Cancer Cell
Article

Proteasomal and Genetic Inactivation of the NF1 Tumor Suppressor in Gliomagenesis

Lauren T. McGillicuddy,[1,2,3] Jody A. Fromm,[1,2,3] Pablo E. Hollstein,[1,2,3] Sara Kubek,[4] Rameen Beroukhim,[2,3,5,6] Thomas De Raedt,[1,2,3,7] Bryan W. Johnson,[1,2,3,7] Sybil M.G. Williams,[1,2,3] Phioanh Nghiemphu,[8] Linda M. Liau,[9] Tim F. Cloughesy,[8] Paul S. Mischel,[10] Annabel Parret,[11] Jeanette Seiler,[11] Gerd Moldenhauer,[12] Klaus Scheffzek,[11] Anat O. Stemmer-Rachamimov,[3,13] Charles L. Sawyers,[14] Cameron Brennan,[15,16] Ludwine Messiaen,[17] Ingo K. Mellinghoff,[4,14,18] and Karen Cichowski[1,2,3,7,*]

[1]Genetics Division
[2]Department of Medicine
Brigham and Women's Hospital, Boston, MA 02115, USA
[3]Harvard Medical School, Boston, MA 02115, USA
[4]Department of Pharmacology, Weill Cornell Medical College, Cornell University, New York, NY 10021, USA
[5]Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA 02142, USA
[6]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA
[7]Ludwig Center, Dana-Farber/Harvard Cancer Center, Boston, MA 02115, USA
[8]Department of Neurology
[9]Department of Neurosurgery
[10]Department of Pathology and Laboratory Medicine
David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA
[11]Structural and Computational Biology and Developmental Biology Units, European Laboratory of Molecular Biology (EMBL), Meyerhofstrasse 1, D-69117 Heidelberg, Germany
[12]Translational Immunology Unit, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
[13]Pathology Department, Massachusetts General Hospital, Boston, MA 02114, USA
[14]Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
[15]Department of Neurosurgery, Weill Cornell Medical College, Cornell University, New York, NY 10021, USA
[16]Department of Neurosurgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
[17]Medical Genomics Laboratory, Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35242, USA
[18]Department of Neurology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
*Correspondence: kcichowski@rics.bwh.harvard.edu
DOI 10.1016/j.ccr.2009.05.009

SUMMARY

Loss-of-function mutations in the *NF1* tumor suppressor result in deregulated Ras signaling and drive tumorigenesis in the familial cancer syndrome neurofibromatosis type I. However, the extent to which *NF1* inactivation promotes sporadic tumorigenesis is unknown. Here we report that NF1 is inactivated in sporadic gliomas via two mechanisms: excessive proteasomal degradation and genetic loss. NF1 protein destabilization is triggered by the hyperactivation of protein kinase C (PKC) and confers sensitivity to PKC inhibitors. However, complete genetic loss, which only occurs when *p53* is inactivated, mediates sensitivity to mTOR inhibitors. These studies reveal an expanding role for NF1 inactivation in sporadic gliomagenesis and illustrate how different mechanisms of inactivation are utilized in genetically distinct tumors, which consequently impacts therapeutic sensitivity.

SIGNIFICANCE

Tumor suppressors are often mutated in human cancer; however, the excessive proteasomal destruction of tumor suppressor proteins also promotes tumorigenesis. Here we show that the NF1 protein is destabilized in sporadic glioblastomas (GBMs) as a consequence of the hyperactivation of PKC. Notably, this destabilization confers sensitivity to PKC inhibitors. In contrast, a separate subset of GBMs that possess *NF1* mutations are insensitive to PKC inhibitors but are sensitive to mTOR inhibitors. These findings reveal a broad role for NF1 inactivation in gliomagenesis and illustrate how different mechanisms of inactivation are utilized in the same tumor type. Moreover, they highlight the importance of elucidating the molecular mechanisms that underlie tumorigenesis, as such knowledge may be essential for developing personalized therapies.

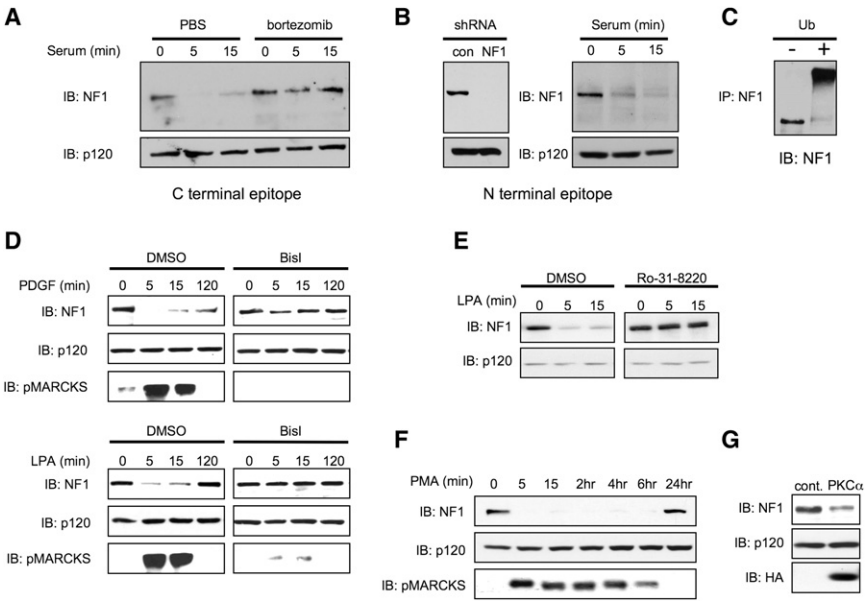


Figure 1. PKC Regulates the Proteasomal Degradation of Neurofibromin

(A) Serum-starved NIH 3T3 cells were pretreated with 1 μM bortezomib or vehicle and stimulated with serum for increasing amounts of time. Immunoblots for neurofibromin and p120 (as a control) are shown. The neurofibromin antibody used was UP69, and recognizes the last eight amino acids of neurofibromin.

(B) Serum-starved NIH 3T3 cells were stimulated with serum for increasing amounts of time. A second antibody (NF1-5.16) that recognizes an epitope within the N terminus of neurofibromin was used. A lentiviral NF1 shRNA construct was used to demonstrate specificity (left).

(C) An in vitro ubiquitination assay was performed as described using immunoprecipitated neurofibromin as a substrate (Cichowski et al., 2003).

(D) Serum-starved NIH 3T3 cells were pretreated with Bis I or vehicle (DMSO) and stimulated with PDGF or LPA for increasing amounts of time. Immunoblots for neurofibromin (UP69) and pMARCKS, a PKC substrate, are shown.

(E) Serum-starved NIH 3T3 cells were pretreated with Ro-31-8220 or vehicle (DMSO) and stimulated with LPA for increasing amounts of time.

(F) Serum-starved NIH 3T3 cells were acutely treated with PMA for increasing amounts of time.

(G) NIH 3T3 cells were infected with a control retrovirus or a retrovirus expressing a constitutively activated PKCα allele tagged with an HA epitope (PKCα). Relative neurofibromin levels were assessed by immunoblot. Ectopic PKC expression was confirmed by an HA immunoblot.

INTRODUCTION

The Ras pathway is commonly deregulated in human cancer (Downward, 2003). Genetic alterations can occur in *Ras* genes, upstream regulators, or downstream effectors. One such regulator is the *NF1* tumor suppressor, which encodes a Ras-GTPase activating protein (RasGAP), referred to as neurofibromin (Martin et al., 1990; Xu et al., 1990). Accordingly, *NF1* inactivation triggers the aberrant activation of the Ras pathway, and loss-of-function mutations in *NF1* underlie the familial cancer syndrome neurofibromatosis type I (NF1) (Basu et al., 1992; DeClue et al., 1992).

NF1 patients develop a diverse set of tumor types, including benign neurofibromas, malignant sarcomas, gliomas, pheochromocytomas, gastrointestinal stromal tumors, and myeloid leukemia (Riccardi, 1992). However, whereas neurofibromin critically regulates Ras in many tissues, it is unknown whether *NF1* inactivation plays a major role in the development of sporadic tumors. *NF1* mutations and genomic alterations have been reported in isolated tumor samples and cell lines of various origins (Andersen et al., 1993; Li et al., 1992; The et al., 1993; Thiel et al., 1995). More recently, heterozygous mutations in *NF1* were observed in larger panels of glioblastomas (Parsons et al., 2008; TCGA Research Network, 2008); however, homozygous mutations were found to be relatively rare (TCGA Research Network, 2008). Thus, our understanding of how *NF1* inactivation may contribute to sporadic tumor development still needs to be refined.

Tumor suppressors are often mutated in human cancer; however, precocious proteasomal degradation of several prominent tumor suppressors, including p53, PTEN, and p27, also contributes to their functional inactivation (Honda et al., 1997; Pagano et al., 1995; Wang et al., 2007). Notably, neurofibromin has been shown to be a direct target of the ubiquitin-proteasome pathway, and its regulated inactivation promotes proliferation in response to growth factors (Cichowski et al., 2003). Therefore, in this study, we sought to identify the signals that trigger neurofibromin degradation and determine whether its destabilization might also play a role in sporadic tumorigenesis.

RESULTS

PKC Activation Is Necessary and Sufficient to Induce Neurofibromin Degradation

We have shown that serum and growth factors trigger the rapid ubiquitination and proteasomal destruction of neurofibromin in many cell types (Cichowski et al., 2003). Accordingly, the specific proteasome inhibitor bortezomib blocks the acute degradation of neurofibromin in NIH 3T3 cells (Figure 1A). Antibodies recognizing distant regions of the protein confirm that neurofibromin is completely degraded and that the loss of immunoreactivity is not due to the masking of a specific epitope after stimulation (Figures 1A and 1B). Notably, neurofibromin is a direct and sensitive target of the ubiquitination machinery, which can be visualized by the accumulation of high-mobility ubiquitinated species both in vitro and in vivo (Figures 1C and 2A; Cichowski et al., 2003). These observations highlight the dynamic and exquisite regulation of neurofibromin by the proteasome.

Previous studies suggest that neurofibromin degradation is necessary for maximal Ras activation triggered by growth factors and that its reexpression is required for the appropriate attenuation of this signal (Cichowski et al., 2003). However, whereas growth factors trigger neurofibromin destruction, nothing is known about the specific signals that mediate this effect. To interrogate the role of individual signaling cascades in this process, NIH 3T3 cells were exposed to pharmacological inhibitors of

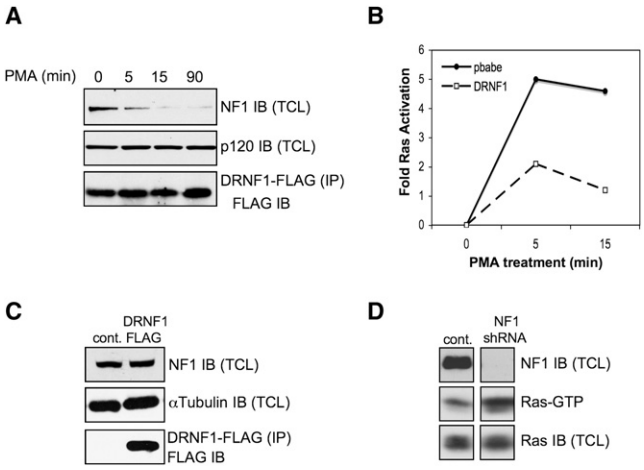


Figure 2. PKC Regulates Ras Activation via Neurofibromin Degradation

(A) Fibroblasts were infected with DRNF1-FLAG. Cells were cultured in 0.25% serum for 24 hr and treated with PMA for increasing amounts of time. Levels of endogenous neurofibromin and p120RasGAP were assessed by immunoblotting (IB) protein from total cell lysates (TCL). DRNF1-FLAG was detected by immunoprecipitating (IP) this fragment from the same lysates, followed by a FLAG immunoblot.

(B) Relative levels of Ras-GTP were assessed in cells expressing DRNF1-FLAG or a control retrovirus (pbabe) in response to PMA as described in (A) by performing a Ras pull-down assay. Ras-GTP levels were quantified as described in Experimental Procedures.

(C) Relative levels of endogenous neurofibromin were assessed in cells expressing DRNF1-FLAG or the pbabe control retrovirus (cont).

(D) Relative levels of Ras activation were assessed in cells infected with a control lentivirus (cont) or a lentivirus expressing an shRNA directed against NF1, in low serum. Ras immunoblots from Ras pull-down assays are shown (Ras-GTP), along with a Ras immunoblot from total cell lysates (Ras IB [TCL]) as loading controls. An NF1 immunoblot from total cell lysates (NF1 IB [TCL]) was performed to confirm knockdown.

PI3K, MEK, and PKC. Only the PKC inhibitor bisindolylmaleimide I (Bis I) blocked neurofibromin degradation, and did so in response to multiple growth factors (Figure 1D; see Figure S1A available online). Bis I efficiently inhibited PKC activity, as it suppressed the phosphorylation of the MARCKS protein, a well-characterized PKC substrate. Two additional PKC inhibitors, Ro-31-8220 and bisindolylmaleimide II, had similar effects (Figure 1E; Figure S1B). Conversely, activation of PKC via phorbol 12-myristate 13-acetate (PMA) rapidly induced neurofibromin degradation (Figure 1F), and did so in multiple cell types (Figure S1C). Neurofibromin levels were restored by 24 hr, at which time PKC was downregulated by a negative feedback loop (Liu and Heckman, 1998). Finally, expression of a constitutively active form of PKCα also decreased neurofibromin protein levels (Figure 1G). Thus, these data indicate that PKC activation is both necessary and sufficient to promote neurofibromin degradation, thus identifying the critical signal that mediates its destruction.

PKC Promotes Ras Activation through the Destabilization of Neurofibromin

PKC is known to promote Ras activation, although the mechanism by which this occurs has not been elucidated (Downward et al., 1990; Marais et al., 1998). To determine whether neurofibromin degradation mediates this effect, we utilized a *d*egradation-*r*esistant fragment of *n*eurofibromin (DRNF1) that is resistant to ubiquitin-mediated degradation in vitro (Cichowski et al., 2003). Whereas endogenous neurofibromin was degraded by PMA in vivo, DRNF1 was not (Figure 2A). Importantly, DRNF1 attenuated Ras activation, indicating that neurofibromin must be degraded to permit normal levels of Ras activity (Figure 2B). In addition, a mutation that impaired RasGAP catalytic activity was impaired in its ability to suppress Ras-GTP levels (Figure S2). Conversely, inactivation of neurofibromin via RNAi was sufficient to activate Ras in the absence of PMA under these conditions (Figure 2D). Taken together, these results suggest that PKC-driven degradation of neurofibromin is an important Ras-regulatory event, dictating both the amplitude and duration of the Ras signal.

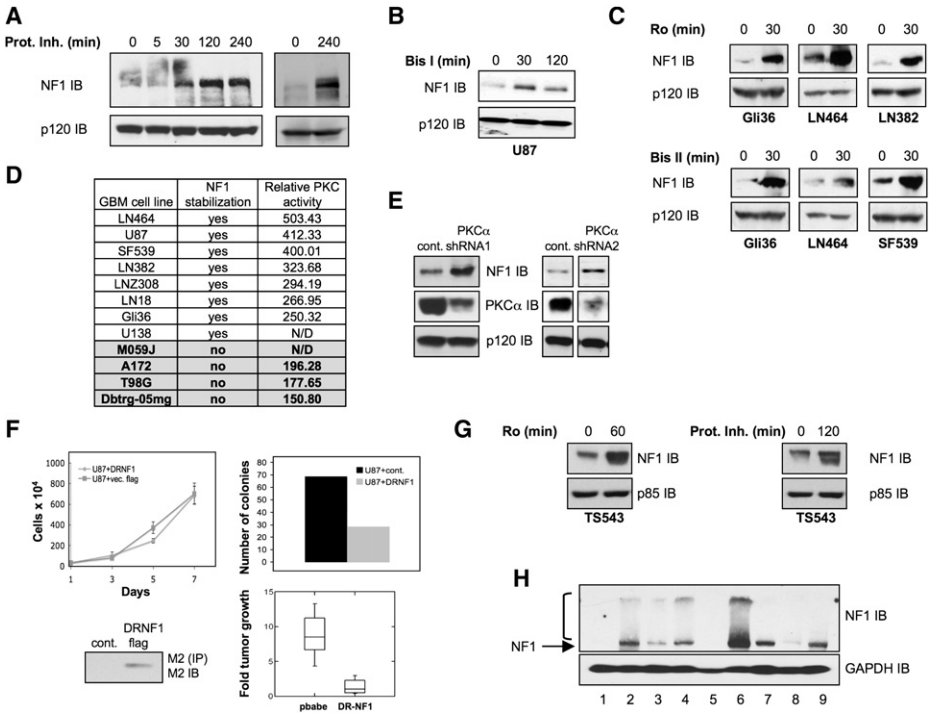
Neurofibromin Is Destabilized by PKC and the Proteasome in Glioblastoma Tumor Cell Lines

Having established the biochemical architecture of the growth factor receptor-PKC-neurofibromin-Ras signaling pathway, we next asked whether its excessive activation might contribute more generally to tumorigenesis. We investigated gliomas because (1) the aberrant activation of PKC has been implicated in gliomagenesis (Mackay and Twelves, 2007), (2) PKC inhibitors exhibit potent effects in preclinical studies in this tumor type and are currently in clinical development (da Rocha et al., 2002; Mackay and Twelves, 2007), and (3) NF1 patients are predisposed to developing gliomas, indicating that *NF1* can function as a tumor suppressor in astrocytic tumors (Riccardi, 1992).

We first investigated whether neurofibromin was constitutively destabilized by the proteasome in human glioblastoma (GBM) cell lines. The GBM-derived U87 cell line has been shown to possess aberrantly high levels of PKCα activity and is sensitive to PKC inhibitors (Yazaki et al., 1996) (Figure 3A, left). Notably, we found that proteasome inhibitors dramatically and rapidly stabilized neurofibromin protein (Figure 3A, left). As with most proteasomal substrates, these inhibitors promoted the initial appearance of higher-mobility polyubiquitinated forms of neurofibromin, manifested as an immunoreactive smear, followed by a dramatic accumulation of unmodified protein, which increased by 5.4-fold (Figure 3A, right). Neurofibromin was rapidly stabilized by multiple PKC inhibitors (Bis I, Ro-31-8220, and Bis II) and stabilization was detected in 8 out of 12 GBM cell lines tested (Figures 3B–3D). Notably, the 3 cell lines that were insensitive to the effects of PKC inhibitors exhibited the lowest levels of PKC activity (Figure 3D; Figure S3). In addition, PKCα shRNA constructs promoted the accumulation of neurofibromin in U87 cells (Figure 3E). Thus, studies utilizing proteasome inhibitors, multiple PKC inhibitors, and PKC shRNA constructs indicate that neurofibromin is destabilized via PKC in a significant subset of human GBM cell lines.

Neurofibromin Destabilization Is Essential for Transformation and Tumorigenesis

We next ascertained the biological consequences of preventing neurofibromin degradation. The nondegradable neurofibromin fragment (DRNF1) did not significantly affect the proliferation of GBM cells (Figure 3F, left), although it inhibited colony growth



GBM cell line	NF1 stabilization	Relative PKC activity
LN464	yes	503.43
U87	yes	412.33
SF539	yes	400.01
LN382	yes	323.68
LNZ308	yes	294.19
LN18	yes	266.95
Gli36	yes	250.32
U138	yes	N/D
M059J	**no**	**N/D**
A172	**no**	**196.28**
T98G	**no**	**177.65**
Dbtrg-05mg	**no**	**150.80**

Figure 3. PKC-Mediated Neurofibromin Instability in Glioblastomas

(A) U87 cells were treated with proteasome inhibitors (1 μM bortezomib and 10 μM MG132) for increasing amounts of time, and neurofibromin levels were assessed by immunoblot. Left: lysates were prepared in the presence of deubiquitinase inhibitor (2 mM NEM) to preserve ubiquitinated species in vitro. Right: lysates were prepared in the absence of NEM to allow quantification using ImageJ software.

(B) U87 cells were exposed to the PKC inhibitor Bis I for increasing amounts of time.

(C) A panel of GBM cell lines was exposed to two additional PKC inhibitors (Ro-31-8220 or Bis II), and neurofibromin levels were assessed by immunoblot.

(D) Table summarizing data acquired using multiple PKC inhibitors (Bis I, Ro-31-8220, Bis II). The cell lines listed in this table were subjected to the same analysis described in (B) and (C). NF1 stabilization observed means that multiple PKC inhibitors promoted an accumulation of neurofibromin. Relative PKC activity was assessed by quantifying the phosphorylation of proteins recognized by PKC-substrate antibodies as previously performed (Ikenoue et al., 2008).

(E) U87 cells were infected with a control lentivirus or lentiviruses expressing one of two shRNA sequences directed against PKCα. PKCα knockdown was confirmed by immunoblot (IB). Neurofibromin levels and p120 levels are shown.

(F) Bottom left: U87 cells were infected with a pbabe retrovirus or a retrovirus that expresses the DRNF1 protein. Expression was confirmed by immunoblot (M2-IP, M2-IB). Top left: proliferation curves; colony number was quantified as described. Error bars represent the standard deviation of cells counted in triplicate wells. Top right and bottom right: box plot representing the fold tumor volume (day 21 versus day 0 of tumor measurement) of tumors formed after subcutaneous injection of U87 cells either expressing the DRNF1-FLAG protein or control retrovirus. Error bars represent the standard deviation of tumor volume (n = 6 for each condition).

(G) Primary GBM neurosphere cultures (TS543) were cultured as described in Experimental Procedures and treated with either the PKC inhibitor Ro-31-8220 (Ro) or the proteasome inhibitors MG132 and bortezomib (Prot. Inh.) as described in (A) for the indicated amounts of time. Neurofibromin levels were assessed by immunoblot (Bethyl). p85 levels are shown as a loading control.

(H) Neurofibromin expression was assessed by immunoblot in tissue from untreated grade IV human tumors. Lysates were prepared in the presence of the deubiquitinase inhibitor NEM. An immunoreactive smear consistent with ubiquitinated protein is shown. GAPDH serves as loading control. Although lane 5 is slightly underloaded, little to no neurofibromin was detected even under the darkest exposures.

in soft agar (Figure 3F, top right) and dramatically suppressed the ability of such cells to form xenograft tumors (Figure 3F, bottom right). These results indicate that neurofibromin destabilization is essential for the transformed and tumorigenic properties of these GBM-derived cells in vitro and in vivo.

Neurofibromin Is Destabilized by the Proteasome in Primary GBM Cultures and Tumor Tissue

To extend these findings to primary tumor cells, we utilized neurosphere cultures derived from a GBM that harbored an amplification of the *PDGFRA* gene (C.B. and I.K.M., unpublished observations), a well-established activator of PKC. Notably, both PKC and proteasome inhibitors resulted in a rapid stabilization of neurofibromin (Figure 3G). We next looked for evidence of proteasomal degradation of neurofibromin in primary human tumor tissue. Neurofibromin immunoblots were performed using tissue from nine grade IV tumors that were resected prior to treatment. Notably, no neurofibromin was detected in two samples (Figure 3H, lanes 1 and 5), and minimal levels were detected in an additional two tumors (lanes 3 and 8). Moreover, in four out of nine samples a high-mobility neurofibromin smear was observed, consistent with the presence of polyubiquitinated species. Thus, these observations support our studies in GBM cell lines and primary cultures and are consistent with the model that neurofibromin is actively ubiquitinated and degraded in a significant subset of human GBMs. These findings differ from

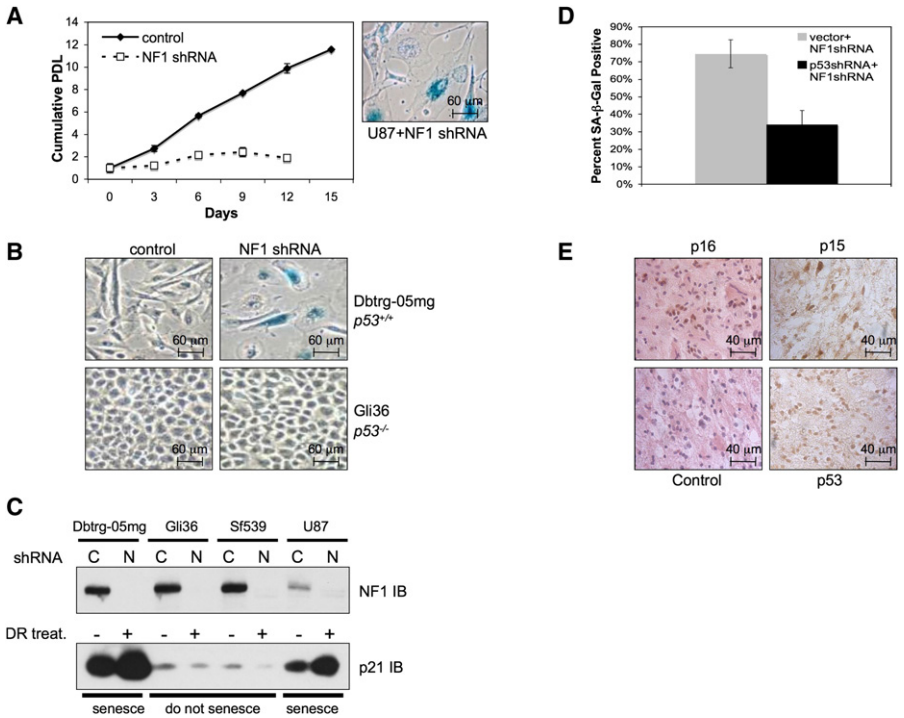


Figure 4. Complete NF1 Inactivation Promotes Senescence In Vitro and In Vivo in Cells with an Intact p53 Pathway

(A) U87 cells were infected with a control or a lentivirus expressing an shRNA directed against NF1. Neurofibromin knockdown was confirmed by immunoblot in (C). Proliferation curves were generated as described. Senescence-associated β-galactosidase (SA-β-gal) activity was assessed as described previously (Dimri et al., 1995).

(B) Dbtrg-05mg or Gli36 cells were infected with a control or a lentivirus expressing an shRNA directed against NF1. Neurofibromin knockdown was confirmed by immunoblot in (C). SA-β-gal assays were performed and phase photographs are shown.

(C) GBM cell lines were infected with a control or a lentivirus expressing an shRNA directed against NF1. Neurofibromin knockdown was confirmed by immunoblot. p53 activity was assessed by examining the levels of p21 in response to doxorubicin (DR) treatment. Cells that became senescent in response to NF1 inactivation are noted (bottom).

(D) Dbtrg-05mg cells were infected with a control (vector) or a lentivirus expressing an shRNA directed against p53. p53 knockdown was confirmed by immunoblot (data not shown). Cells were then infected with a control lentivirus (control) or a lentivirus expressing an shRNA directed against NF1. SA-β-gal assays were performed and the percentage of SA-β-gal-expressing cells was quantified as described. Error bars represent the standard deviation of SA-β-gal-positive cells counted in triplicate wells.

(E) Paraffin sections of pilocytic astrocytomas from NF1 patients were stained with a control antibody (control, counterstained with H&E), a p53 antibody (no counterstain), a p16 antibody (counterstained with H&E), or a p15 antibody (no counterstain).

the genetic observation that only 2.9% of sporadic glioblastomas possess homozygous mutations in *NF1* (TCGA Research Network, 2008), and provide a mechanistic explanation for the more frequent loss of expression observed by western analysis.

Complete *NF1* Loss Triggers Cellular Senescence in GBM Cells with an Intact p53 Pathway

In the course of our studies, we assessed the effects of RNAi-mediated neurofibromin loss in GBM cell lines. Unexpectedly, ablation of neurofibromin in U87 and Dbtrg-05mg cells triggered cellular senescence, as demonstrated by a potent growth arrest, a large flattened morphology, and the detection of senescence-associated β-galactosidase (SA-β-gal) activity (Figures 4A and 4B). In contrast, senescence was not observed in Gli36, SF539, U251, T98G, M059J, and U138 cells (Figure 4B and data not shown). Although we have previously shown that *NF1* loss triggers cellular senescence of normal human diploid fibroblasts (Courtois-Cox et al., 2006), it was nevertheless surprising that the inactivation of *NF1* triggered such a response in human tumor cell lines that possess many additional genetic alterations. Notably, however, all senescing cell lines expressed wild-type *p53* and retained a functional p53 pathway, as illustrated by their induction of p21 (*CDKN1A*) in response to doxorubicin (Figure 4C). In contrast, all the GBM-derived cell lines that did not senesce in response to *NF1* inactivation harbored *p53* mutations and/or a defective p53 response (Figure 4C; Ikediobi et al., 2006; Van Meir et al., 1994). Moreover, when p53 was inactivated by shRNA prior to the ablation of *NF1* expression, the senescence response was mitigated (Figure 4D and data not shown).

Astrocytomas from NF1 Patients Express Markers of Senescence

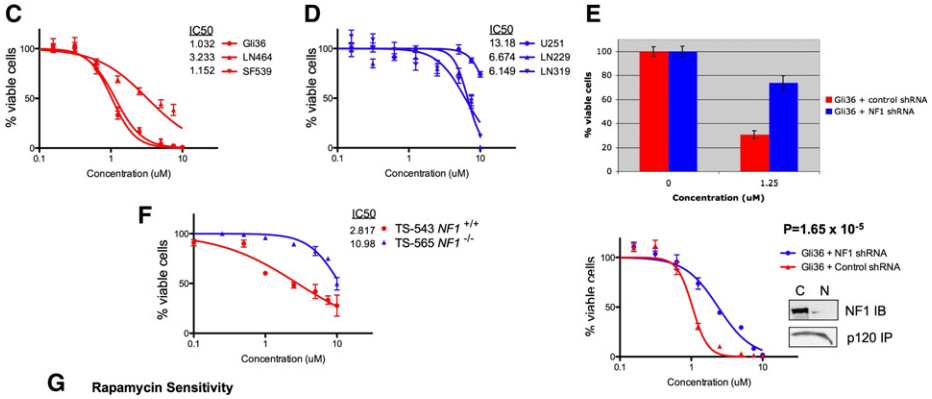
Oncogene-induced senescence is thought to restrain tumorigenesis by preventing progression of benign tumors to malignancy (Narita and Lowe, 2005). Notably, whereas NF1 patients are predisposed to developing gliomas, the majority are benign pilocytic astrocytomas that rarely, if ever, progress (Rodriguez et al., 2008). Moreover, many tumors spontaneously stop growing and

A

GBM cell line	*NF1* mutation	consequence
U251	c.2033dupC no WT copy present	premature stop codon
LN319	c.274_276delAAAinsT no WT copy present	premature stop codon
LN229	total gene deletion	no expressed protein

B

primary GBM	*NF1* mutation	consequence
Glioma 217	c.6789_6792delTTAC no WT copy present	premature stop codon
Glioma 311	c.4103delT no WT copy present	premature stop codon
Glioma 211	c.896_897dupTT c.3496G>T	premature stop codon exon skipping



G Rapamycin Sensitivity

GBM cell line	*NF1*	IC50 [nM]	IC50 [nM] after rescue
U251	-/-	1.21	90.0
LN319	-/-	0.84	>>200
LN229	-/-	8.04	113.4

Figure 5. *NF1* Genomic Alterations and Drug Sensitivity in Glioblastomas

(A) Table detailing detected mutations in *NF1* in GBM cell lines.

(B) Table detailing detected mutations in *NF1* in primary GBM tumor samples.

(C) IC_{50} curves were generated using three $NF1^{+/+}$ (red) GBM cell lines treated with Ro-31-8220 or vehicle for 18 hr before assessing viability by a Cell Titer-Glo Luminescent Cell viability assay. Error bars represent the standard deviation from eight replicates.

(D) IC_{50} curves were generated using three $NF1^{-/-}$ (blue) GBM cell lines treated with Ro-31-8220 or vehicle for 18 hr before assessing viability by a Cell Titer-Glo Luminescent Cell viability assay. Error bars represent the standard deviation from eight replicates.

(E) Gli36 cells were infected with a control (C, red) or a lentivirus expressing an shRNA directed against NF1 (N, blue) and were treated with Ro-31-8220 or vehicle for 18 hr before assessing the percentage of viable cells. An immunoblot confirming knockdown is shown. The top panel illustrates the dramatic difference in viability observed at 1.25 μM Ro-31-8220. Error bars represent the standard deviation from eight replicates.

(F) Primary GBM cultures that are $NF1^{+/+}$ (red, TS543) or $NF1^{-/-}$ (blue, TS565) were treated with Ro-31-8220 or vehicle for 24 hr before assessing the percentage of viable cells as described. Error bars represent the standard deviation from cells plated in triplicate.

(G) $NF1^{-/-}$ GBM cell lines were infected with a control retrovirus or a retrovirus expressing the DRNF1 protein (rescued). Cells were selected with puromycin prior to seeding for drug sensitivity assay. IC_{50} values were calculated as described.

a subset regress. Therefore, we hypothesized that loss of *NF1* in these astrocytic lesions might be restricting tumor development by triggering a senescence response. Two pilocytic astrocytomas from NF1 patients were obtained. $p15^{INK4b}$, a reported marker of oncogene-induced senescence, and $p16^{INK4a}$, which is also commonly detected in senescent tissue, were both highly expressed in these tumors (Figure 4E and data not shown) (Collado et al., 2005; Michaloglou et al., 2005). Tumors also express p53, suggesting that this pathway is activated in these lesions. SA-β-gal activity could not be assessed in these archived samples; nevertheless, the expression of p15, p16, and p53 support the hypothesis that these tumors are restrained by oncogene-induced senescence. Interestingly, in previous studies, Parada and colleagues had shown that inactivating mutations in *Nf1* and *p53* cooperate to promote the development of GBMs in mice, but only when *p53* inactivation precedes, or is coincident with, *Nf1* loss (Zhu et al., 2005). Our findings suggest a potential mechanistic explanation for these observations and provide insight into the constraints governing glioma evolution in NF1 patients.

Homozygous *NF1* Mutations Are Present in GBM Cell Lines and in Primary Tumors that Harbor p53 Mutations

The data shown in Figure 3 suggest that excessive PKC activity and subsequent proteasomal degradation is one mechanism by which neurofibromin can be inactivated in GBMs. However, we noted that 3 of 15 GBM cell lines that we were studying did not express detectable levels of neurofibromin protein, even in the presence of PKC and proteasome inhibitors (data not shown). Notably, all three cell lines (U251, LN229, and LN319) harbored *p53*-inactivating mutations (Ikediobi et al., 2006; Tallen et al., 2008; Van Meir et al., 1994). Because p53 inactivation appeared to be permissive for total inactivation of *NF1*, we assessed the status of the *NF1* gene in each cell line. All three cell lines harbored loss-of-function and/or recurrent mutations found in NF1 patients, and no wild-type *NF1* was present (Figure 5A).

In NF1 patients, single point mutations, small insertions, and deletions are known to occur throughout the *NF1* gene (Messiaen et al., 2000). As such, we reasoned that subtle *NF1* mutations might be missed by standard genomic analysis. To examine changes in *NF1* copy number, we examined data

from single-nucleotide polymorphism analysis that was performed on archived tumor tissue from 141 spontaneous human tumors (Beroukhim et al., 2007). None of these tumors exhibited complete loss of the *NF1* locus, consistent with other reported findings (Jensen et al., 1995). However, 23 (16%) exhibited single copy loss at the *NF1* locus (Beroukhim et al., 2007). We then utilized these tissue samples in a directed sequencing effort. Based on our analysis of cell lines, human tumors from NF1 patients, and previous mouse modeling studies, we reasoned that null mutations in *NF1* might only be tolerated in the absence of functional p53. Therefore, we sequenced the *NF1* gene in 14 of the 23 GBM tissue samples that also possessed *p53* mutations (Beroukhim et al., 2007).

Three out of the 14 tumors with p53 alterations (21%) possessed homozygous null *NF1* mutations (Figure 5B). Importantly, all of these mutations were recurrent in NF1 patients and/or resulted in a truncation before or within the catalytic GAP-related domain. In tumor 217, a mutation in exon 37 (c.6789_6792 delTTAC) was detected, resulting in a null *NF1* allele that has been observed in NF1 patients (Pros et al., 2008). Tumor 311 possessed a mutation in exon 23-2 (c4103 delT) resulting in a premature stop codon. In tumor 211, multiplex ligation-dependent probe amplification analysis indicated that both *NF1* alleles were present; however, two independent, inactivating mutations were detected, one in exon 7 (c896-897 dupTT) and the other in exon 20 (c3496G > T), also representing a recurrent patient mutation. Thus, the detection of null *NF1* mutations in 3/15 GBM cell lines and 3/14 primary tumors indicates that genetic inactivation of both *NF1* copies does occur in spontaneous human gliomas. While this manuscript was under consideration, heterozygous alterations of the *NF1* gene (deletions and point mutations) were reported to occur in GBMs in two other studies (Parsons et al., 2008; TCGA Research Network, 2008). However, the latter study also reported that only a small fraction of tumors, 6/203 (2.9%), possessed null mutations in both *NF1* alleles. The data in the present study extend these observations by demonstrating that mutations in *NF1* and *p53* are coincidental in this tumor type and provide a mechanistic explanation for why complete genetic loss of *NF1* necessitates p53 inactivation. The observation that benign pilocytic astrocytomas from NF1 patients express markers of senescence and robust p53 expression provides additional in vivo evidence to support the hypothesis that p53 plays a critical role in restricting the tumorigenic effects of *NF1* inactivation.

The Mechanism by which NF1 Is Inactivated Imparts Differential Sensitivities to Therapeutic Agents

The data described thus far suggest that NF1 may be suppressed either by excessive proteasomal degradation of the NF1 protein triggered by excessive PKC activity or by genetic inactivation of the *NF1* gene. To investigate whether these distinct mechanisms of inactivation might impact the sensitivity to specific therapeutic agents, the effects of PKC inhibitors on tumor cell lines were assessed. Importantly, the GBM cell lines in which we found neurofibromin to be destabilized by PKC were very sensitive to the PKC inhibitor Ro-31-8220 (Figure 5C), whereas cells that genetically lacked *NF1* were relatively insensitive to PKC inhibition (Figure 5D). The difference in IC_{50} values between genotypes was found to be statistically significant as determined by a Mann-Whitney U test ($p = 0.05$). In addition, inactivation of *NF1* via an shRNA-expressing lentivirus significantly decreased the response of normally sensitive cells to PKC inhibitors (extra sum of squares F test, 1.65×10^{-5}) (Figure 5E). A similar differential sensitivity of genetically wild-type versus *NF1* mutant cell lines was observed with a second PKC inhibitor (Figure S4). In addition, U87 cells were specifically sensitive to genetic ablation of PKCα (Figure S5).

The sensitivity to PKC inhibitors was also assessed in the primary GBM culture TS543, in which we found neurofibromin to be destabilized by PKC, in comparison to a second GBM culture (TS565), in which we detected two *NF1* mutations (c2195G > T, c470G > T) and a complete loss of neurofibromin protein expression (Figure S6). Consistent with data from GBM cell lines, the TS543 cultures were exquisitely sensitive to PKC inhibitors, whereas the TS565 cultures were relatively insensitive (extra sum of squares F test, $p = 4.657 \times 10^{-6}$) (Figure 5F). Taken together, these data suggest that PKC may represent a potential therapeutic target in GBMs in which the *NF1* gene is intact and the protein is destabilized.

However, we and others have previously shown that neurofibromin critically regulates the mTOR pathway and that *NF1* inactivation confers sensitivity to mTOR inhibitors in other cell types (Dasgupta et al., 2005; Johannessen et al., 2005). Therefore, in an effort to identify a therapeutic agent that might be effective on GBMs that harbor null genetic mutations in *NF1*, the effect of the mTOR inhibitor rapamycin was assessed. Whereas GBM cells that genetically lack *NF1* exhibited a significantly decreased sensitivity to PKC inhibitors, we found that these cells were exquisitely sensitive to the mTOR (mTORC1) inhibitor rapamycin (Figure 5G). *NF1*-deficient cells were also sensitive to the inactivation of the mTOR pathway via RNAi-mediated suppression of raptor, which is exclusively a component of the mTORC1 complex (Figure S7). Conversely, the reintroduction of a neurofibromin fragment into *NF1* null cells promoted rapamycin resistance, indicating that *NF1* loss was responsible for the observed sensitivity (Figure 5G). Notably, in a recent phase I clinical trial, rapamycin was shown to exhibit antitumor activity in a subset of PTEN-deficient glioblastomas (Cloughesy et al., 2008). These studies suggest that *NF1* mutations may be an additional genetic event that may impact the sensitivity of sporadic glioblastomas to mTOR inhibitors.

DISCUSSION

The *NF1* tumor suppressor is mutated in the familial cancer syndrome neurofibromatosis type I (Cawthon et al., 1990; Wallace et al., 1990). Nevertheless, gross genomic loss of *NF1* does not appear to be a common event in spontaneous human tumors. The studies in this report reveal a critical role for NF1 inactivation in the development of sporadic human gliomas and provide a framework for understanding how proteasomal degradation and complete genetic inactivation of *NF1* contribute to this process (Figure 6).

PKC Regulates Neurofibromin Stability

Prior to this work, neurofibromin was known to be a direct target of the proteasome; however, the precise signals that mediate its destruction had not been identified (Cichowski et al., 2003). In

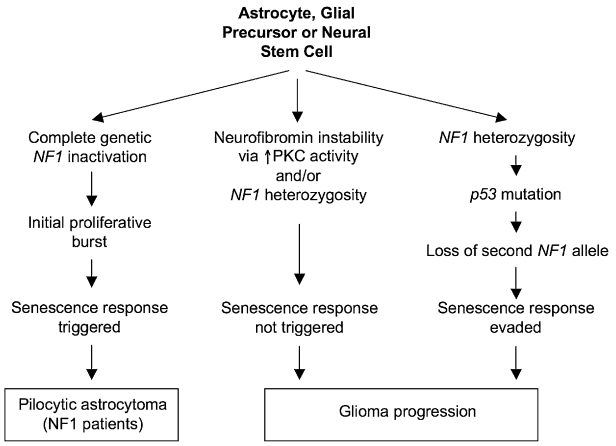


Figure 6. Model Illustrating How Complete Genetic Loss and Proteasomal Degradation of NF1 Functions in Gliomagenesis

We hypothesize that NF1 inactivation participates in gliomagenesis via three distinct mechanisms. Left: because NF1 patients are born with an *NF1* mutation, the majority of tumors that arise are likely to be driven by a second hit mutation in the remaining *NF1* allele. This event would be predicted to result in an initial burst of proliferation and ultimately the development of pilocytic astrocytomas. If the p53 pathway is intact (expected in the majority of cases), tumors undergo senescence and are unable to progress. Middle: however, neurofibromin can also be excessively destabilized by PKC and the proteasome. Heterozygous mutations in *NF1* may further decrease protein levels in some tumors. Importantly, neurofibromin instability promotes tumorigenesis but is not sufficient to trigger a senescence response. Data generated from GBM cell lines and primary cultures further suggest that tumors with an intact *NF1* gene, but destabilized protein, may be sensitive to PKC inhibitors. Right: heterozygous deletions or mutations in *NF1* also occur. We hypothesize that mutations in the *p53* pathway would be permissive for a second hit mutation in *NF1*. Such tumors would effectively evade the senescence response. Our preclinical studies further suggest that *NF1*-deficient GBMs may be sensitive to mTOR inhibitors.

this study, we show that activation of PKC is both necessary and sufficient for neurofibromin degradation. This finding has important implications for normal signal transduction pathways as well as tumorigenesis. Specifically, these studies demonstrate that the NF1 tumor suppressor is a critical mediator of Ras activation downstream of PKC and experimentally illustrate that robust Ras activation requires the coordinated regulation of GEFs (guanine nucleotide exchange factors) and GAPs. Notably, the intensity of the Ras signal is tightly regulated and is critical for specifying biological responses to growth factors (Marshall, 1995). The observation that numerous receptors can trigger neurofibromin degradation suggests that in vivo the extent of neurofibromin degradation and consequential Ras activation may be dictated by the intensity of combined GFR/PKC signals.

Deregulated Signal Transduction Pathways in Gliomagenesis

We have also demonstrated that the PKC-neurofibromin signaling axis is aberrantly activated in spontaneous gliomas and that neurofibromin destabilization is required for the tumorigenic properties of tumor cells in vitro and in vivo. Notably, numerous GFRs have been implicated in glioma development (Furnari et al., 2007). Importantly, the majority, if not all of these receptors, activate PKC. PKC activity has also been independently shown to be hyperactivated in gliomas, presumably due to the aberrant activation of these receptors (da Rocha et al., 2002). However, it remains to be determined whether genetic alterations in PKC itself may also occur in some tumors (Mackay and Twelves, 2007). Interestingly, *Ras* mutations do not occur in gliomas; however, Ras-GTP levels have been shown to be elevated (Feldkamp et al., 1999; Furnari et al., 2007). Our data suggest that the excessive destruction of neurofibromin is one mechanism that contributes to pathogenic levels of Ras activation in this tumor type.

Therapeutic Implications

These findings have important clinical implications. Because multiple GFRs have been shown to be concomitantly activated in a single glioma, it has been suggested that combination therapies designed to target multiple receptors may be required for effective treatment (Stommel et al., 2007). By extension, these studies suggest that PKC inhibitors may provide a potential therapy for gliomas that genetically retain *NF1* by simultaneously suppressing tumorigenic signals from multiple activated receptors. Notably, PKC inhibitors have been shown to be effective in preclinical studies of GBMs in vitro and in vivo and are currently in clinical development (da Rocha et al., 2002; Mackay and Twelves, 2007). Our data reveal one mechanism by which tumor cells are sensitized to these agents and indicate that PKC may represent an important therapeutic target in GBMs in which the *NF1* gene is intact and the protein is destabilized.

NF1, p53, and Senescence

Whereas these data indicate that proteasomal inactivation of neurofibromin contributes to gliomagenesis, we have also detected *NF1* mutations in cells and tumor tissue from sporadic human gliomas. Two recent studies have also observed mutations in the *NF1* gene in GBMs (Parsons et al., 2008; TCGA Research Network, 2008); however, complete genetic loss was reported to be rare (TCGA Research Network, 2008). Thus, our findings suggest that NF1 inactivation may play an even broader role in glioma development than was previously recognized. These studies also provide a biological framework for understanding how different mechanisms of inactivation may be required during tumor evolution. Whereas proteasomal reduction and perhaps even heterozygosity may promote tumorigenicity, this tissue type appears to be very sensitive to complete inactivation of *NF1* and, in the latter context, p53 plays an important role in restricting tumorigenesis. Genomic analysis of human tumor tissue, cellular studies, the senescent properties of pilocytic astrocytomas from NF1 patients, and mouse modeling efforts (Zhu et al., 2005), all suggest that homozygous null *NF1* mutations are only tolerated in the absence of an intact p53 pathway. Thus, the present study demonstrates that *NF1* and *p53* mutations are coincidental in sporadic human gliomas and provides mechanistic insight into why the order of mutational events influences glioma progression. This model also highlights why proteasomal inactivation (perhaps coupled with genetic heterozygosity) may represent a more prevalent mechanism of NF1 inactivation in gliomas.

It should be noted that NF1 shares many functional similarities with PTEN, which is also thought to promote tumorigenesis via genetic inactivation and enhanced proteasomal degradation.

Moreover, like *NF1*, complete inactivation of *PTEN* early in the tumorigenic process (in the prostate) appears to limit tumor development by promoting cellular senescence (Chen et al., 2005). Such observations suggest that exquisitely sensitive protective mechanisms have evolved to respond to aberrant oncogenic signals above a certain threshold, resulting from complete, but not partial, inactivation of these tumor suppressors. Thus, studies aimed at further elucidating this tumor suppressive response, as well as defining additional proteins that contribute to the proteasomal regulation of these tumor suppressors, may identify new genes that restrict or promote human cancer. Finally, because such protective responses exist, different mechanisms of inactivation may be selected for during the course of tumor development, which may depend on the complement of genetic alterations already present. Moreover, our data suggest that defining the mechanism by which a tumor suppressor is inactivated may ultimately be important for choosing appropriate therapies.

EXPERIMENTAL PROCEDURES

Cell Culture

NIH 3T3 cells were cultured in DMEM supplemented with calf serum. IMR90, RT4, A172, U138, M059J, U87, U251, Gli36, SF539, Dbtrg-05mg, human umbilical vein endothelial cells (HUVECs), and mouse embryonic fibroblasts (MEFs) were cultured in DMEM supplemented with fetal calf serum.

Human Subjects

The collection and analysis of human tissue samples was approved by the Institutional Review Boards of the University of California, Los Angeles, and Memorial Sloan-Kettering Cancer Center. Informed consent was obtained from all subjects. For tissues obtained in Figures 3H and 4E, the Office of Research Protections at Brigham and Women's Hospital ruled that this study does not represent human subject research due to exemption 32 CFR 219.101(b)4: the research involved collection or study of existing pathological specimens that are publicly provided without identifiers.

TS Cultures

Tumor sphere (TS) cell cultures were isolated from primary human glioblastomas by disassociation into a single cell suspension with Accumax (Innovative Cell Technologies). Cells were filtered through a 100 μm filter, and then washed and plated in NeuroCult NS-A proliferation media (Stem Cell Technologies) supplemented with EGF (20 ng/ml; R&D Systems), FGF (10 ng/ml; R&D Systems), and heparin (2 μg/ml).

Degradation Time Courses and PKC Inhibition

NIH 3T3 and IMR90 cells were plated in serum-free medium at 5×10^5 cells/10 cm plate. HUVEC, RT4, and GBM cell lines were plated in 1% serum, and primary GBM cultures were plated in the media described above. After 18 hr, cells were treated with either 1 μM Bis I, 1 μM Bis II, 2.5–5 μM Ro-31-8220 (Calbiochem), or 1 μM Go 6976 (LC Laboratories) or vehicle. In Figure 1, cells were pretreated with inhibitors 30 min before adding 6 μM LPA, 20 ng/ml PDGF, or 10 ng/ml PMA. Cells were lysed at specified time points with 1% SDS boiling lysis buffer or RIPA buffer.

Immunoblotting and Immunoprecipitations

The following antibodies were used: neurofibromin (C-terminal antibodies: UP69, a polyclonal antibody raised against a KLH-conjugated peptide, RNSIK KIV [used for all experiments except Figure 3G]; Courtois-Cox et al., 2006) and A300-140A (Bethyl Laboratories). N-terminal antibody: Ab NF1-5.16 (a monoclonal antibody provided by K. Scheffzek and G. Moldenhauer); pMARCKs (Calbiochem); HA (12CA5; Roche), PKCα (BD Biosciences); p21 WAF1/cip-1 (c-19; Santa Cruz); phospho-PKC motif 2351 (Cell Signaling), and GAPDH (Cell Signaling) (Johannessen et al., 2005).

Retroviral and Lentiviral Infections

Retroviral and lentiviral infections were performed as described (Johannessen et al., 2005). PKCαCAT-HA and DRNF1-FLAG were introduced into the pbabe retroviral vector. The DRNF1-FLAG construct possesses a FLAG epitope tag and encodes amino acids 1175–1535. This protein fragment retains RasGAP activity and has been shown to be resistant to ubiquitination and degradation in vitro (Cichowski et al., 2003). The lentiviral pLKO vector containing the following shRNAs were used: NF1#2(H9), 5′-TTATAAATAGCCTGGAAAAGG-3′; PKCα1 (e1), 5′-CCGTCTTAACACCACCTGA TCTCGAGATCAGGTGGTGTTAA GACGG-3′; PKCα #2(D12), 5′-ATGGAACTCAGGCAGAAATTCT CGAGAGAA TTTCTGCCTGAGTTCCAT-3′. The retroviral pMSCV-pM vector containing the following shRNA was used: p53 shRNA, 5′-GGCCTGACTCAGACTGACATT-3′.

Ras Activation Analysis

Cells were cultured in the presence of 0.25% serum, and 10 ng/ml of PMA was added for increasing amounts of time. Ras-GTP levels were detected using a Ras activation assay, following the manufacturer's instructions (Pierce Biotechnology). Ras-GTP activation was quantified by using Photoshop to assess the amount of Ras that was pulled down with the Ras binding domain, divided by total levels of Ras in the lysates. Values at 5 and 15 min were then normalized to control to establish levels of fold Ras activation.

Proteasome Inhibitor Treatment

GBM cell lines and primary neurosphere cultures were treated with 1 μM bortezomib (LC Laboratories) or 10 μM MG132 (Boston Biochem) and 1 μM bortezomib as specified and lysed at designated time points.

Soft Agar Assay

Soft agar assays were performed as described (Johannessen et al., 2005).

Subcutaneous Implantation

Animal procedures were approved by the Center for Animal and Comparative Medicine at the Harvard Medical School in accordance with the NIH Guild for the Care and Use of Laboratory Animals and the Animal Welfare Act. Subcutaneous implantations in athymic male nude mice (nu/nu; 6 weeks old) were carried out as previously described (Berger et al., 2004). Briefly, 2.5×10^6 of U87 cells expressing pbabe DRNF1-FLAG or pbabe vector in 100 μl of PBS were injected subcutaneously (six injections per condition). Two weeks after injection (day 0), the width and length of the tumor were measured every 2-3 days by caliper, and the volume was calculated with the following formula: volume = (length × width^2)π/6.

Growth Curves

U87 cells expressing pbabe DRNF1-FLAG or pbabe vector were plated in triplicate at 5×10^5 in a 6-well dish and counted every other day. Dbtrg-05mg cells expressing p53 shRNA and NF1 shRNA or p53 shRNA and vector were plated in triplicate at 2×10^5 and counted on day 1, 7, and 9. For the cumulative population doubling assay, 2×10^5 cells were plated in triplicate in 6-well dishes. Cells were counted and reseeded at a density of 2×10^5 every 3 days for five passages. Population doublings were calculated as described (Courtois-Cox et al., 2006).

SA-β-Gal Activity

SA-β-gal activity was performed as described and percentages were assessed by counting at least 300 cells (Dimri et al., 1995).

Immunohistochemistry

Immunohistochemistry for p16 was performed as described using the p16INK4a antibody (Ab-7, MS-1064-P; Neomarkers), the p53 antibody (D07; Signet Laboratories), or the p15 antibody (C-20, sc-612; Santa Cruz Biotechnology) (Courtois-Cox et al., 2006).

NF1 Mutational Analysis

The entire NF1 coding region was amplified in five overlapping RT-PCR fragments and used as the template for direct sequencing as described (Messiaen et al., 2000). Copy number analysis by multiplex ligation-dependent probe amplification was performed as described (Wimmer et al., 2006). The nomenclature of the mutations is based on NF1 mRNA sequence NM_000267.1, with

1 being the first nucleotide of the ATG start codon. The NF1 exons are named according to the most widely used nomenclature adapted by the researchers and diagnostic labs, which does not use strictly consecutive numbers.

IC_{50} Determinations and Viability Assays

NF1$^{+/+}$ and *NF1*$^{-/-}$ GBM cell lines (3×10^3) were plated in replicates of eight in a 96-well dish. Cells were treated with 0, 0.625, 1.25, 2.5, 4, 5, 7.5, or 10 μM Ro-31-8220 or vehicle for 18 hr before assessing viability by adenosine triphosphate quantification using a Cell Titer-Glo Luminescent Cell viability assay kit (Promega). Gli36 cells were infected with NF1#2 shRNA or vector and selected in puromycin for 2 days. Cells (3×10^3) were plated in 96-well dishes and IC_{50} values were determined as described above. To calculate IC_{50} values for GBM neurospheres TS543 and TS565, cells were plated in growth factor-free media and treated with the specified PKC inhibitor at the concentrations indicated for 24 hr. Cells were evaluated for viability and cell counts using a Vi Cell XR analyzer (Beckman Coulter).

Rapamycin Sensitivity Assays

Twenty thousand cells were seeded per well of a 6-well dish. Twenty-four hours later, cells were treated with various concentrations of rapamycin in triplicate. Log-phase cells treated with rapamycin or vehicle were isolated after three doubling periods, and viable cells were counted.

Statistical Analysis

All numerical data including error bars represent the mean ± the standard deviation. Specific statistical tests for each experiment are described in the text.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00175-5.

ACKNOWLEDGMENTS

We thank Wade Harper, Steve Elledge, Gerard Evan, and Bill Hahn for helpful discussions and Maria Debiec-Rychter for samples. This work was supported by grants from the Brain Tumor Funders' Collaborative (I.K.M., T.F.C., and P.S.M.), NCI (R01 CA111754-01) (K.C.), DOD (W81XWH-08-1-0136) (K.C.), The Children's Tumor Foundation (L.T.M., S.M.G.W., and A.P.), and Landesstiftung Baden-Wuerttemberg, Germany (K.S.).

Received: September 25, 2008
Revised: April 3, 2009
Accepted: May 12, 2009
Published: July 6, 2009

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