Journal of Cellular Biochemistry 102:811–819 (2007)

All in the Family: Using Inherited Cancer Syndromes to Understand De-Regulated Cell Signaling in Brain Tumors

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Abstract The cell signaling pathways that are tightly regulated during development are often co-opted by cancer cells to allow them to escape from the constraints that normally limit cell growth and cell movement. In this regard, de-regulated signaling in cancer cells confers a number of key tumor-associated properties, including increased cell proliferation, decreased cell death, and increased cell motility. The identification of some of these critical signaling pathways in the nervous system has come from studies of inherited cancer syndromes in which affected individuals develop brain tumors. The study of brain tumors arising in patients with neurofibromatosis 1 (NF1), neurofibromatosis 2 (NF2), and tuberous sclerosis complex (TSC) has already uncovered several key intracellular signaling pathways important for modulating brain tumor growth. An in-depth analysis of these intracellular signaling pathways will not only lead to an improved understanding of the process of brain tumorigenesis, but may also provide important molecular targets for future therapeutic drug design. J. Cell. Biochem. 102: 811–819, 2007. © 2007 Wiley-Liss, Inc.

Key words: neurofibromatosis 1; NF1; neurofibromin; neurofibromatosis 2; NF2; merlin; schwannomin; tuberous sclerosis complex; TSC1/hamartin; TSC2/tuberin; brain tumor signaling

The development and maintenance of the mammalian brain is a tightly regulated, highly choreographed process involving complex relationships between neighboring cells. During brain development, immature cells receive numerous signals that govern cell fate decisions, including whether to divide, die, or migrate to another brain region or niche. Each of these extracellular cues is transduced by intracellular signaling cascades involving the activation and/or inactivation of protein signaling intermediates, beginning at the plasma membrane and often culminating in changes in DNA transcription within the nucleus. These extracellular signals may involve the binding of a specific ligand (e.g., growth factor) to a cell surface receptor containing an intracellular signaling domain (e.g., receptor tyrosine kinases; RTKs). Alternatively, transduction of these environmental cues may occur via transmembrane proteins that facilitate interactions with other proteins present within the cytoplasm and result in signal propagation.

Tumors of the nervous system can arise de novo or develop in individuals with an inherited predisposition to tumor formation (Table I). While the majority of brain tumors are not the result of an inherited cancer syndrome, understanding the genetic basis of those brain tumors that develop in the context of familial syndromes provides important insights into tumorigenesis. Since familial cancer syndromes result from inherited mutations in a single gene (a "tumor suppressor" gene), identifying these genes often uncovers key intracellular signaling pathways that govern normal growth when properly regulated and lead to tumorigenesis when rendered non-functional by mutations. In this review, we will discuss three representative inherited cancer syndromes in which affected individuals develop nervous system tumors: neurofibromatosis 1 (NF1), neurofibromatosis 2 (NF2), and tuberous sclerosis complex (TSC).

NEUROFIBROMATOSIS TYPE 1

NF1 is an autosomal dominant inherited cancer syndrome which affects 1 in 2,500–3,000 individuals [Friedman, 1999]. The most common

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Received 26 June 2007; Accepted 28 June 2007

DOI 10.1002/jcb.21506

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TABLE I. Familial Brain Tumor Predisposition Syndromes

Syndrome	Gene	Nervous system tumor
Neurofibromatosis type 1	*NF1*	Optic glioma, astrocytoma, neurofibroma, MPNST
Neurofibromatosis type 2	*NF2*	Schwannoma, meningioma, neurofibroma, ependymoma
Tuberous sclerosis complex	*TSC1, TSC2*	Subependymal giant cell astrocytoma
von Hippel Lindau	*VHL*	Hemangioblastoma
Li-Fraumeni	*TP53*	Astrocytoma
Nevoid basal cell carcinoma	*PTCH*	Medulloblastoma

features include pigmentary abnormalities (café-au-lait macules), freckling in the armpits and groin, and hamartomas of the iris (Lisch nodules). In addition, individuals with NF1 are also prone to the development of peripheral and central nervous system tumors. The most common tumor is the neurofibroma, a benign peripheral nerve tumor composed of neoplastic Schwann cells [Gutmann et al., 1997]. A subset of neurofibromas (plexiform neurofibromas) can undergo malignant transformation into malignant peripheral nerve sheet tumors (MPNSTs), which are often fatal. The second most common tumor is the optic pathway glioma, an astrocytoma that involves the optic nerve, optic radiations, and chiasm [Listernick et al., 1997]. These tumors can cause blindness or endocrine abnormalities when they invade into the hypothalamus.

The *NF1* Gene

Linkage analysis and positional cloning resulted in the identification of the *NF1* gene on chromosome 17q11.2 [Viskochil et al., 1990; Marchuk et al., 1991]. The *NF1* gene spans over 350 kb of genomic DNA and encodes an 11–13 kb messenger RNA. The protein product of the *NF1* gene, neurofibromin, is composed of 2,818 amino acids and has a molecular mass of 220–250 kDa. In the nervous system, neurofibromin is expressed in the cytoplasm of neurons, oligodendrocytes, Schwann cells, and astrocytes.

As described for other inherited cancer syndromes, individuals with NF1 are born with a mutated or a non-functional copy of the *NF1* gene in all cells of their body. *NF1*-associated tumors develop only when the remaining normal *NF1* gene undergoes somatic mutation, leading to complete loss of neurofibromin function [Knudson, 1971]. In this regard, loss of *NF1* gene expression in a Schwann cell would result in neurofibroma formation, while *NF1* inactivation in a glial cell leads to optic glioma.

Neurofibromin Function

Initial sequence analysis of neurofibromin revealed that it contains a small domain that shares significant homology with proteins belonging to the guanosine triphosphatase (GTPase) activating protein (GAP) family, involved in the negative regulation of small GTPase proteins like Ras. In addition, neurofibromin also has non-Ras functions, including the ability to positively modulate intracellular cyclic AMP levels.

Ras-dependent signaling. Neurofibromin has been shown to play a central role in regulating Ras-dependent intracellular signaling pathways (Fig. 1). Ras exists in two conformations: an active, GTP-bound form and an inactive GDP-bound form. Like other GAP molecules, neurofibromin accelerates the hydrolysis of GTP-bound Ras to result in the inactivation of Ras. Ras hyperactivation has been identified in numerous malignant tumors and promotes increased cell proliferation. Consistent with this observation, loss of neurofibromin results in increased Ras activation [Xu et al., 1990; Bollag et al., 1996]. Moreover, inhibition of Ras reverses the increased proliferation observed in *Nf1*-deficient mouse cells, including astrocytes and Schwann cells, further underscoring the importance of neurofibromin's Ras regulation in cell growth control.

Increased Ras activity results in the activation of several downstream signaling cascades, including the Raf-ERK and PI3K-mTOR pathways. Ras activation has been shown to increase the mitogen activated protein kinase (MAPK) pathway by increasing the activation of Raf protein kinase and its downstream effectors MAP/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK). Aberrant Ras activation also leads to hyperactivation of phosphoinositide-3-kinase (PI3K), which in turn leads to increase in the activation of protein kinase B (Akt) and its effector, mammalian

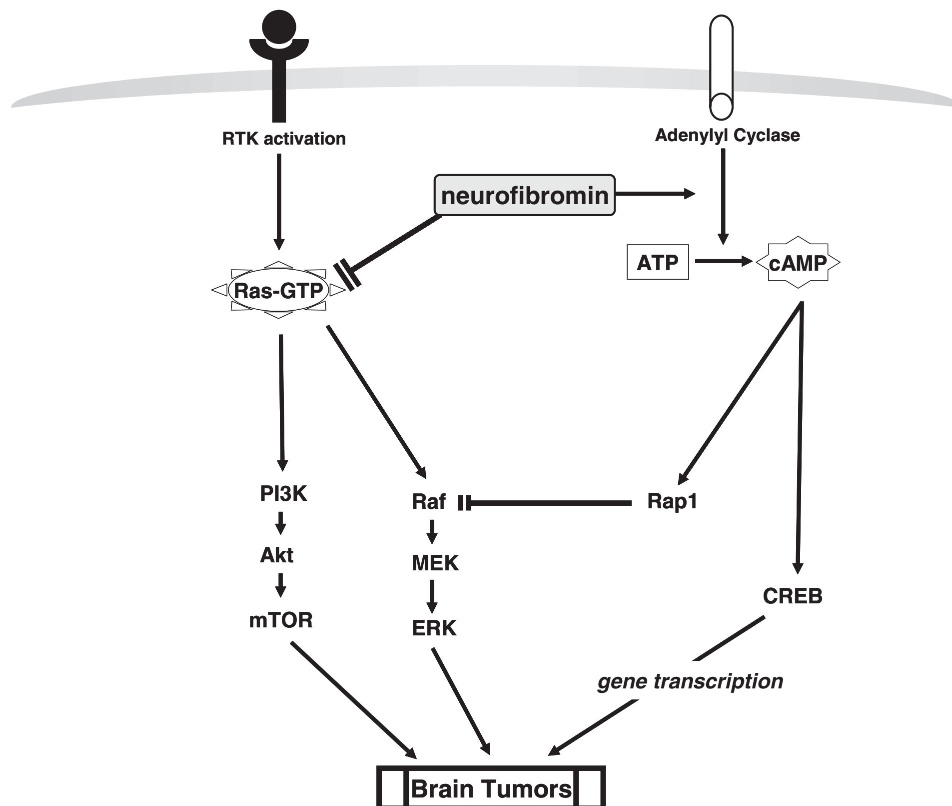

Fig. 1. Neurofibromin-regulated signaling in brain tumors. Neurofibromin functions as a negative regulator of Ras-GTPase activation as well as a positive regulator of intracellular cAMP generation. As a Ras regulator, upon RTK activation, neurofibromin inhibits Ras activation, and its downstream effectors, PI3K-Akt-mTOR, and Raf-MEK-ERK. As a cAMP regulator, it promotes the conversion of ATP to cAMP by adenylyl cyclase. Increased cAMP levels in the cell are growth inhibitory in part by inhibiting MEK activity through Rap1-GTPase, as well as by controlling cAMP-mediated transcription through cAMP response element binding protein (CREB).

Target of Rapamycin (mTOR). Recent studies have shown that treatment of *Nf1*-deficient cells with rapamycin, an mTOR inhibitor, reverses the mTOR hyperactivation and the increased proliferation associated with neurofibromin loss [Dasgupta et al., 2005; Johannessen et al., 2005].

cAMP-dependent signaling. Although less studied, neurofibromin also regulates cyclic adenosine monophosphate (cAMP)-dependent signaling (Fig. 1). Intracellular cAMP levels are controlled by the conversion of adenosine triphosphate (ATP) to cAMP by adenylyl cyclase (AC). While the exact mechanism underlying neurofibromin regulation of cAMP generation has not been elucidated, cAMP levels, and AC activity are significantly lower in *Nf1*-deficient brain cells [Tong et al., 2002] and neurofibromin positively regulates cAMP production at the level of AC [Dasgupta et al., 2003]. As increased intracellular cAMP in astrocytes is associated with decreased cell growth, loss of neurofibromin expression leads to decreased cAMP generation and increased cell growth.

Nf1 Mouse Models

In an effort to understand the molecular and cellular pathogenesis of tumor formation in NF1, several genetically-engineered mouse models have been developed. Complete *Nf1* inactivation in mice (*Nf1*−/− mice) is embryonic lethal, as a result of cardiac abnormalities [Jacks et al., 1994]. Mice heterozygous for a targeted mutation in the *Nf1* gene (*Nf1*+/− mice) are viable, but do not develop either neurofibromas or optic gliomas. To circumvent the embryonic lethality associated with complete neurofibromin loss, Parada and colleagues developed *Nf1* conditional knockout mice using the Cre-LoxP technology. Using these mice, *Nf1* inactivation in Schwann cells and glial precursors has been achieved using specific

promoters to drive Cre recombinase expression. Surprisingly, *Nf1* loss in Schwann cells or glia is insufficient for either neurofibroma or glioma formation in vivo, suggesting that additional factors are required for tumorigenesis [Bajenaru et al., 2002; Zhu et al., 2002]. To more accurately recapitulate the human condition, *Nf1*+/− mice were generated that lack neurofibromin expression in either Schwann cell or glial precursors. This second generation of *Nf1* genetically engineered mice has yielded robust models for plexiform neurofibroma [Zhu et al., 2002] and optic glioma [Bajenaru et al., 2003]. Moreover, these mice have been employed to identify the critical signaling pathways important for regulating neurofibromin growth regulation in vivo [Dasgupta et al., 2005] as well as the contribution of signals from the tumor microenvironment that regulate *Nf1*−/− glial cell growth [Munchhof et al., 2006; Daginakatte and Gutmann, 2007].

NEUROFIBROMATOSIS TYPE 2

Neurofibromatosis type 2 (NF2) is also an autosomal dominant inherited cancer predisposition syndrome affecting approximately 1 in 38,000 individuals worldwide [Evans et al., 1992]. NF2 is characterized by the development of many nervous system tumors, including cranial, spinal, and peripheral nerve schwannomas, as well as meningiomas, and ependymomas. Schwannomas involving both eighth cranial nerves (bilateral vestibular schwannomas) are the hallmark of the disease, and commonly lead to balance and hearing problems. Meningiomas are the second most common tumor seen in patients with NF2. Lastly, ependymomas (glial cell tumors) are detected in 5–33% of affected individuals [Parry et al., 1994].

The *NF2* Gene

Family linkage and genetic mapping studies localized the *NF2* gene to chromosome 22q12.2. The *NF2* gene spans 110 kb of genomic DNA and contains 17 exons. The protein product of the *NF2* gene is a 595 amino acid residue protein of approximately 66–69 kDa, called merlin or schwannomin [Rouleau et al., 1993; Trofatter et al., 1993]. Merlin is expressed in Schwann cells, glial cells, and neurons in the nervous system. Amino acid sequence analysis of the predicted protein revealed three structural regions: a unique carboxyl terminal domain, an α-helix domain, and a highly conserved amino terminal domain known as the FERM (Four-point one, Ezrin, Radixin, and Moesin) domain. The presence of a FERM domain placed merlin in the 4.1 superfamily of proteins, previously shown to link plasma membrane proteins to the actin cytoskeleton [Sun et al., 2002].

The ability of merlin to function as a negative growth regulator is determined in part by the formation of intramolecular associations that allow the amino terminus of merlin to bind with its carboxyl terminal end [Sherman et al., 1997]. This folding is regulated by phosphorylation of merlin on residue Serine-518, such that phosphorylated merlin cannot form a productive intramolecular complex and is functionally inactive [Shaw et al., 2001; Rong et al., 2004].

Merlin Function

Efforts to define how merlin regulates cell growth relevant to tumorigenesis initially focused on proteins that interact with merlin and might function as its critical effectors. There have been over 10 merlin-interacting proteins identified; however, the relationship between these binding partners and merlin growth suppression has not been firmly established to date. These partners include membrane-associated and intracellular molecules. Furthermore, merlin also plays a role in intracellular signaling.

Membrane-linked signaling. The association between merlin and plasma membrane signaling complexes coupled with the observation that merlin regulates contact inhibition growth, suggests that merlin might integrate signals from specialized membrane structures to control cell growth involving both receptor tyrosine kinase molecules and transmembrane co-receptors (Fig. 2). In this fashion, merlin has been hypothesized to regulate cell growth by integrating receptor tyrosine kinase (RTK) and CD44, the hyaluronic acid receptor, signaling [Morrison et al., 2001; Scoles et al., 2002].

Engagement of CD44 by extracellular signals, such as hyaluronic acid, may stimulate cell growth partly through the formation of a signaling complex involving CD44 and receptor tyrosine kinase (RTK) molecules, such as the epidermal growth factor receptor [Curto et al., 2007]. Under conditions of contact

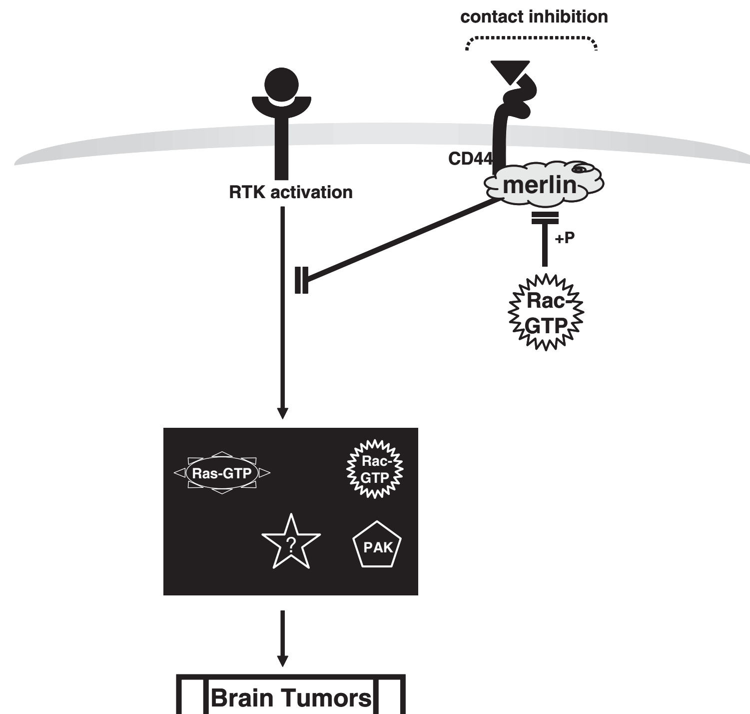

Fig. 2. Merlin-regulated signaling in brain tumors. Merlin functions as a regulator of contact inhibition signaling from the plasma membrane in part by modulating signal transduction from transmembrane sensors, like CD44. Merlin binding to CD44 regulates RTK signaling, such that loss of merlin results in increased RTK activity and increased RTK pathway activation through a number of downstream signaling intermediates. In addition, merlin function is controlled by Rac1-mediated phosphorylation.

inhibition (e.g., cell–cell contact), active hypophosphorylated merlin binds to CD44 and limits RTK molecule signaling. Thus, loss of merlin in tumors, due to bi-allelic *NF2* gene inactivation, leads to increased RTK signaling and promotes cell growth. Merlin can also be inactivated by phosphorylation, resulting in reduced merlin binding to CD44 binding and increased cell proliferation.

In addition to interactions with RTK and CD44 receptors, merlin has been shown to regulate the formation of adherens junctions [Lallemand et al., 2003]. Adherens junctions are specialized subcellular structures that are thought to mediate cell–cell interactions and may be important for transducing contact inhibition growth arrest signals when cells contact neighboring cells. In *Nf2*-deficient cells adherens junctions do not form properly and there is a loss of contact inhibition growth arrest, suggesting that merlin is required to transduce signals from the extracellular environment.

Intracellular signaling. Studies comparing normal human Schwann cells with human schwannoma cells revealed increased membrane ruffling, which could be reversed by pharmacological inhibition of a small Ras-like GTPase, called Rac1. Further studies also demonstrated that merlin negatively regulates Rac1-dependent signaling and that the phenotypes observed in *Nf2*-deficient cells are similar to those observed in cells expressing activated Rac1 [Shaw et al., 2001]. In addition, merlin has been shown to suppress the recruitment of Rac1 to the plasma membrane. The mechanism underlying Rac1 regulation of cell growth in *Nf2*−/− cells has not been completely elucidated, but may involve phosphorylation of p21-activated kinase (PAK). It should be noted that other studies have implicated different downstream signaling pathways, including Ras-dependent, Rac/PAK mediated Raf-MEK-ERK signaling [Jin et al., 2006].

The existence of numerous binding partners for merlin and the possibility of cross-talk between different intracellular signaling pathways have complicated the identification of downstream targets for merlin growth regulation. For this reason, we favor a model in which merlin transduces extracellular signals present at specialized subcellular structures to

modulate RTK signaling in a cell type-specific and context-dependent fashion. In this regard, the consequence of merlin loss in any given cell type reflects not only the intracellular signaling pathways most critical for modulating cell growth in that cell, but also which extracellular signals are present. Further work will be required to provide experimental proof for this hypothesis.

Nf2 Mouse Models

Conventional *Nf2* knockout (*Nf2*−/−) mice die during early embryogenesis due to a failure to form extra-embryonic ectoderm and initiate gastrulation [McClatchey et al., 1997]. As was true for *Nf1*+/− mice, *Nf2*+/− mice do not develop the tumors most frequently seen in patients with NF2. To circumvent the embryonic lethality associated with complete *Nf2* gene inactivation, Giovannini and colleagues developed conditional *Nf2* mice. Loss of merlin expression in Schwann cell precursors or meningeal cells results in the formation of schwannomas and meningiomas, respectively [Giovannini et al., 2000; Kalamarides et al., 2002]. Interestingly, unlike *Nf1* mouse models, there does not appear to be a requirement for *Nf2* heterozygosity in the tumor environment, suggesting that stromal cells may have a limited role in the pathogenesis of NF2-associated tumors.

TUBEROUS SCLEROSIS COMPLEX

TSC is an autosomal dominant disorder which affects approximately 1 in 7,000 individuals [Gomez et al., 1999]. The main feature of the disorder is the formation of hamartomas in multiple organs, including the kidneys, lung, skin, heart, and brain. TSC causes severe neurological disorders including mental retardation, epilepsy, and autism. In the central nervous system, individuals with TSC develop subependymal nodules, subependymal giant cell astrocytomas (SEGAs), and cortical tubers [Crino et al., 2006]. Cortical tubers are hamartomas containing significant numbers of abnormal glia and enlarged neurons. The number and size of the tubers has been suggested to correlate with TSC disease severity [Kwiatkowski and Manning, 2005]. SEGAs are glial neoplasms that typically form within the lateral ventricles and may lead to neurologic symptoms as a result of obstruction of cerebrospinal fluid flow.

The *TSC* Genes

Familial linkage studies resulted in the identification of two distinct TSC-associated loci on chromosomes 9 and 16 (9q34 and 16p13), which encode the *TSC1* and *TSC2* genes, respectively [European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al., 1997]. Functional loss of the *TSC1* or *TSC2* genes leads to identical clinical manifestations. Similar to NF1 and NF2, which require biallelic inactivation for tumor development, formation of TSC-associated tumors also requires inactivation of both alleles of either the *TSC1* or *TSC2* gene.

The *TSC1* gene encompasses 55 kb of genomic DNA and encodes for a mRNA of approximately 8.6 kb, while the *TSC2* gene encompasses 40 kb of genomic DNA and encodes for a transcript of approximately 5.5 kb. Hamartin, the *TSC1* gene product, is an 1,100 amino acid, 140 kDa cytoplasmic protein whereas the *TSC2* gene product, tuberin, is an 1,800 amino acid, 250 kDa molecule [Crino et al., 2006]. Tuberin, like neurofibromin, contains a small domain with GTPase activating protein (GAP) homology (see below). Hamartin is not homologous to any known protein, but contains several coiled-coil domains that mediate interactions with other intracellular proteins, including tuberin. In this fashion, hamartin, and tuberin physically interact to produce a single signaling complex. Loss of either hamartin or tuberin expression leads to a failure to form the tuberin–hamartin complex and results in abnormal growth regulation.

Tuberin/Hamartin Function

Since tuberin contains a GAP domain, the role of tuberin in GTPase regulation was studied first. Numerous studies have demonstrated that tuberin inactivates the Ras-like GTPase, Rheb (Ras homolog enriched in brain), such that in the absence of a functional tuberin–hamartin complex, Rheb-GTP levels are increased [Garami et al., 2003; Inoki et al., 2003]. Since Rheb can directly activate mTOR signaling, the role of the *TSC* proteins in mTOR signaling has been intensively investigated. Loss of either tuberin or hamartin results in increased mTOR pathway activation in multiple different cell types. Furthermore, inhibition of mTOR activity with rapamycin restores normal growth regulation [Kwiatkowski and

Manning, 2005], suggesting that the main function of the tuberin–hamartin complex is to negatively regulate mTOR signaling.

Studies in *Drosophila* demonstrated that hamartin and tuberin function downstream of RTKs [Potter et al., 2001; Tapon et al., 2001]. Activation of RTK signaling results in increased PI3-K activity and leads to Akt-mediated phosphorylation of tuberin (Fig. 3). Phosphorylation of tuberin disrupts tuberin–hamartin binding and releases Rheb from tuberin-mediated inactivation. In this fashion, increased RTK signaling abrogates the tuberin–hamartin complex function and results in increased Rheb and mTOR pathway activity [Tee et al., 2003].

TSC Mouse Models

Similar to *Nf1* and *Nf2*, conventional *Tsc* knockout mice die during mid-embryogenesis [Kobayashi et al., 1999, 2001]. Mice heterozygous for *Tsc1* and *Tsc2* are viable, but do not develop the nervous system abnormalities seen in children with TSC. *Tsc1* conditional knockout mice have been developed to facilitate studies on tumorigenesis in the brain. While conditional inactivation of the *Tsc1* gene in glial cells results in abnormal hippocampal neuronal organization and epilepsy [Uhlmann et al., 2002; Wong et al., 2003], these mice do not develop subependymal giant cell astrocytomas. Future genetically engineered mice are currently under development in multiple laboratories that might more fully recapitulate the central nervous system tumor phenotypes observed in patients with TSC.

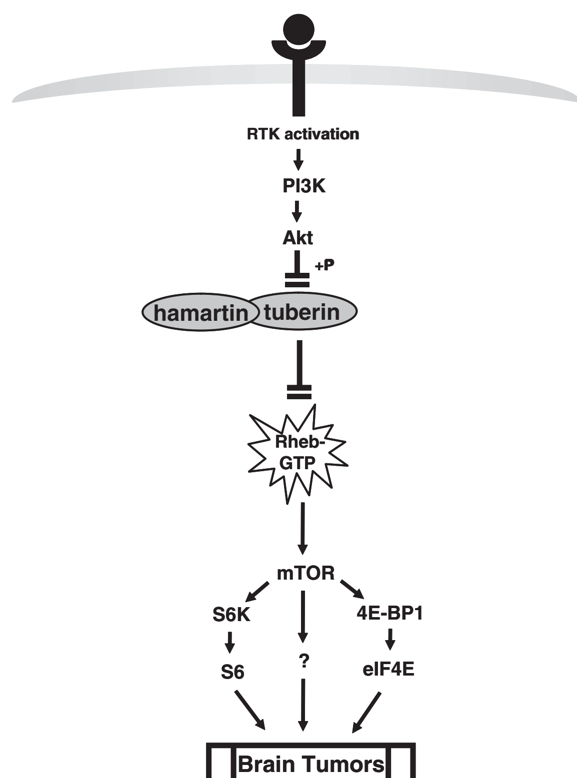

Fig. 3. Hamartin- and tuberin-regulated signaling in brain tumors. RTK activation results in increased PI3K and Akt activity and Akt-mediated tuberin phosphorylation. Tuberin phosphorylation disrupts the tuberin–hamartin complex and leads to increased Rheb activity as a result of the loss of tuberin Rheb GAP function. Rheb then stimulates mTOR activity and leads to increased mTOR pathway signaling through eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) as well as the ribosomal S6 kinase (S6K). Other mTOR downstream signaling intermediates may also contribute to cell growth.

FROM BENCH TO BEDSIDE

Identifying the critical intracellular signaling pathways that promote brain tumor formation and progression provides new opportunities to develop refined therapies for these deadly human cancers. Over the past decade, new treatments for brain tumors have been discovered based on insights derived from studying inherited cancer syndromes. In particular, the finding that loss of function mutations in the *NF1* and *TSC* genes result in increased mTOR signaling has led to the development of clinical trials targeting mTOR hyperactivation. The use of rapamycin (and rapamycin analogs) in preclinical studies as well as early clinical studies in humans with NF1 and TSC have yielded promising results [Franz et al., 2006]. In addition, mTOR inhibitors have also been employed to treat recurrent high-grade gliomas arising in the general population [Galanis et al., 2005]. Future applications that target other signaling pathways de-regulated in these and other inherited nervous system cancer syndromes may similarly result in the development of therapies that alone or in combination are effective at inhibiting brain tumor growth.

ACKNOWLEDGMENTS

We apologize to those investigators whose work we did not cite due to space limitations.

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