$ mice was significantly earlier than that of qk^v/qk^v mice. qk^v/qk^v mice began demonstrating stress-induced seizures at 12 weeks of age, whereas qk^v/qk^v; *p53+/-* mice and qk^v/qk^v; $p53^{-/-}$ mice were observed to have seizures as early as 4 weeks of age. As expected, mice heterozygous for the qk^v mutation did not demonstrate tonic-clonic seizures or hindlimb shaking. As adults, qk^v/qk^v; *p53-/-* mice showed an ataxic-like phenotype not observed in their qk^v/qk^v littermate controls, with their ataxia becoming more severe with age. Cerebellar morphology was assessed by immunostaining of P30 coronal brain sections with an antibody against Calbindin, a Purkinje neuron-specific marker. Immunostaining of the brains of qk^v/qk^v; *p53-/-* mice revealed Purkinje cell body loss as well as defects in Purkinje cell arborization (Figure 2A). Purkinje cell defects were not observed in P15 mice, suggesting these neuronal defects were not due to failure in neuronal migration at earlier stages of development (Figure 2B). However,

misplaced Purkinje neurons have occasionally been observed in qk^v/qk^v mice (Suzuki and Zagoren 1975). Interestingly, QKI-5 was found to be expressed by Purkinje cells, contrary to previous findings (Hardy et al. 1996) (Figure 3). Immunostaining was abolished when sections were incubated with QKI-5-specific antibody pre-absorbed with peptide, indicating that reactivity was indeed specific for QKI-5. QKI-6 and-7 expression was restricted to glial cells, in agreement with previous findings (Hardy et al. 1996). These results suggest that QKI is indeed expressed by neuronal cells and therefore may play a role in neuronal maintenance and survival.

qk^v/qk^v; $p53^{-/-}$ mice demonstrate a reduced survival rate and altered tumor tropism

Kaplan-Meier curve analysis was performed in order to compare mouse survival rates (Figure 4). In agreement with previous observations, (Sidman et al. 1964) none of the qk^v/qk^v mice cohort succumbed to tumor formation at the end of the 261 days observation period. Overall, qk^v/qk^v; $p53^{-/-}$ mice were found to have a reduced survival time compared to the other groups, with a 50% survival rate of 117 days. qk^v/qk^v; $p53^{-/-}$ mice showed a significantly reduced survival time compared to $qk^v/+$; $p53^{-/-}$ mice (p=0.0135) as well as $p53^{-/-}$ mice (p=0.0443) according to the log rank/Mantel Cox test. $qk^v/+$; $p53^{-/-}$ mice demonstrated a similar survival curve to $p53^{-/-}$ mice, with 50% survival rates of 136 and 133 days, respectively.

Previous studies have shown that the majority of $p53^{-/-}$ mice develop tumors of the lymphatic system and soft tissue sarcomas, with only one instance of a brain tumor reported in a $p53^{+/-}$ mouse (Donehower et al. 1992; Jacks et al. 1994). Similarly, the $p53^{-/-}$ and $qk^v/+$; $p53^{-/-}$ mice we generated developed primarily sarcomas and lymphomas, with no occurrence

of any brain malignancies (Table 1). In contrast, none of the qk^v/qk^v; $p53^{-/-}$ mice demonstrated any occurrence of sarcomas, lymphomas, or tumors previously described in *p53-/-* mice (Table 1). Several qk^v/qk^v; $p53^{-/-}$ and qk^v/qk^v; $p53^{+/-}$ mice displayed neurological symptoms characteristic of hydrocephaly. Brains examined from these mice showed enlarged lateral ventricles with an accumulation of cerebrospinal fluid, compression of cortical layers, and deterioration of the surrounding myelinated tracts. The brain of one qk^v/qk^v; *p53-/-* mouse showed increased vascularization of the cerebellum and abnormal gross cerebellar architecture. Further histological analysis of cerebellar sections revealed granule cell layer invasion of the surrounding parenchyma, consistent with medulloblastoma (Figure 5). Cytological analysis confirmed the presence of medulloblastoma, as tumor cell nuclei were densely packed and polygonal in shape. Tumor cells were also found to express markers of astrocytic (GFAP), neuronal (NFH) and vascular (CD31) lineage. Cells expressing markers of multiple lineages indicate cells of a stem cell precursor nature, consistent with primitive neuroectodermal tumors such as medulloblastoma.

QKI regulates *gli1* mRNA expression

Medulloblastoma has not previously been observed to occur spontaneously in either *p53-/-* or qk^v/qk^v mice, however we documented one case of medulloblastoma in a cohort of eleven qk^v/qk^v; *p53-/-* mice. Medulloblastoma has been shown to arise from mutations in ptch/SHH signaling, thus we investigated the possible involvement of QKI in this pathway.

Human *gli1*, the downstream effecter of SHH signaling, was found to harbor two *tra GLI* elements (TGE) in the 3'UTR of its mRNA. It has previously been shown that QKI-6 is capable of binding mouse and human *gli1* mRNA specifically through the TGE. (Lakiza et

al. 2005). To assess its potential role in translational control of *gli1* expression, we used a Luciferase based reporter assay with eight tandem Gli binding sites derived from the Hepatocyte Nuclear Factor-3beta (HNF-3β) enhancer region (previously described by (Cheng and Bishop 2002). The insert portion of the plasmid encoding human *gli1* (*hgli1*) received from Dr. Steven Cheng was sequenced to verify the presence of the 3'UTR. Indeed, the plasmid contained the full length human cDNA and the presence of the TGEs in the *gli1* 3'UTR was confirmed. Human embryonic kidney (HEK) 293 cells, which lack endogenous QKI and Gli1 expression, were transfected with plasmids expressing the luciferase reporter assay, *hgli1*, and myc-tagged QKI-6. As negative controls, the luciferase assay was performed with empty vector myc-pcDNA as well as a plasmid expressing *gli3*, which acts mainly as a transcriptional repressor and has been shown to lack the TGE sequence in its 3'UTR (Cheng and Bishop 2002; Lakiza et al. 2005).

As expected, *gli3* expression demonstrated basal transcriptional activity as compared with empty vector myc-pcDNA (Figure 6A). Increasing levels of QKI-6 demonstrated a negligible effect on either myc-pcDNA or Gli3 transfected cells. In the absence of QKI-6, Gli expression resulted in high transcriptional activity, in agreement with previous findings (Cheng and Bishop 2002). QKI-6 demonstrated a negative effect on *gli1* transcriptional activity, and was repressed in a dose-dependent manner (Figure 6A). Since the luciferase reporter construct contained only Gli1 enhancer sites in its promoter, it is unlikely that the decrease in Gli1 transcriptional activity is due to QKI-6 acting as a transcriptional repressor. A Western blot was performed to verify Gli1 protein levels as well as expression of myc-QKI-6, which was visualized using anti-myc (9E10) that recognizes the myc epitope tag. A Western blot for Sam68 was also performed as a loading control. Indeed, Gli1 protein levels

were observed to decrease following QKI-6 expression in a dose dependent manner (Figure 6B). These findings are consistent with QKI-6 acting to negatively regulate *gli1* expression at the translational level.

In order to determine if *gli1* regulation by QKI-6 is dependent on RNA binding, we used a mutant QKI-6 with an amino acid substitution in the KH domain. The KH domain is directly responsible for RNA binding and contains several evolutionarily conserved motifs (Galarneau and Richard 2005). The embryonic lethal ENU mutation qk^{kt} was determined to be due to a substitution of valine at position 157 for glutamic acid (V157E) (Cox et al. 1999). V157 is shared among all mammalian QKI isoforms and is highly conserved among vertebrate QKI homologs as well as other STAR binding proteins (Zorn et al. 1997). Since its initial characterization, the V157E mutation has been shown to abrogate binding of RNA targets and is therefore absolutely essential for protein function (Larocque et al. 2002). As expected, the mutated QKI-6 (QKI-6:V157E) fails to repress *gli1* expression and shows comparable Gli1 transcriptional activity to the empty vector myc-pcDNA negative control (Figure 7).

We also investigated the possibility of Gli1 regulation by the other QKI isoforms, QKI-5 and -7. In contrast with QKI-6, QKI-5 expression did not result in decreased levels of Gli1 and showed luciferase activities similar to cells transfected with Gli1 alone (Figure 8). Expression of QKI-7, which shares a greater homology with QKI-6 on the RNA and protein level, reduced *gli1* expression to a lesser extent than QKI-6. However, the reduction in *gli1* expression was still significant according to the paired two-tailed Student's t-Test ($p = 0.005$).

ERK1/2 activation is up-regulated in the absence of QKI

We examined the possibility that QKI may modulate other pathways implicated in tumorigenesis. Although there is currently no evidence for QKI as a tumor suppressor in clinical medulloblastoma, *qkI* has been shown to be frequently lost in human GBM. This led us to examine signaling pathways that are commonly altered in human GBM, such as the EGFR pathway (Feldkamp et al. 1999). EGFR signaling results in the downstream activation of multiple mitogen activated protein kinases (MAPK) and ultimately phosphorylation of the transcription factors ERK1/2. In cancer, the EGFR pathway is often up-regulated and activation of the pathway can be directly assessed by examining ERK1/2 phosphorylation. We asked whether QKI could modulate the EGFR pathway by knocking down QKI expression in human GBM cell lines and evaluating any possible differences in ERK1/2 activation.

To assay the activation of the EGFR pathway, we first starved cells in cell culture medium supplemented with 0.1% bovine calf serum (BCS) for 16 hours, followed by stimulation with 10% BCS for one hour. As controls, we performed both starvation of cells without stimulation as well as neither starvation nor stimulation. As shown in Figure 9, only cells that were both starved and stimulated with BCS showed ERK1/2 phosphorylation, consistent with EGFR pathway activation. A Western blot for total ERK1/2 was performed in order to confirm specific activation of ERK1/2. As shown in Figure 9, total ERK1/2 levels were constant in comparison with the loading control, Sam68. QKI knockdown efficiency was verified by immunoblotting with anti-QKI-5,-6, and -7 antibodies. QKI levels were markedly reduced in QKI siRNA treated cells as compared to siGFP treated cells. QKI knockdown cells showed an increased level of phospho-ERK1/2 compared to siGFP treated

cells, indicating that endogenous QKI suppresses ERK1/2 activation in GBM cells. These results suggest that QKI plays an inhibitory role in the ERK activation, and therefore loss of QKI may play a role in EGFR-mediated tumorigenesis.

In order to try to validate our findings in an *in vivo* setting, we performed immunostaining for phospho-ERK1/2 on qk^v/qk^v and qk^v/qk^v;*p53-/-* P30 brains. ERK activation was not observed in any region of the brain of *wt* littermate controls (Figure 10). However, in qk^v/qk^v;*p53-/-* mice, several areas of the corpus callosum in the cortical region displayed positive phospho-ERK1/2 reactivity. This was also less frequently observed in qk^v/qk^v mice in the same regions. Phospho-ERK staining co-localized with DAPI staining in both qk^v/qk^v and qk^v/qk^v; *p53-/-* brains (Figure 10, arrows), consistent with ERK translocation to the nucleus upon activation. These findings suggest that QKI negatively regulates the ERK pathway.

Discussion

The qk^v mouse is a well characterized model of dysmyelination, with defects in myelination being specifically attributed to altered balance of *qkI* isoform expression in oligodendrocytes (Chenard and Richard 2008). QKI-6/7 have been shown to up-regulate $p27^{kip1}$ mRNA in OPCs, leading to cell cycle exit and differentiation into mature oligodendrocytes (Larocque et al. 2005). In addition to $p27^{kip1}$, the terminal differentiation of OPCs into mature oligodendrocytes has been shown to be dependent on p53 *in vitro* (Billon et al. 2004). In order to further elucidate the genetic link between *qkI* and *p53*, we have bred the qk^v/qk^v mice into a *p53-/-* background. Our study indicates that qk^v/qk^v; *p53-/-* mice do not have decreased corpus callosum thickness or MBP-staining compared to qk^v/qk^v littermate controls, suggesting that the qk^v-associated myelination defect is not further impaired by the loss of *p53*. These findings indicate that *qkI* does not cooperate with *p53 in vivo* to regulate the oligodendrocyte differentiation pathway. The *C. elegans* homolog of QKI, GLD-1, is known to associate with the *p53 cep-1* mRNA and regulate its activity (Schumacher et al. 2005). Consistent with these results, a bioinformatics analysis has identified *p53* as a putative target of QKI. The *p53* mRNA harbors a putative QRE within its 3'UTR (TACTAACnnnnGAAG). These findings indicate that QKI may regulate *p53* mRNA levels *in vivo*. Thus, the absence of *qkI* in addition to *p53* would not result in an altered phenotype as the loss of *p53* would affect the same pathway as QKI. Consistent with these observations, qk^v/qk^v; *p53-/-* mice did not demonstrate an increased severity of hypomyelination compared to qk^v/qk^v mice.

qk^v/qk^v; *p53-/-* mice displayed Purkinje-cell defects in the cerebellum characterized by dendritic arborization defects and cell body loss, indicating that qkI is required for

neuronal cell. However, the exact mechanism by which this phenotype occurs has yet to be elucidated. We have shown that, contrary to previous findings (Hardy et al. 1996), Purkinje cells neurons express the QKI-5 isoform. QKI-5 expression in Purkinje cells was not altered in qk^v/qk^v or qk^v/qk^v; *p53-/-* mice compared to littermate controls, indicating that Purkinje cell degeneration could not be due to altered QKI expression. Cerebellar defects have previously been documented in mice homozygous for the qk^v mutation. Three month old qk^v/qk^v mice displayed axonal swellings in both the Purkinje and granular cell layer, indicative of axonal injury (Suzuki and Zagoren 1975). Axonal swelling is characteristic of inflammatory lesions in multiple sclerosis and experimental autoimmune encephalitis, suggesting that axonal pathology is secondary to myelination defects (Raine and Cross 1989; Trapp et al. 1998). While my work showed that the qk^v dysmyelinating phenotype was not exacerbated on a *p53-/-* background, Purkinje cell degeneration was only observed in qk^v/qk^v; *p53-/-* mice and not qk^v/qk^v controls, suggesting the neuronal pathology that was not due to myelination defects. Interestingly, qk^v/qk^v; *p53-/-* mice displayed ataxia at about one month of age that was not observed in their qk^v/qk^v counterparts. Disruption in normal cerebellar architecture is one cause of ataxia (Vaillant and Monard 2009), thus Purkinje cell degeneration may be related to the ataxic-like phenotype observed in qk^v/qk^v; *p53-/-* mice.

Altered tumor spectrum in qk^v/qk^v; *p53-/-* mice

qk^v/qk^v; *p53-/-* mice failed to develop tumors characteristic of *p53-/-* mice, but demonstrated a reduced survival rate compared to both $qk^v/+$; *p53-/-* and *p53-/-* mice. One case of medulloblastoma was documented in a cohort of 11 qk^v/qk^v; *p53-/-* mice, while 2 other mice demonstrated signs of hydrocephaly. One cause of hydrocephaly is obstruction of CSF circulation, such as intra-ventricular brain tumors including astrocytoma (Isaacs

2002). Hydrocephaly may also be caused by tumors of the choroid plexus, resulting in an overproduction of CSF (Weiss et al. 2002). *qk*^{v}/*qk*^{v}; *p53-/-* mice did not demonstrate signs of intra-ventricular astrocytoma, however they were not analyzed for choroid plexus tumors. Therefore, it remains a possibility that the underlying cause of hydrocephaly observed in *qk*^{v}/*qk*^{v}*; p53-/-* mice could have been due to tumors of the choroid plexus. Proper examination of the brain was not possible for approximately half of the *qk*^{v}/*qk*^{v}*; p53-/-* cohort, as tissue autolysis was too advanced at the time of necropsy. The majority of *p53-/-* and *qk*^{v}/+; *p53-/-* mice in our study succumbed to thymic lymphoma, demonstrating clear symptoms including difficulty breathing due to tumor-induced compression of the lungs, enlarged ribcages, and lethargy due to tumor burden. Necropsy of these mice revealed highly vascularized, enlarged thymuses that completely encompassed the heart and the majority of the lungs, and in some instances, extended outside the ribcage into the subcutaneous layers. These symptoms were not observed in any of the *qk*^{v}/*qk*^{v}; *p53-/-* mice before death, and necropsy revealed normal thymic morphology. Thus, while *qk*^{v}/*qk*^{v}; *p53-/-* mice evidently exhibit an altered tumor spectrum compared to *p53-/-* mice, the main cause of death remains an object of speculation. Several possible explanations exist for the altered tumor tropism and reduced survival rate observed in *qk*^{v}/*qk*^{v}; *p53-/-* mice. The *qk*^{v}/*qk*^{v} mutation results in brain-specific loss of *qkI* isoforms *-6/7*, resulting in preferential sensitization of the nervous system to tumorigenesis in the absence of *p53*. As mentioned earlier, our post-mortem pathological observation methods were limited, thus it is possible that *qk*^{v}/*qk*^{v} mice developed brain tumors that remained undetected.

p53+/- and *p53-/-* mice have been used as a background strain to breed with other mutant strains of mice to produce entirely novel tumor spectrums that were not found in mice

with a mutation in *p53* alone (Huse and Holland 2009). Mice heterozygous for the tumor suppressors Neurofibromatosis type I (*nf1)* and *p53* developed GBM at a high frequency, whereas neither *p53*+/- or *nf1*+/- mice showed any incidence of astrocytoma (Donehower et al. 1992; Gutmann et al. 1999; Reilly et al. 2000). Likewise, *ptch1*+/-; *p53*-/- mice succumbed to medulloblastoma formation with a 95% incidence before 12 weeks of age, although no primitive neuroectodermal tumors had previously been observed in *p53*-/- mice (Wetmore et al. 2001). Similarly, the combined loss of *qkI-6/7* and *p53* in the brain could result in altered tumor susceptibility compared to *p53*-/- mice.

QKI is implicated in medulloblastoma formation

The brain tumor confirmed histologically in our qk^{v}/qk^{v}; *p53*-/- cohort was the PNET, medulloblastoma. The Jacks group observed only one instance of a brain malignancy out of a cohort of *p53* heterozygous mice (n=40), whereas another study did not observe any tumors of the nervous system in a cohort of *p53*-/- mice (n= 35) or *p53*+/- (96) mice (Donehower et al. 1992; Jacks et al. 1994). The *p53*+/- brain tumor in the Jacks study was identified as an ependymoma, a tumor originating from the walls of the ventricular system (Poppleton and Gilbertson 2007). Therefore, the qk^{v}/qk^{v}; *p53*-/- mice display a different tumor tropism that has not been previously observed in either parent mutant strain. *p53* mutations contribute to approximately 10% of human medulloblastoma cases (Kleihues et al. 1997); in addition, many mouse models of medulloblastoma have been developed by the combination of a specific tumor suppressor mutation and the loss of *p53* (Huse and Holland 2009). Although *qkI* has been mapped to the common fragile site 6q25-26 in GBM, loss of 6q25-26 in medulloblastoma is rare (Thompson et al. 2006), suggesting that somatic *qkI* mutations in clinical medulloblastoma do not confer any specific tumorigenetic advantage. QKI has been

shown to be expressed in post-natal neural progenitor cells of the SVZ (Hardy 1998b), suggesting that QKI may also be expressed in other populations of multi-potent precursor cells including the rostral rhombic lip progenitor cells that give rise to granule neuronal precursor cells. It has not been determined if the qk^{v} mutation affects QKI expression in neural progenitor cells including granule neuronal precursor cells, therefore it remains a possibility that the loss of *qkI-6/7* in the absence of *p53* could provide sufficient oncogenic stress to induce transformation.

Granule neuronal precursor cell proliferation is known to be regulated by SHH signaling, and SHH pathway effectors are frequently mutated in medulloblastoma (Guessous et al. 2008). The downstream effector of SHH signaling, Gli1, has increased expression in medulloblastoma and is essential for medulloblastoma formation in *ptc1*+/- mice (Kimura et al. 2005). We have shown that QKI-6/7 is a negative regulator of *gli1* mRNA expression, and is therefore implicated in normal cerebellar development as well as SHH-induced medulloblastoma formation. We showed that QKI-6 directly regulates *gli1* expression through its RNA-binding KH domain. Although we did not determine if *gli1* mRNA levels were affected, we showed that Gli levels were significantly decreased at the protein level. GLD-1, the QKI homology in *C. elegans*, has been previously shown to bind and inhibit the expression of the *gli1* homolog, *tra-2*. The same study also demonstrated that the decrease in tra-2 luciferase activites due to GLD-1-mediated inhibition was not a result of altered RNA levels, suggesting that *tra-2* expression is controlled at the translational level (Jan et al. 1999). Inhibition of *gli1* expression by QKI-6 has been shown to be dependent on the evolutionarily conserved TGE, which functions in post-transcriptional regulation (Lakiza et al. 2005). In support of these observations, we showed that the nuclear isoform QKI-5 had

no effect on *gli1* levels, suggesting that inhibition of *gli1* expression takes place in the cytoplasm, where mRNA translation occurs. Thus, it is likely that QKI-6 acts in a similar fashion to GLD-1 in suppressing *hgli1* expression. The exact mechanism by which QKI-6 mediates *gli1/tra-2* translational repression has yet to be elucidated; however several possible models have been proposed. It is possible that QKI-6 binds translation initiation factors and suppresses their activity, or that the TGE/QKI-6 interaction results in sequestration of *gli1* mRNA from cell translational machinery. STAR proteins are hypothesized to regulate mRNA poly(A) tail length, as the presence of TGEs correlates with shorter poly(A) tails (Jan et al. 1997).

In addition to negatively regulating *gli1* expression, QKI is also implicated in other pathways that are frequently altered in medulloblastoma. QKI has been shown to up-regulate the CDK inhibitor $p27^{kip1}$ in differentiating oligodendrocytes leading to cell cycle arrest, and therefore may play a similar role in other cell types (Larocque et al. 2005). Indeed, $p27^{kip1}$ has been shown to be strongly expressed in post-mitotic granule neurons, whereas $p27^{kip1}$expression is low in proliferating granule neuron precursor cells (Miyazawa et al. 2000). These findings suggest that $p27^{kip1}$ may also play a role in inhibiting GCNP proliferation in the cerebellum. Deregulation of $p27^{kip1}$ is a frequent event in a variety of cancers, including medulloblastoma (Chu et al. 2008). Recently, it was shown that mice heterozygous for *ptch1* on a $p27^{kip1-/-}$ background have a significantly higher tumor incidence than *ptch1*+/- mice that with *wt* $p27^{kip1}$ (Ayrault et al. 2009). In addition, *ptch1*+/-; $p27^{kip1-/-}$ medulloblastomas demonstrated increased invasiveness and proliferation capacity compared to medulloblastomas from *ptch1*+/- mice. These findings indicate that *ptch1* LOH in the absence of $p27^{kip1}$ contributes to increased tumorigenecity and medulloblastoma formation.

Alternatively, QKI may contribute to medulloblastoma formation by negatively regulating the WNT pathway. We did not analyze the qk^{v}/qk^{v}; *p53-/-* medulloblastoma for SHH activation; thus it remains a possibility that medulloblastoma formation was due to alterations in other pathways including WNT signaling. Recently, it was shown that QKI-5 and -6 are capable of binding the 3'UTR of β-catenin mRNA to negatively regulate its expression. Over-expression of QKI was also found to decrease levels of nuclear and cytoplasmic β-catenin in gastric carcinoma cells, indicative of WNT pathway suppression (Yang et al. 2009). Taken together, these findings reveal another possible mechanism by which QKI may negatively regulate tumor formation.

QKI is implicated in EGFR regulation

6q25-27 is a common fragile site in glioblastoma and includes the *qkI* gene (Mulholland et al. 2006). In addition to its mapping to a region of genomic instability, *qkI* mRNA transcripts have been shown to be down-regulated specifically in GBM (Li et al., 2002). It is likely that QKI functions as a tumor suppressor in GBM, however the exact mechanism has yet to be elucidated. We provide evidence that QKI negatively regulates ERK1/2 activation, and is therefore implicated in EGFR signaling. EGFR amplification occurs in over 30% of primary GBM (Collins and James 1993), thus loss of QKI could provide another mechanism by which to activate EGFR signaling. We have shown that QKI knockdown results in increased ERK1/2 activation in GBM cells, however the exact mechanism has yet to be determined. Based on the consensus QRE sequence, we have identified putative QKI-binding sites in multiple proteins that regulate MAPK activation, including phosphoprotein enriched in astrocytes 15 (*pea15*), *nf1*, and pleiotrophin (*ptn*). *ptn* was also identified by SELEX analysis as a putative QKI target (Galarneau and Richard 2005). In parallel, QKI

homolog HOW was found to bind and repress the translation of midkine and pleiotrophin heparin-binding growth factor (Miple) mRNA, the pleiotrophin homolog in *Drosophila* (Toledano-Katchalski et al. 2007). Over-expression of Miple in the Drosophila mesoderm resulted in increased ectopic expression of MAPK. QKI may play a similar role in mammalian cells by repressing translation of *ptn* mRNA. Taken together, these results indicate a novel pathway by which QKI may regulate tumorigenesis, including GBM formation.

Summary

QKI has been identified as a candidate tumor suppressor in multiple independent studies; however the pathways it may potentially regulate has yet to be elucidated. We present evidence that QKI is implicated in normal cerebellar development and medulloblastoma formation by down-regulating *gli1* expression at the RNA level. We also present evidence that QKI suppresses ERK1/2 activation, suggesting that QKI may be involved in preventing other CNS tumors such as GBM by negatively regulating the EGFR pathway. Introduction of the qk^{v}/qk^{v} mutation in the *p53-/-* background causes an increase in neurological defects such as ataxia and seizures. In addition, loss of p53 leads to Purkinje cell body and dendrite degeneration. Our study indicates that QKI proteins may interact with p53 *in vivo* to regulate neuronal maintenance and survival.

Figures

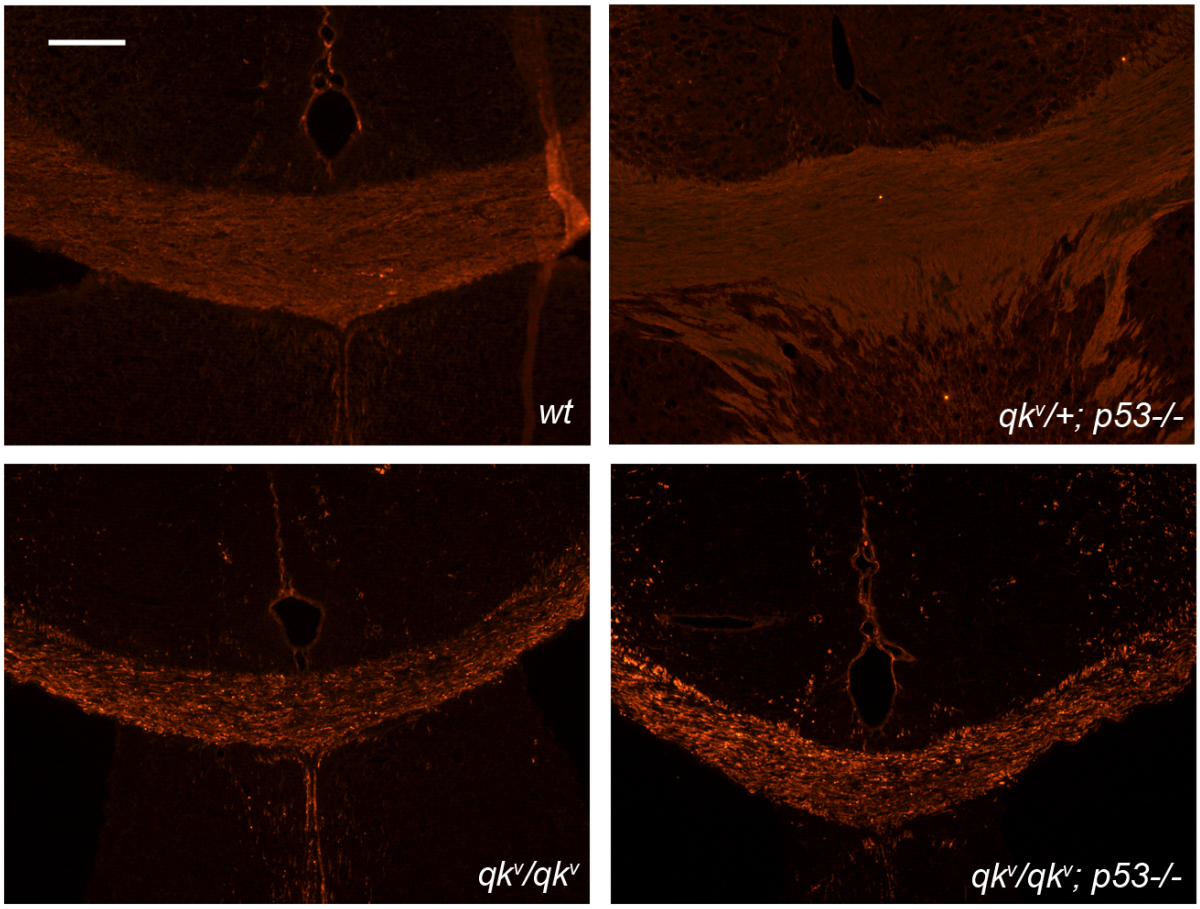
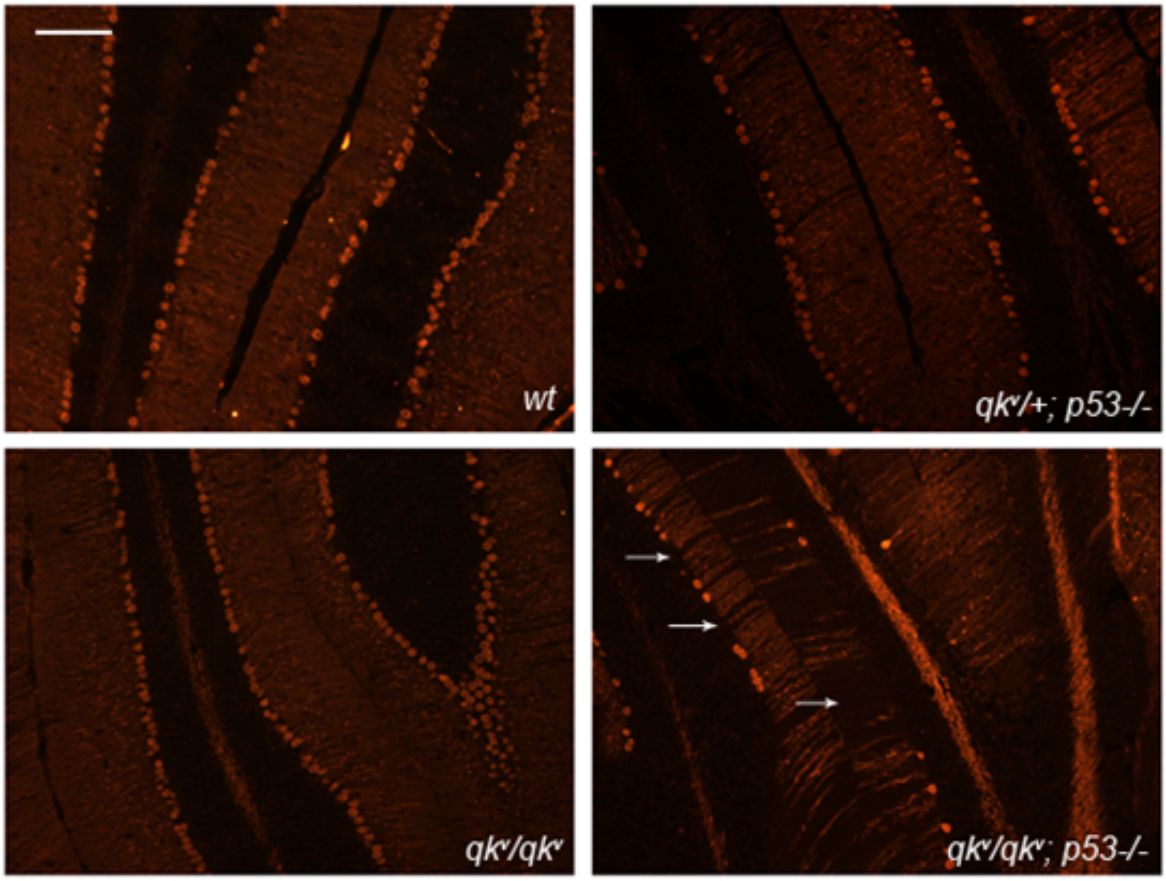


Figure 1. *qk*^{v}-associated hypomyelination is not exacerbated on a *p53-/-* background.

Brains were frozen over acetone-dry ice and cryostat sectioned at a thickness of 10 microns. Coronal sections of age and sex-matched mouse cortexes were stained with anti-MBP antibody. Scale bar represents 100 microns.

A



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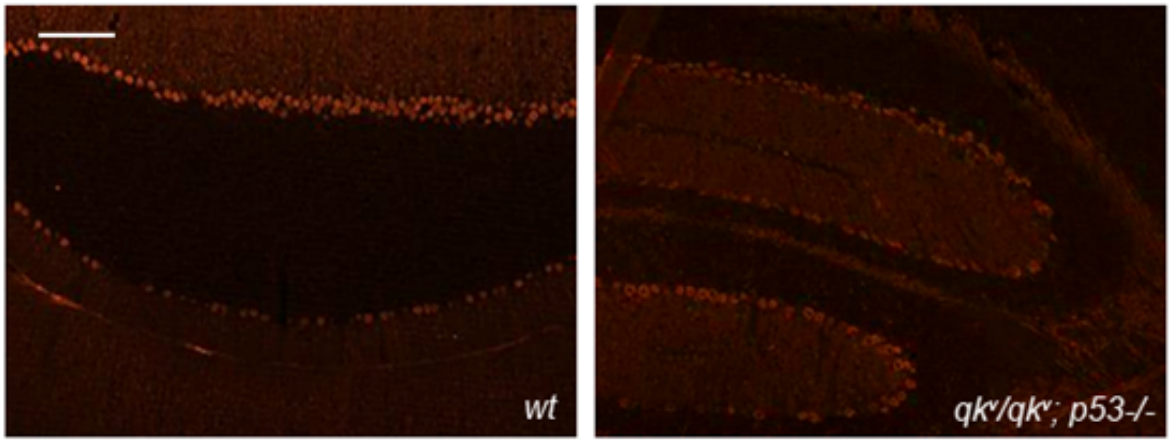


Figure 2. *qk^v/qk^v; p53-/-* mice display Purkinje cell defects.

(A) *qk^v/qk^v*; *p53-/-* mice display Purkinje cell degeneration and dendritic arbor loss by P30 (arrows), whereas control littermates exhibit normal cerebellar architecture. Sections were stained with anti-Calbindin-D-28K antibody. Scale bar represents 100 microns.

(B) *qk^v/qk^v*; *p53-/-* show normal Purkinje cell morphology of the cerebellum at P14. Coronal sections of the cerebellum were stained with anti-Calbindin-D-28K antibody. Scale bar represents 100 microns.

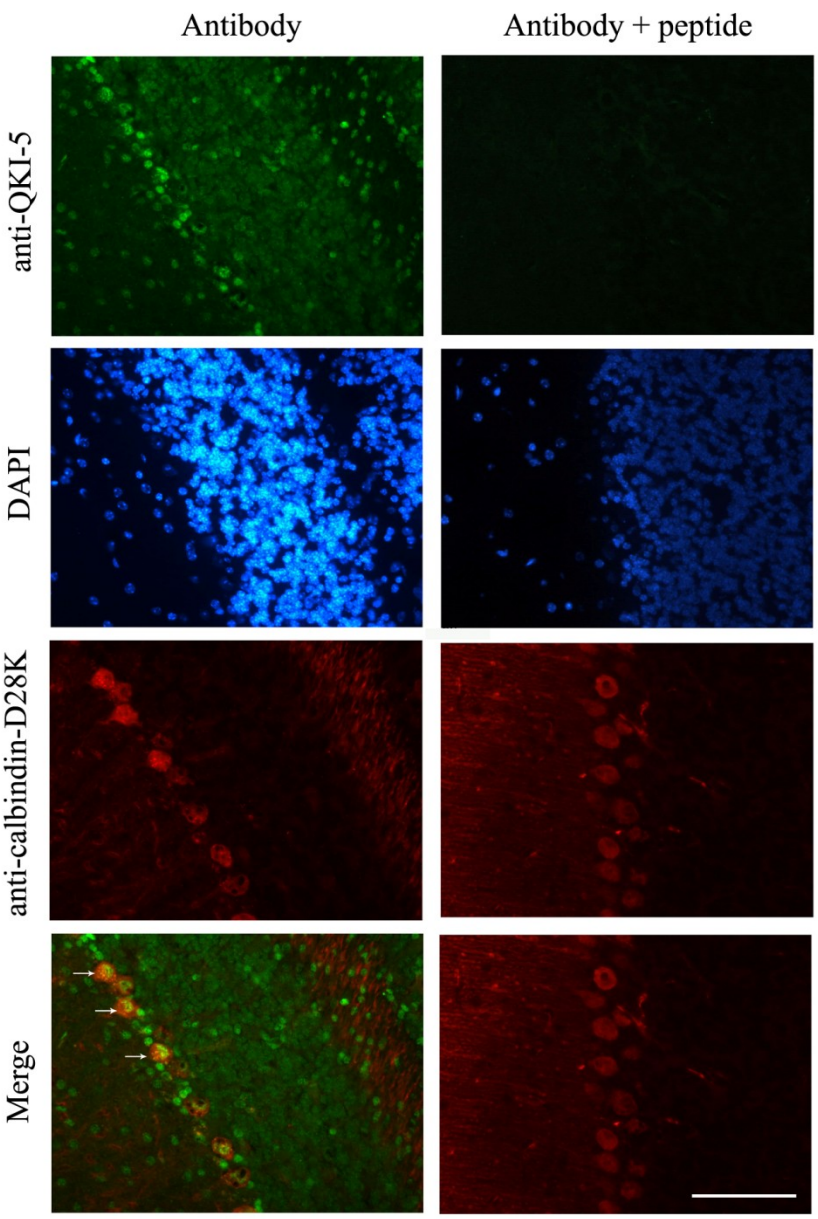


Figure 3. Purkinje neurons express QKI-5. Coronal sections of *wt* mouse cerebellum were immunostained with antibodies against QKI-5 and calbindin. To verify staining specificity, sections were stained in parallel with antibody pre-absorbed with QKI-5. Purkinje cells show nuclear QKI-5 expression (arrows). Scale bar represents 50 microns.

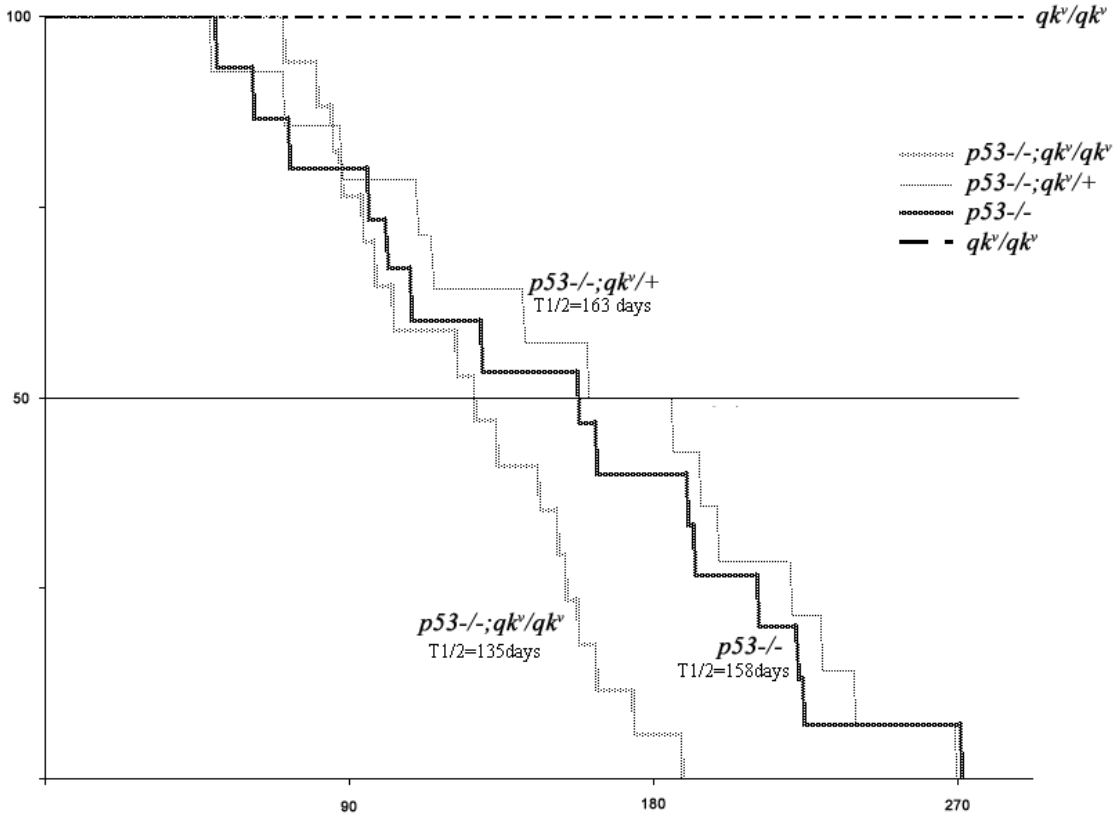


Figure 4. *qk*^{v}/*qk*^{v} homozygous mice show a reduced survival rate in the absence of p53.

qk^{v}/*qk*^{v}; *p53*$^{-/-}$ mice (*n* = 11) were observed to have a median survival rate of 119 days, significantly lower compared to *qk*^{v}/+ ; *p53*$^{-/-}$ mice (142 days, *n* = 12) and *p53*$^{-/-}$ mice (145 days, *n* = 9) according to the log rank/Mantel Cox test (*p* = 0.0135 and *p* = 0.443, respectively). The loss of survival was measured by mice that were found dead or that had to be sacrificed due to illness according to the guidelines of the Canadian Animal Care Committee.

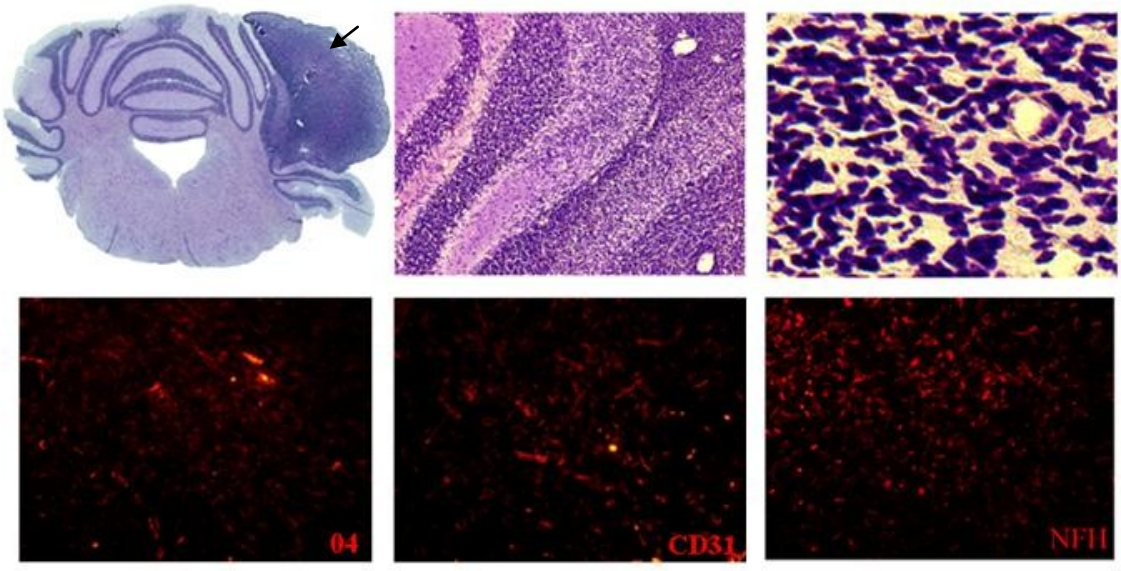


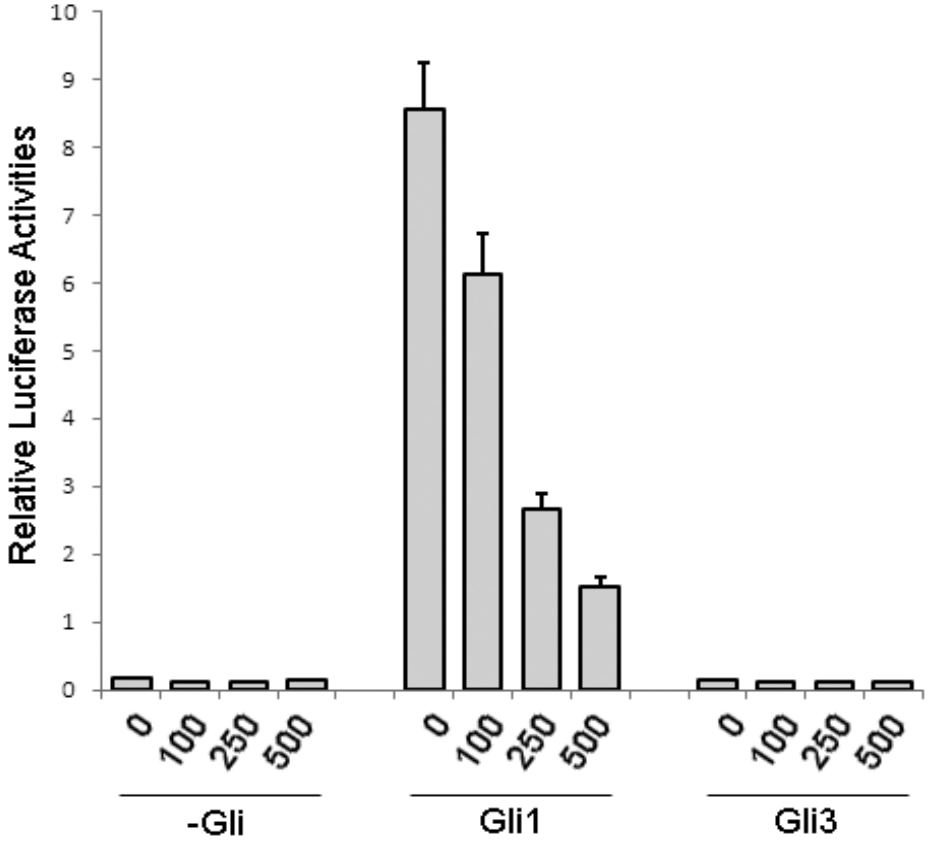
Figure 5. *qk^{v}/qk^{v}; p53$^{-/-}$* mice develop cerebellar tumors.

(A) Low-power magnification showing macroscopic appearance of medulloblastoma occurring in the cerebellum, with an arrow denoting the tumor area. Section was stained with H&E.

(B) Higher magnification of medulloblastoma, demonstrating the invasion of the molecular layer by the over proliferation of granular like tumor cells. Section was stained with H&E.

(C) Histopathology of medulloblastoma cells, showing densely packed polygonal nuclei with scant cytoplasm.

(D,G,H) Immunocytochemistry performed on the medulloblastoma reveals tumor reactivity to GFAP, NFH, and CD31, specific markers for different cell lineages.


Relative Luciferase Activities
10
9
8
7
6
5
4
3
2
1
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0
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Gli1
Gli3

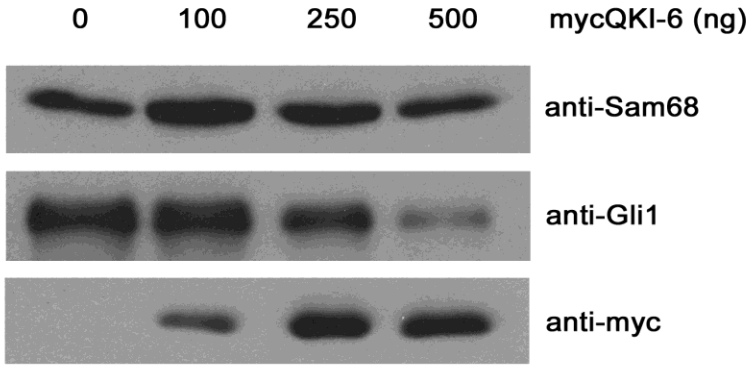
0
100
250
500
mycQKI-6 (ng)
anti-Sam68
anti-Gli1
anti-myc

Figure 6. QKI-6 regulates Gli1 expression in a dose-dependent manner.

(A) A luciferase reporter under the control of an 8x Gli response element was used to assay Gli expression. HEK 293 cells were transfected with vectors expressing the luciferase reporter and full length Gli1 cDNA along with increasing concentrations of myc-tagged QKI-6. Gli1 luciferase activity decreases with increasing concentrations of QKI-6 compared to controls empty vector myc-pcDNA and Gli3. Luciferase activities for 100, 250, and 500 ng of transfected QKI-6 are significant compared to empty vector myc-pcDNA according the paired two-tailed Student's t-Test ($p = 0.02$, $p = 0.002$, $p = 0.003$, respectively).

(B) Western blot analysis was performed to verify Gli1 expression. Gli1 expression levels decrease with corresponding increasing levels of QKI-6, as detected with an antibody directed against the myc tag. Sam68 was used as a loading control.

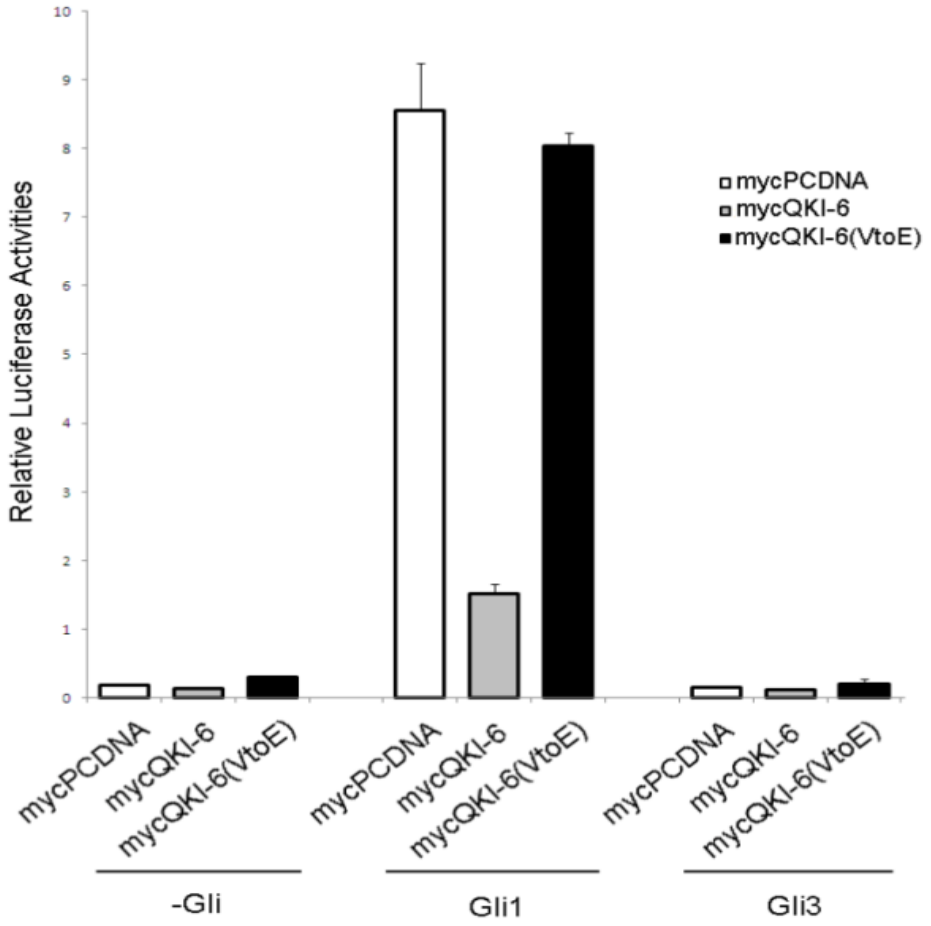

Figure 7. The QKI-6 RNA binding domain is necessary for Gli1 regulation.

A luciferase reporter under the control of an 8x Gli response element was used to assay Gli expression. An RNA binding defective QKI-6 was created by making a mutation in the KH domain, changing valine157 to glutamic acid. 293 HEK cells were transfected with vectors expressing the luciferase reporter, Gli1, QKI-6, and QKI-6:V157E with empty vector myc-pcDNA and Gli3 serving as controls. QKI-6 expression results in significantly decreased Gli1 luciferase activites ($p < 0.001$), whereas the QKI-6:V157E mutant fails to repress Gli1 expression.

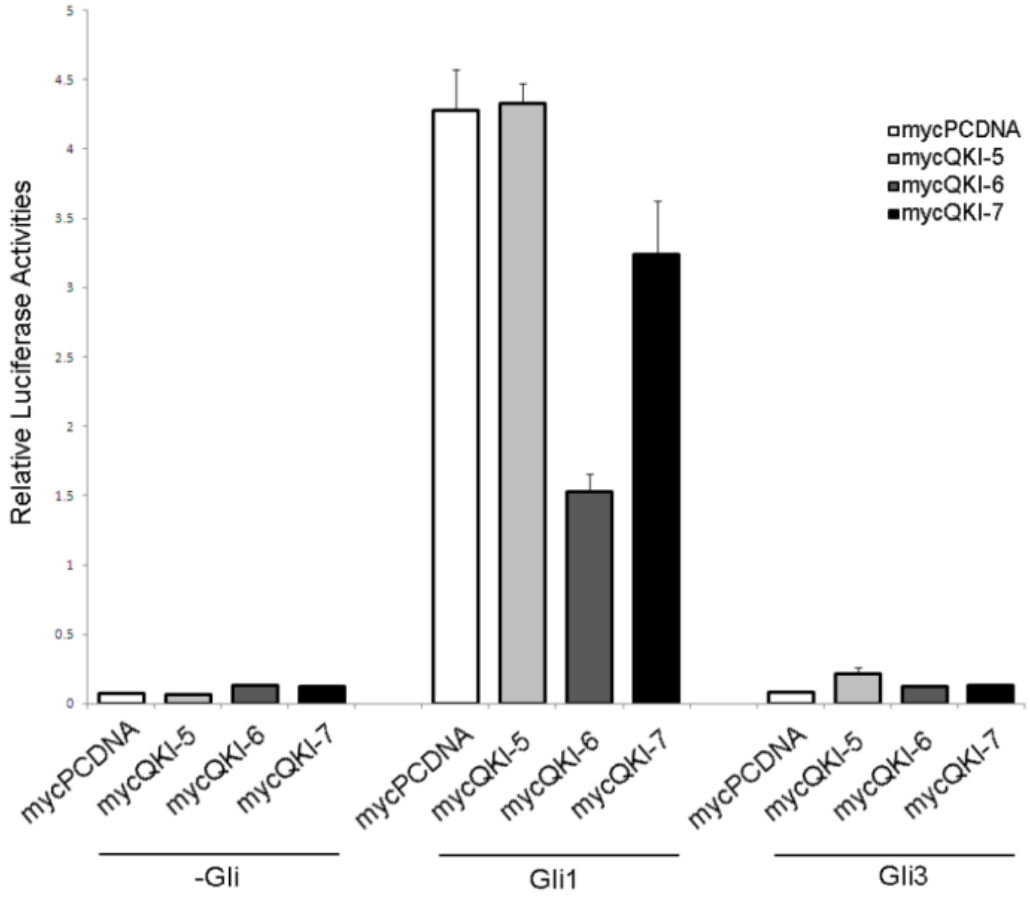

Figure 8. Gli1 regulation is QKI isoform-dependent.

293 HEK cells were transfected with vectors expressing the luciferase reporter, Gli1, QKI-5, QKI-6, and QKI-7 with empty vector myc-pcDNA and Gli3 serving as controls. In contrast with QKI-6, QKI-5 fails to repress *gli1* expression. QKI-7 represses Gli1 expression at a lesser extent compared to QKI-6, however repression is still significant compared to controls according the paired two-tailed Student's t-Test ($p = 0.005$)

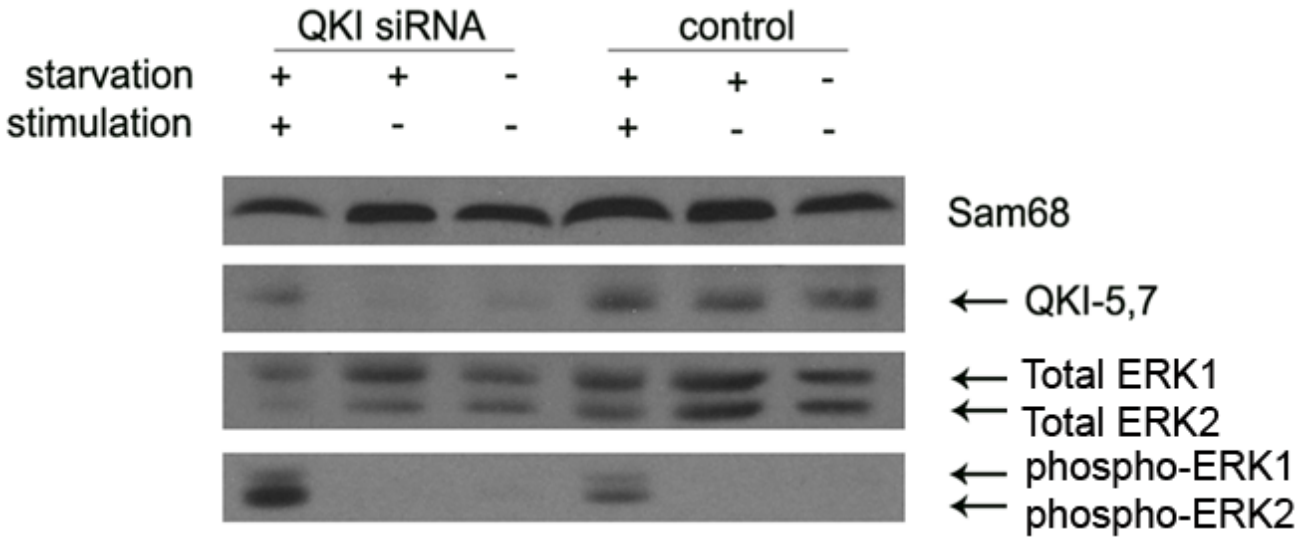

Figure 9. QKI negatively regulates ERK1/2 activation in Glioblastoma cells.

ERK1/2 activation is up-regulated in QKI knockdown U373 GBM cells. QKI knockdown efficiency was verified by immunoblotting with anti-QKI-5,-6, and -7 antibodies. Cells were serum starved for 16 h in DMEM supplemented with 0.1% bovine calf serum and stimulated for 1 h with DMEM supplemented with 10% BCS. Sam68 was used as a loading control.

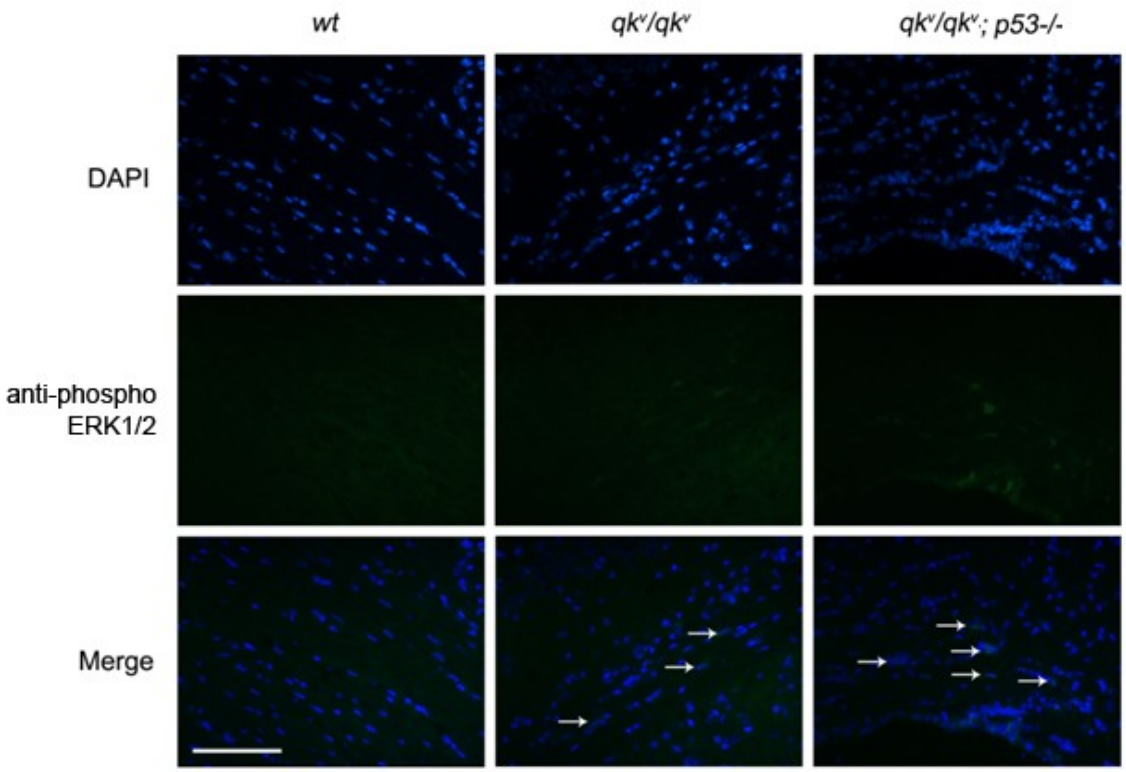

Figure 10. ERK1/2 activation is up-regulated in *qk*^{v}/*qk*^{v} mice.

Coronal sections including the corpus callosum were stained with a phospho-specific antibody for phospho-ERK1/2. Phospho-ERK1/2 immunostaining showed positive nuclear reactivity in *qk*^{v}/*qk*^{v} and *qk*^{v}/*qk*^{v}*; p53-/-* mice (arrows). Scale bar represents 100 microns.

Table 1. Tumors in qk^v/qk^v; *p53-/-* mice [a]

Genotype	Total no. of mice	Brain tumors	Hydrocephaly	Subcutaneous	Thymic lymphoma	Intraperitoneal	Other
p53-/-;qk^v/qk^v	11	1(11%)	2(18%)	0	0	0	0
p53+/-;qk^v/qk^v	33	0	5(15%)	0	0	0	0
p53-/-;qk^v/+	12	0	0	2(15%)	8(61%)	3(23%)	0
p53-/-	9	0	0	0	5(55%)	3(33%)	1(11%)

a. tumors were classified according to tumor location.

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