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Sensitivity of Glioblastomas to Clinically Available MEK Inhibitors Is Defined by Neurofibromin 1 Deficiency

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Abstract

Loss of neurofibromin 1 (NF1) leads to hyperactivation of RAS, which in turn signals through the RAF/MEK/ERK and phosphoinositide 3-kinase (PI3K)/mTOR pathways to regulate cell growth and survival. Because NF1-deficient acute myeloid leukemias are sensitive to MEK inhibitors, we investigated here whether NF1-deficient glioblastoma multiforme (GBM) would respond to MEK inhibition. In 19 GBM cell lines, we found that treatment with the clinically available MEK inhibitors PD0325901 or AZD6244 decreased levels of phospho-ERK, the downstream effector of MEK, regardless of NF1 status. However, growth inhibition occurred only in a subset of NF1-deficient cells, in association with decreased levels of cyclin D1, increased levels of p27, and G₁ arrest. As a single agent, PD0325901 suppressed the growth of NF1-deficient, MEK inhibitor-sensitive cells *in vivo* as well. Mechanistically, NF1-deficient, MEK inhibitor-sensitive cells were dependent upon the RAF/MEK/ERK pathway for growth and did not activate the PI3K pathway as a mechanism of acquired resistance. Importantly, NF1-deficient cells intrinsically resistant to MEK inhibition were sensitized by the addition of the dual PI3K/mTOR inhibitor PI-103. Taken together, our findings indicate that a subset of NF1-deficient GBMs may respond to MEK inhibitors currently being tested in clinical trials.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W.L. See, T. Nicolaides, R.O. Pieper

Development of methodology: W.L. See, I.-L. Tan, J. Mukherjee

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.L. See, I.-L. Tan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W.L. See, I.-L. Tan, J. Mukherjee, T. Nicolaides, R.O. Pieper

Writing, review, and/or revision of the manuscript: W.L. See, I.-L. Tan, J. Mukherjee, T. Nicolaides, R.O. Pieper

Study supervision: R.O. Pieper

Introduction

Glioblastoma multiforme (GBM) is the most aggressive and fatal adult human brain cancer, and over 10,000 new cases are diagnosed in the United States each year. Molecular characterization suggests that there are 4 GBM subtypes, which are each associated with a unique set of genetic alterations and prognoses (1–4). This subtyping has increased interest in the development of therapies targeted to specific genetic alterations and which could be more effective than current approaches.

Of the 4 GBM subtypes (proneural, neural, classical, and mesenchymal), the mesenchymal subtype is perhaps of the most interest. This subcategory, which comprises roughly 20% of GBM, is associated with a high incidence of p53 and *PTEN* mutations, a relative absence of *IDH* or *EGFR* mutation/ amplification, and poor prognosis (2–4). A defining feature of the mesenchymal subset is mutations and/or deletions in the gene encoding neurofibromin 1 (NF1; 2, 4), suggesting that this subtype may be uniquely amenable to agents that target pathways driven by NF1 loss.

The loss of NF1, however, activates a variety of pathways, any of which could contribute to gliomagenesis. NF1 is a regulator of the GTP-binding protein RAS that cycles between the active GTP-bound and inactive GDP-bound forms (5). RAS GTP/GDP cycling is positively regulated by GTP exchange factors (GEF), which promote the exchange of GDP for GTP and negatively regulated by GTPase-activating proteins (GAP), such as NF1, that promote the hydrolysis of GTP to GDP. Loss of NF1 can therefore enhance RAS activation and promote signaling down a variety of RAS effector pathways, the most well characterized being the RAF/MEK/ERK pathway. RAF kinase becomes active upon binding to RAS-GTP and initiates the MEK/ERK phosphorylation cascade, leading to increases in gene transcription of cell-cycle regulators such as cyclin D1 to promote cell growth and survival. Suppression of the cell-cycle inhibitor p27 is in part mediated by cyclin D1 binding and activation of cyclin-dependent kinases (CDK) and acts to further promote cell-cycle progression (6). RAS-GTP can also interact with and enhance kinase activity of the p110 α catalytic subunit of phosphoinositide 3-kinase (PI3K) that converts PIP2 to PIP3, an action that is reversed by the lipid phosphatase PTEN (7). PIP3 leads to membrane recruitment and activation of AKT, which in turn leads to activation of the serine/threonine kinase mTOR. mTOR then phosphorylates the downstream effectors 4EBP1 and S6K, resulting in enhanced mRNA translation and negative feed back regulation of PI3K signaling (8, 9). In addition to the RAF/MEK/ERK and PI3K pathways, RAS-GTP also signals down the Ral-GDS pathway (10) making any of these signaling systems potentially important and targetable in NF1-deficient GBM.

Identification of key downstream effectors that drive tumor growth in NF1-deficient GBM is critical, given the large number of pathways and effectors potentially activated by NF1 loss. Although RAS itself is a logical target, effective RAS inhibitors are not available. The selective RAF inhibitors Vemurafenib (PLX4032) and GSK2118436 are clinically available and effective in melanomas with activating mutations in BRAF (11). They fail, however, to inhibit ERK phosphorylation and can paradoxically increase ERK signaling in cells lacking BRAF mutations (as is the case in most GBM). Inhibitors of mTOR are also widely

available, although their usefulness is limited by the loss of the S6K-mediated negative feedback loop that can increase AKT activation in response to mTOR inactivation (12). Dual PI3K/mTOR inhibitors alleviate problems caused by mTOR-induced feedback inhibition but are ineffective at shutting down RAF/MEK/ERK signaling (12). Clinically available inhibitors of MEK in contrast effectively block MEK-induced ERK activation. Furthermore, acute myeloid leukemias (AMLs) driven by NF1 loss, as well as tumors with activating mutations in RAS, are selectively sensitive to inhibitors of MEK (13–16), suggesting that the RAF/MEK/ERK pathway may be of particular importance in tumors with deregulated RAS activity. Little is known, however, about the role of RAF/MEK/ERK signaling in the growth of NF1-deficient GBM, or about the MEK inhibitor sensitivity in these tumors.

In this study, we identify a panel of NF1-deficient GBM cell lines and show that the subset of NF1-deficient GBM cells dependent on RAF/MEK/ERK signaling for growth is exquisitely sensitive to the single-agent MEK inhibitors PD0325901 or AZD6244. In addition, we show that intrinsically MEK inhibitor-resistant NF1-deficient GBM cells can be sensitized to MEK inhibitors by addition of the dual PI3K/mTOR inhibitor PI-103. Together, these data suggest that NF1-deficient GBMs are an ideal target for MEK inhibitors or MEK inhibitor-based combination therapies.

Materials and Methods

Cells and cell culture reagents

Human GBM cell lines were obtained from American Tissue Culture Collection (ATCC; LN229, A172, T98G, MO59J, LN18, U87, U138) or the UCSF BTRC Tissue Core (U251, U373, SF188, U343, SF126, SF210, SF268, SF295, SF539). GBM43 human xenograft cells were provided by David James (UCSF) and LN319 GBM cells were provided by Karen Cichowski (Dana-Farber/Harvard Cancer Center). ATCC uses the Pro-mega Powerplex Systems to authenticate cell lines. All cell lines were not reauthenticated but were passaged for less than 6 months upon receipt in DMEM-H21 supplemented with 10% FBS (JRSscientific) and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂ incubator. PD0325901 and AZD6244 were purchased from Selleck Chemicals and PI-103 was purchased from Calbiochem.

Isolation of MEK inhibitor-resistant GBM cells

LN229 GBM cells were cultured in 1 µmol/L PD0325901 and media was replaced every 3 days for 8 weeks or until colonies formed. Colonies were isolated using sterile cloning cylinders (Corning) and expanded as clonal populations in 100 nmol/L PD0325901.

Lentiviral production and infection

PTEN shRNA lentiviral production and infection—Scramble or PTEN short hairpin RNA (shRNA) pLKO.1, VSV-G, and VPR plasmids (Open Biosystems) were cotransfected at a 1:0.9:0.1 ratio into 293T packaging cells using Fugene6 (Roche). Lentiviral supernatants were harvested at 48 and 72 hours posttransfection. LN229 GBM cells were plated at 2.5×10^5 cells/well on 6-well plates overnight and infected with

lentiviral supernatants in the presence of 8 µg/mL polybrene (Sigma-Aldrich) for 24 hours. Infected LN229 GBM cells were selected in 0.5 µg/mL puromycin for at least 1 week and expanded as polyclonal populations.

Luciferase lentiviral production and infection—pSIN-luciferase, VSV-G, and Gag-pol plasmids were cotransfected at a ratio of 1.5:1:1 into 293T packaging cells using Fugene6 (Roche). Lentiviral supernatants were harvested at 48 and 72 hours posttransfection, filtered (0.45 µm), and centrifuged at 40,000 × g for 1.5 hours. LN229 and U251 GBM cells were plated at 2.5 × 10 cells/well on 6-well plates overnight and infected with 1 µL concentrated lentivirus diluted in 1 mL of media containing 8 µg/mL polybrene (Sigma-Aldrich) for 24 hours. Infected LN229 and U251 GBM cells were screened for luciferase expression by incubation with 150 µg/mL D-luciferin (Gold Biotechnologies) and bioluminescence imaging using a Xenogen IVIS Lumina system (Caliper).

Immunoblotting

GBM cells were washed in cold PBS and lysed in buffer containing 20 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 300 mmol/L sucrose, 6 mmol/L MgCl₂, 1 mmol/L EGTA, 0.5% Triton X-100, 1× PhosStop (Roche), and protease inhibitor cocktail (Roche). Following centrifugation (14,000 rpm, 10 minutes), protein concentration was measured, and protein (50 µg) was subjected to SDS-PAGE using 4% to 20% Tris-Glycine gradient gels (Invitrogen). Following transfer onto Immuno-Blot PVDF membranes (Bio-Rad Laboratories), blots were incubated in blocking buffer (1× TBS containing 5% milk and 0.05% tween-20, 1 hour), incubated with primary antibodies diluted in blocking buffer (overnight, 4°C), washed in 1× TBST, incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnologies) diluted in blocking buffer (1 hour, room temperature), then washed in 1× TBST. Antibody binding was detected using ECL reagents (Amersham Pharmacia Biotech). Primary antibodies were obtained from Cell Signaling (phospho-ERK, ERK, phospho-AKT S473, AKT, PTEN, cyclin D1, cleaved-PARP, Bim, BCL-2, MCL-1) or Santa Cruz Biotechnologies (NF1-D, p27, β-actin).

Cell growth assays

Cells (3,000 cells/well, 96-well plates) were incubated with 100 µL media containing PD0325901 or AZD6244 at the indicated doses for 5 days, then incubated with Alamar Blue reagent (Invitrogen; 20 µL/well, 1 hour), after which fluorescence (A540/620) was measured using a microplate reader (BioTek Instruments). Cell growth was determined by normalizing fluorescence to media only controls.

Fluorescence-activated cell-sorting analysis

Cells (1 × 10 cells/well, 6-well plates) were incubated with the indicated kinase inhibitors for 5 days. Floating and trypsinized cells were fixed in 70% EtOH, stained in PBS containing 20 µg/mL propidium iodide (Sigma-Aldrich) and 100 µg/mL RNase A (Roche), and subjected to flow cytometry using a FACSCalibur (BD Biosciences). Cell cycle and sub-G1 analysis was conducted using FlowJo software (TreeStar).

Animal studies

Immunodeficient mice (nu/nu; Charles River) were injected intracranially with 3×10^5 luciferase-expressing LN229 or U251 GBM cells as described (17). Tumor growth was monitored weekly by treating mice with D-luciferin (150 mg/kg i.p.; Gold-Biotechnology) and measuring bioluminescence using a Xenogen IVIS Bioluminescence imaging station (Caliper). Twenty-one days postintracranial injection, mice harboring logarithmically-growing tumors were treated with vehicle or 10 mg/kg PD0325901 once daily for 4 weeks. Tumor growth was calculated by normalizing luminescence measurements to pretreatment values.

Results

NF1 deficiency is associated with MEK inhibitor sensitivity in GBM cell lines

To identify NF1-deficient GBM cells, we obtained a panel of 18 GBM cell lines and conducted immunoblot analysis of neurofibromin 1 expression. Of the 18 cell lines, 6 (LN229, U373, LN319, U251, SF188, and GBM43), contained no detectable NF1 protein (Fig. 1A), and were defined as NF1-deficient. Consistent with these results, 4 of the cell lines (LN229, LN319, U251, SF188) were previously reported to contain NF1 homozygous mutations and/or deletions (18, 19).

To determine if NF1-deficient GBM cells are selectively sensitive to MEK inhibitors, we exposed the panel of 19 GBM cell lines to PD0325901 (0–10 $\mu\text{mol/L}$) or AZD6244 (0–10 $\mu\text{mol/L}$) for 5 days and measured cell growth/viability. None of the 13 NF1-proficient GBM cell lines exhibited growth suppression in response to single-agent MEK inhibitors (Fig. 1B and C). Two of the 6 NF1-deficient GBM cell lines, however (LN229 and U373) were exquisitely sensitive (Fig. 1B and C), with IC_{50} values in the nanomolar range (PD0325901 $\text{IC}_{50} = 30 \text{ nmol/L}$, AZD6244 $\text{IC}_{50} = 300 \text{ nmol/L}$). All cells examined regardless of MEK inhibitor sensitivity exhibited a sustained (24 hours) decrease in levels of phospho-ERK, the downstream target of MEK, following a 100 nmol/L exposure of PD0325901 (Fig. 2A). In the MEK inhibitor-sensitive cells, however, this drug exposure also suppressed levels of cyclin D1 (Fig. 2A, lower band), a downstream effector of ERK and positive cell cycle regulator, and increased levels of p27 (Fig. 2A), a negative cell-cycle regulator. Similar results were obtained after exposure to a growth-suppressing concentration of AZD6244 (500 nmol/L; Supplementary Fig. S1). In contrast, the MEK inhibitor-mediated inhibition of phospho-ERK levels in NF1-deficient, MEK inhibitor-resistant cells (LN319, U251, SF188, GBM43) was dissociated from effects on cyclin D1 and, in 3 of 4 cases, p27 (Fig. 2A). Similar results were observed in NF1-proficient GBM cell lines (data not shown). Consistent with these results, treatment with PD0325901 increased the percentage of cells in G1 in the MEK inhibitor-sensitive LN229 and U373 GBM cells but had little effect on MEK inhibitor-resistant U251, LN319, SF188, and GBM43 cells (Fig. 2B). Together, these results suggest that MEK inhibitor-induced suppression of phospho-ERK alters cell-cycle progression and cell growth, but only in MEK inhibitor-sensitive GBM cells.

MEK inhibition results in variable apoptotic responses in NF1-deficient GBM cells

To determine if LN229 and U373 cells undergo apoptosis in response to MEK inhibition, these cells as well as 4 NF1-deficient, MEK inhibitor-resistant GBM cell lines were incubated with 100 or 1,000 nmol/L PD0325901 for 5 days and fluorescence-activated cell-sorting (FACS) analysis was conducted to determine the percentage of cells with a sub-G₁ (apoptotic) DNA content. The percentage of sub-G₁ LN229 cells significantly increased in response to 100 and 1,000 nmol/L PD0325901 (Fig. 3A), although there was no significant increase in apoptosis in U373 cells or in 3 of 4 NF1-deficient, MEK inhibitor-resistant GBM cells. Consistent with these results, upregulation of Bim expression and cleaved-PARP was detected in LN229 cells treated with PD0325901, but not in PD0325901-treated U373 cells (Fig. 3B). The levels of the prosurvival factor BCL-2, but not MCL-1, were dramatically increased in U373 cells (Fig. 3B), suggesting that NF1-deficient tumors containing low levels of BCL-2 may be more sensitive to MEK inhibitor-induced apoptosis.

The MEK inhibitor PD0325901 suppresses the growth of NF1-deficient GBM intracranial xenografts

To determine if PD0325901 inhibits the growth of NF1-deficient GBM cells *in vivo*, luciferase-expressing LN229 or U251 cells were injected intracranially into nude mice and allowed to reach logarithmic growth, after which the mice were treated with vehicle or a daily oral dose of PD0325901 (10 mg/kg) and monitored by bioluminescence imaging for tumor growth. Treatment with PD0325901 suppressed levels of phospho-ERK, the downstream effector of MEK, for up to 24 hours after drug exposure in tumors derived from either LN229 (MEK inhibitor-sensitive) or U251 (MEK inhibitor-resistant) cells (Fig. 4A), consistent with our *in vitro* data. Suppression of cyclin D1 and upregulation of p27 in response to PD0325901 treatment was more variable in intracranial LN229 tumors (Fig. 4A). PD0325901 exposure suppressed the growth of LN229 tumors (Fig. 4B) and increased the survival of LN229-bearing animals (Fig. 4C), but had no significant effect on the intracranial growth of U251 cells or the survival of U251 tumor bearing mice (Fig. 4B and C), again consistent with our *in vitro* findings (Fig. 1B).

NF1-deficient, MEK inhibitor-sensitive GBM cells are dependent on the RAF/MEK/ERK pathway for growth

Our *in vitro* and *in vivo* studies showed that a subset of NF1-deficient GBM cells were exquisitely sensitive to MEK inhibitors, suggesting that these cells are dependent on the RAF/MEK/ERK pathway for growth. To test this idea, we chronically treated LN229 GBM cells with 1 μ mol/L PD0325901 for 6 to 8 weeks until resistant colonies formed. Three resistant colonies were then isolated, expanded as clonal populations, and screened for MEK inhibitor sensitivity and effects of drug selection on target inhibition. All 3 clonal populations (1,182, 1,183, and 1,187) were at least 10-fold less sensitive to either PD0325901 or AZD6244, as compared with parental cells, with IC₅₀ values of \sim 1 μ mol/L for PD0325901 and \sim 10 μ mol/L for AZD6244 (Fig. 5A and B). To determine if this resistance was because of hyperactivation of the target RAF/MEK/ERK pathway or through activation of alternative pathways, we conducted immunoblot analysis to examine the ability of the drug to modulate its downstream targets. Although a 100 nmol/L exposure to

PD0325901 suppressed levels of phospho-ERK, decreased levels of cyclin D1, and increased levels of p27 in the parental LN229 cells (Fig. 5C), a 10-fold higher dose was required in all 3 PD0325901-resistant clones to suppress phospho-ERK and cyclin D1. In addition, PD0325901-resistant clones were extremely resistant to drug-induced effects on p27, and in no case did the resistant clones exhibit increased phospho-AKT relative to parental controls. These results suggest that NF1-deficient, MEK inhibitor-sensitive GBM cells are dependent on the RAF/MEK/ERK pathway for growth, and that PI3K activation is not a mechanism by which these cells develop resistance.

Inhibition of PI3K/mTOR signaling enhances sensitivity to MEK inhibition in a subset of NF1-deficient GBM

Although NF1-deficient, MEK inhibitor-sensitive GBM cells depend on the RAF/MEK/ERK pathway for growth, loss of NF1 can cause RAS to signal down both RAF/MEK/ERK and PI3K/mTOR pathways. We therefore initiated studies to determine if inhibition of PI3K signaling enhanced sensitivity to MEK inhibition in MEK inhibitor-sensitive cells or in NF1-deficient GBM cells that do not respond to single-agent MEK inhibitors. First, we infected LN229 cells with a lentivirus encoding 1 of 2 shRNAs targeting PTEN, a tumor suppressor that negatively regulates the PI3K pathway and is commonly mutated/deleted in GBM (2, 4, 7), after which we monitored effects of PTEN loss and AKT activation on MEK inhibitor sensitivity. Introduction of either of 2 shRNAs significantly reduced PTEN levels relative to that noted in scramble controls, and levels of phospho-AKT were also significantly increased in the PTEN-knockdown cells (Fig. 6A). Knockdown of PTEN did not, however, alter PD0325901 sensitivity (Fig. 6B), suggesting that PI3K pathway activation does not confer resistance to MEK inhibition in LN229 cells.

To further address the role of PI3K signaling in MEK inhibitor sensitivity, NF1-deficient GBM cells were incubated with PD0325901 (100 nmol/L), a dual PI3K/mTOR inhibitor (PI-103, 500 nmol/L), or both drugs in combination, after which effects on cell growth and target inhibition were monitored. Treatment with PD0325901 alone reduced LN229 and U373 cell growth and suppressed phospho-ERK and cyclin D1 levels (Fig. 6C and D; Supplementary Fig. S2A). Treatment with PI-103, at a dose that suppressed phospho-AKT levels without altering phospho-ERK or cyclin D1 levels (Fig. 6D; Supplementary Fig. S2A), did not significantly enhance growth suppression in response to MEK inhibition in LN229 and U373 cells (Fig. 6C), consistent with dependency on the RAF/MEK/ERK pathway for growth in these cells. In contrast, treatment with PD0325901 resulted in a modest reduction in LN319, U251, and GBM43 growth that was significantly enhanced by addition of PI-103 (Fig. 6C). Sensitivity to PI-103 was not associated with PTEN status (Fig. 6C). In MEK inhibitor-resistant GBM43 cells, cyclinD1 levels were not altered in response to either PD0325901 or PI-103 alone but did decrease in response to the combination of PD0325901 and PI-103 (Fig. 6D), consistent with the drug-induced effects on cell growth (Fig. 6C). Similar results were obtained with U251 and LN319 GBM cells (Supplementary Fig. S2A). Apoptosis, as measured by sub-G₁ DNA content, was not significantly induced in NF1-deficient GBM cells except in LN229 cells treated with PD0325901 (Supplementary Fig. S2B). Treatment of NF1-proficient GBM cells with PD0325901 resulted in a modest reduction in cell growth that was not consistently enhanced by treatment with PI-103, and

drug treatment did not induce apoptosis except in U138 cells (Supplementary Fig. S3A–S3C). Together these results suggest that suppression of PI3K signaling has little effect on MEK inhibitor-sensitive cells that rely on RAF/MEK/ERK signaling for growth, but enhances sensitivity to the growth suppressive effect of MEK inhibitors in the majority of NF1-deficient, MEK inhibitor-resistant GBM cells, which appear to rely on both the RAF/MEK/ERK and PI3K/mTOR signaling pathways.

Discussion

In this study, we show that a subset of NF1-deficient GBM cells dependent on RAF/MEK/ERK signaling for growth is exquisitely sensitive to the clinically available MEK inhibitors PD0325901 or AZD6244 as single agents. In addition, we show that even intrinsically MEK inhibitor-resistant NF1-deficient GBM cell lines are sensitized to MEK inhibitors by addition of the dual PI3K/mTOR inhibitor PI-103. Together, these data suggest that the NF1-deficient GBMs may be optimal targets for individualized therapy using MEK inhibitors or MEK inhibitor-based approaches.

The present studies suggest that NF1-deficient GBM cells are highly dependent on RAF/MEK/ERK signaling alone or in combination with PI3K/mTOR signaling for growth. These results differ somewhat from other studies in NF1-deficient tumors. As an example, NF1-deficient optic gliomas (OPG) seem to depend primarily on mTOR signaling and are sensitive to inhibitors of mTOR in preclinical studies (20–23). This could reflect a difference in the cell of origin as NF1 inactivation leads to mTOR-mediated increases in proliferation and differentiation in astrocytes and neural stem cells derived from the brain stem (the site of origin of OPGs), but not from the neocortex (the site of most GBM; refs. 20, 24–27). Alternatively, NF1^{+/-} microglial cells that contribute to OPG formation may favor mTOR signaling over RAF/MEK/ERK signaling (28–30). These findings therefore emphasize that the targetable pathway may differ depending on the tumor subgroup of interest.

A second unique aspect of the studies is the relative uniformity of ways in which cells acquire MEK inhibitor resistance. All 3 LN229 MEK inhibitor-resistant clones examined exhibited hyperactivation of ERK signaling (Fig. 5C) that was associated with upregulation of EGFR and PDGFR β and lack of MEK1 mutations (see W.L. and colleagues, in preparation), suggesting that this may be a preferred mechanism of resistance. Acquired MEK inhibitor resistance in NF1-deficient AML and B-Raf mutated melanoma has also been reported to result from reactivation of ERK signaling (11, 15). Other studies in B-Raf mutated melanomas, however, show that acquired RAF inhibitor resistance can also result from ERK-independent mechanisms that activate parallel pathways (11). Mechanisms of resistance beyond RAF/MEK/ERK pathway hyperactivation could be found if a wider range of NF1-deficient, acquired MEK inhibitor-resistant clones was examined. From a therapeutic standpoint, however, the lack of variability in mechanism of resistance noted here might be a favorable sign.

Another unique aspect of the results presented is the high degree of variability in MEK inhibitor sensitivity across the NF1-deficient GBM cells examined. Although only 2 of 6

NF1-deficient GBM cell lines were exquisitely sensitive to the single-agent MEK inhibitors, NF1-deficient AML in mice, by contrast, are invariably sensitive to PD0325901, with only a few exceptions (Kevin Shannon, personal communication). The MMLV insertion that drives these NF1-deficient AML may occur in a restricted fashion and the NF1-deficient GBM cell lines used in our study may be a more genetically heterogeneous population. Better understanding of tumor heterogeneity in NF1-deficient GBM will therefore be important for identification of the MEK inhibitor-sensitive subset and optimal use of MEK inhibitors in this population of tumors.

Our studies also provide an explanation for MEK inhibitor sensitivity as well as a framework for understanding NF1-regulated signaling. In this study, we found at least 3 distinct RAS signaling groupings in NF1-deficient GBM cell lines (Fig. 7A – C). In NF1-deficient, MEK inhibitor-sensitive LN229 and U373 cells, PD0325901-mediated ERK inhibition caused a decrease in the levels of cyclin D1 that was unaffected by inhibition of the PI3K/mTOR pathway with PI-103 (Fig. 6C and D), suggesting that in these cells ERK activity directly regulates the levels of cyclin D1, most likely through canonical alterations in transcription (ref. 31; Fig. 7A). In contrast, in NF1-deficient, MEK inhibitor-resistant GBM cells, suppression of both ERK and PI3K/mTOR activity was required to decrease the levels of cyclin D1 (Fig. 6C and D), perhaps through transcriptional and/or translational mechanisms (Fig. 7B). Alternatively, ERK has been reported to cross-talk with the mTOR pathway (32–37) and this convergence could lead to regulation of cyclin D1 exclusively at the level of mRNA translation (Fig. 7C). Our preliminary data support the latter possibility because in MEK inhibitor-resistant GBM43 cells, both PI-103 and PD0325901 are required to completely suppress the levels of both phospho-S6, the downstream effector of S6K, and cyclin D1. Studies are currently underway, therefore, to determine if pathways regulating S6 activity may be targets for the treatment of MEK inhibitor-resistant GBM.

These studies also have implications for the clinical use of MEK inhibitors in GBM. MEK inhibitors are clinically available, and as such the major limitation in designing a clinical trial is selecting the right patient population. Screening for NF1 deficiency by DNA sequencing or copy number analysis is possible although not necessarily feasible. Alternatively, identification of biomarkers for MEK inhibitor sensitivity would be very useful. These studies suggest that MEK inhibitor-induced alterations in the levels of cyclin D1 and p27 are indicative of response, and these could be useful biomarkers if trials included a biopsy or surgical resection after initial drug treatment. Although our data show that the MEK inhibitor response in NF1-deficient GBM cells is mostly cytostatic, low levels of BCL-2, as showed in LN299 cells (Fig. 3B), could also be a biomarker for MEK inhibitor-induced apoptotic response in NF1-deficient GBM. Alternatively, MEK inhibitor-based trials could be designed to include a PI3K/mTOR inhibitor, addition of which enhances MEK inhibitor sensitivity (13, 14, 16, 36, 38, 39), and may target a broader GBM population. Given the dismal prognosis of the mesenchymal subtype of GBM, these approaches could be extremely useful for this difficult to treat subgroup of brain tumors.

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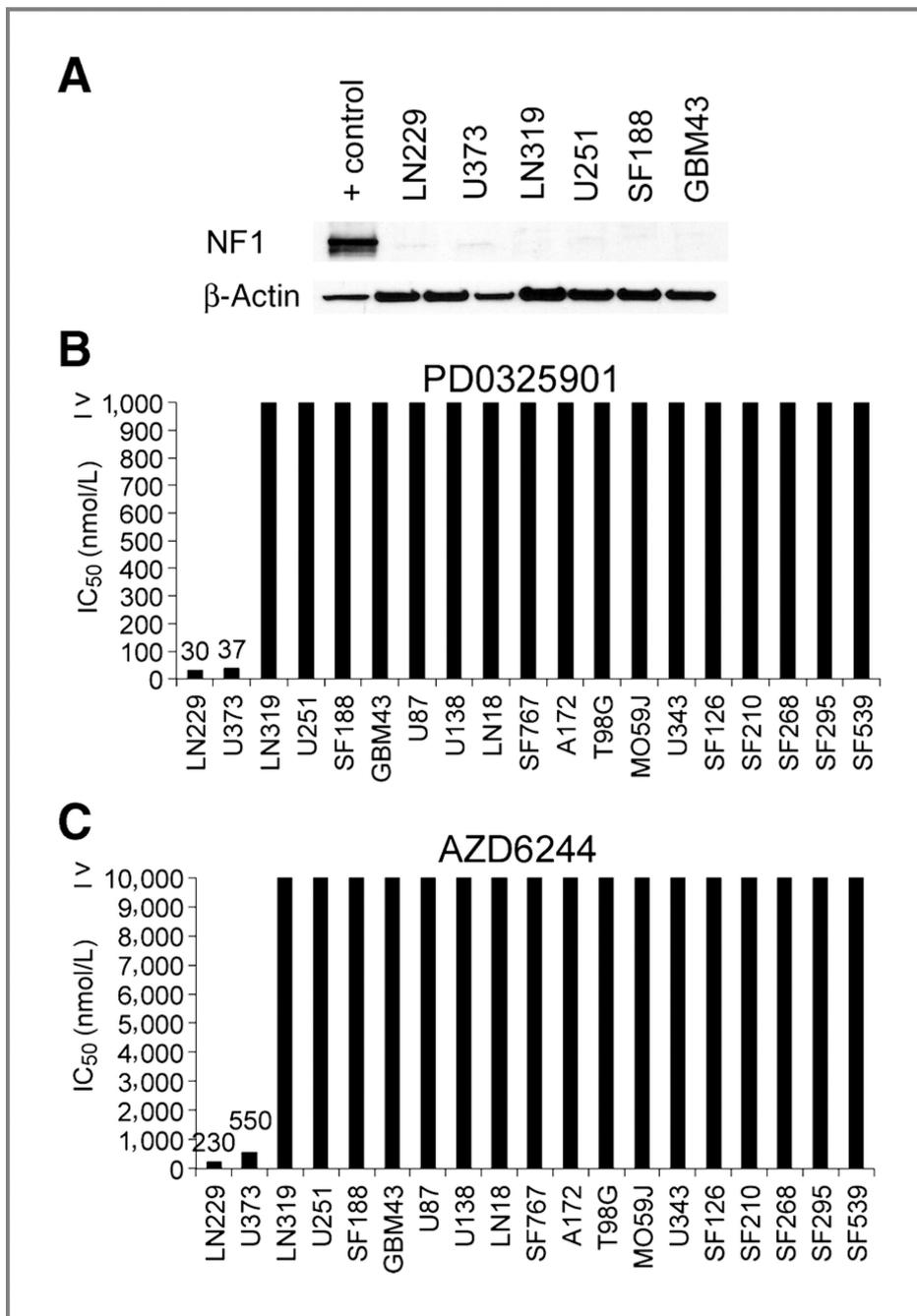


Figure 1. NF1 deficiency is associated with MEK inhibitor sensitivity. A, NF1-deficient GBM cells were subjected to immunoblot analysis using NF1- and β-actin-specific antibodies. + control, 293T cells. B and C, GBM cells were treated with 0 to 10 μmol/L PD0325901 (B) or 0 to 10 μmol/L AZD6244 (C) for 5 days, and IC₅₀ values were determined using Alamar Blue assay. Values are the mean of 3 independent experiments conducted in triplicate.

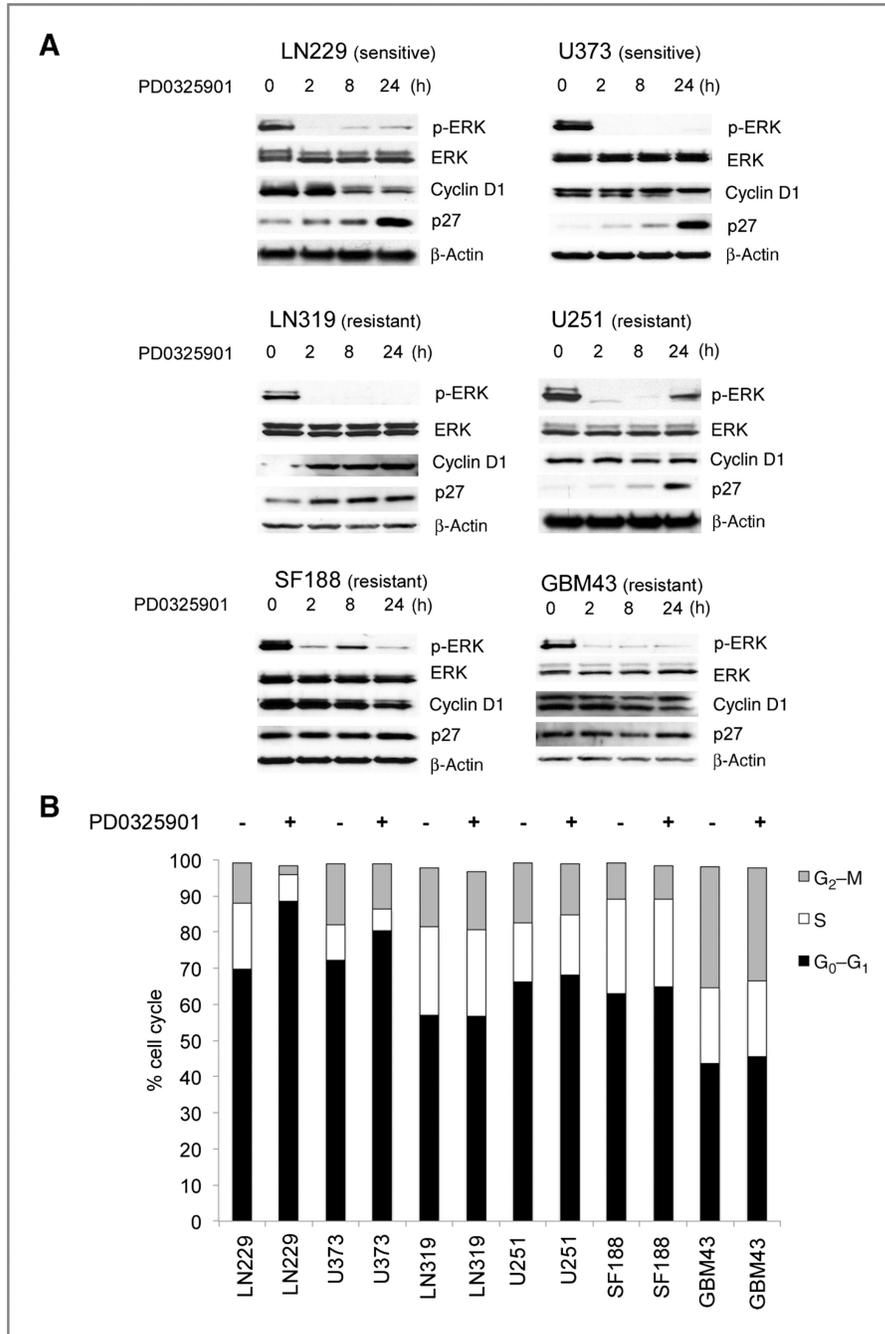
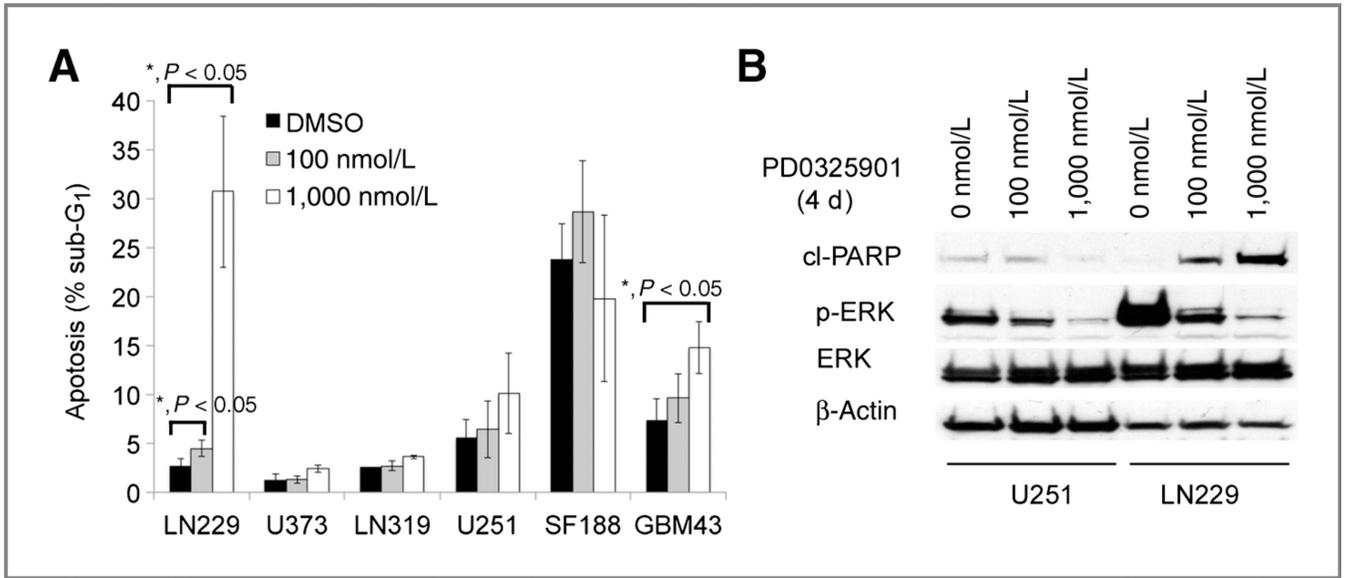


Figure 2. LN229 and U373 NF1-deficient GBM cells undergo G₁ arrest in response to PD0325901. A, NF1-deficient GBM cells were treated with 100 nmol/L PD0325901 for up to 24 hours and subjected to immunoblot analysis using the indicated antibodies. B, NF1-deficient GBM cells were treated with dimethyl sulfoxide or 100 nmol/L PD0325901 for 5 days, and FACS analysis was conducted to determine cell-cycle distribution. Values are the mean of 3 independent experiments conducted in duplicate.

**Figure 3.**

NF1-deficient LN229 GBM cells undergo apoptosis in response to PD0325901. A, NF1-deficient GBM cells were treated with PD0325901 for 5 days, and FACS analysis was conducted to determine the percentage of cells with sub-G₁ (apoptotic) DNA content. Values are the mean of 3 independent experiments conducted in duplicate. Bar, SEM. *P* value determined by Student *t* test. B, NF1-deficient U251 or LN229 GBM cells were treated with PD0325901 for 4 days and subjected to immunoblot analysis using the indicated antibodies.

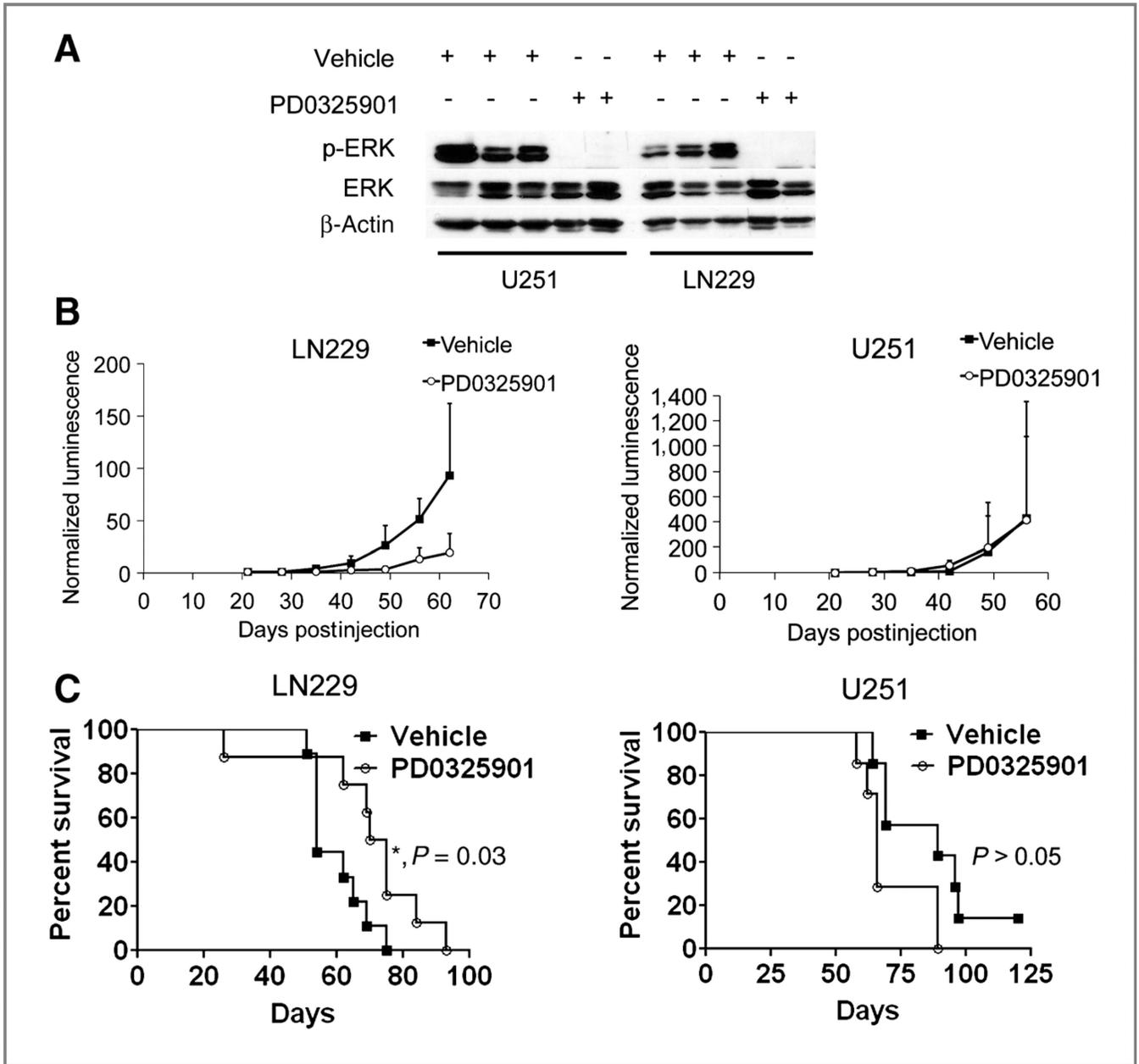


Figure 4. PD0325901 suppresses the growth of NF1-deficient LN229 GBM intracranial xenografts. A, mice harboring LN229 or U251 intracranial xenografts were treated with vehicle or 10 mg/kg PD0325901 for 24 hours. Animals were sacrificed and tumors were harvested and subjected to immunoblot analysis using the indicated antibodies. B, mice harboring luciferase expressing LN229 or U251 intracranial xenografts were treated with vehicle (LN229, $n = 9$; U251, $n = 7$) or 10 mg/kg PD0325901 (LN229, $n = 8$; U251, $n = 7$) every day for 4 weeks, and tumor growth was monitored by measuring bioluminescence. Bar, SD. C, percentage of survival of mice from B. P value was determined by log-rank test using Prism software.

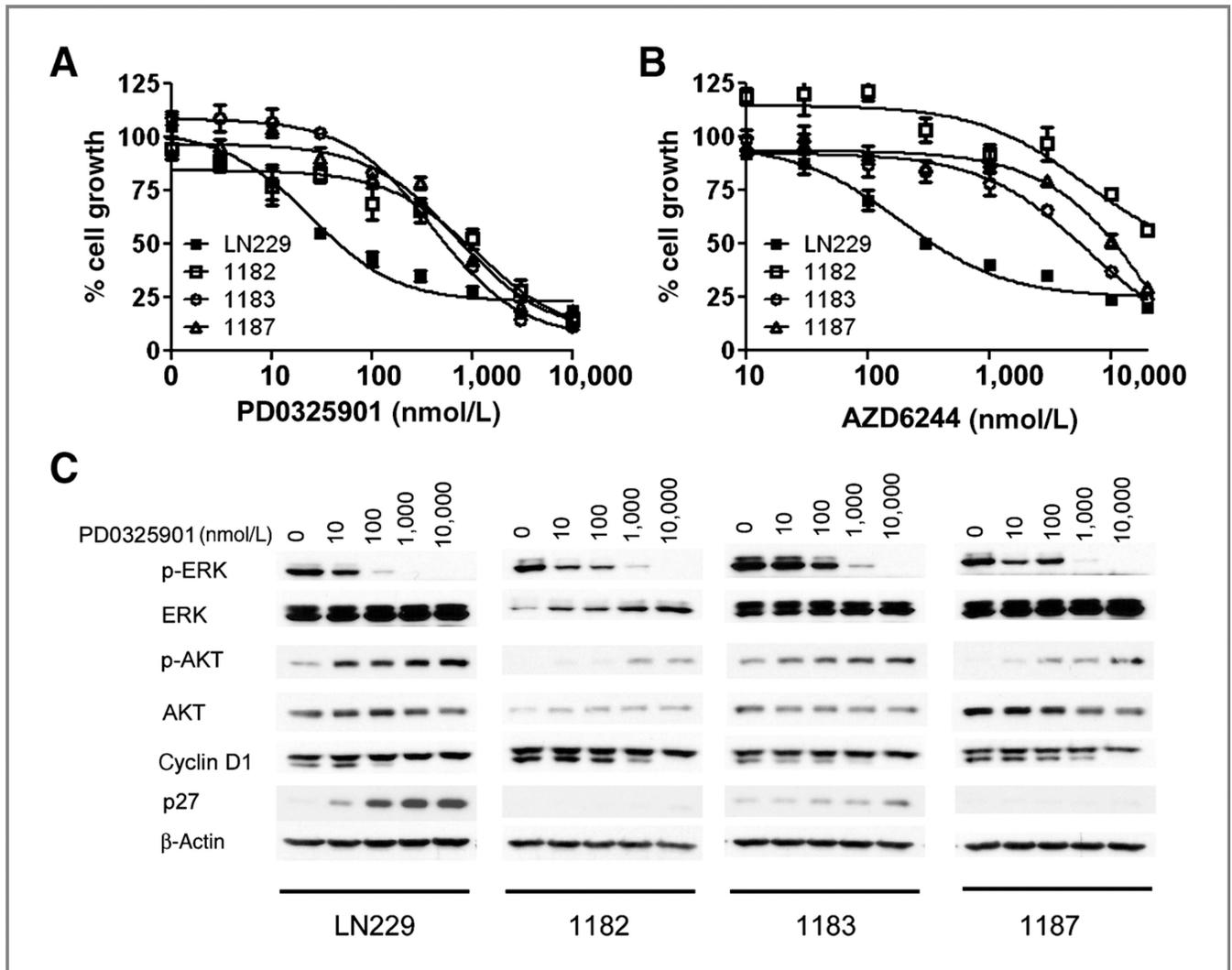


Figure 5. LN229 MEK inhibitor-resistant clones hyperactivate ERK signaling. A and B, NF1-deficient LN229 cells were chronically treated with 1 μ mol/L PD0325901 for 6 to 8 weeks, and resistant clones were isolated and expanded as clonal populations. Parental and resistant clonal populations (1182, 1183, and 1187) were treated with increasing doses of PD0325901 (A) or AZD6244 (B) for 5 days, and cell growth was determined using the Alamar Blue assay. Graph represents the mean of 3 independent experiments conducted in triplicate. C, parental or MEK inhibitor-resistant clonal populations (1182, 1183, and 1187) were treated with increasing doses of PD0325901 for 24 hours and subjected to immunoblot analysis using the indicated antibodies.

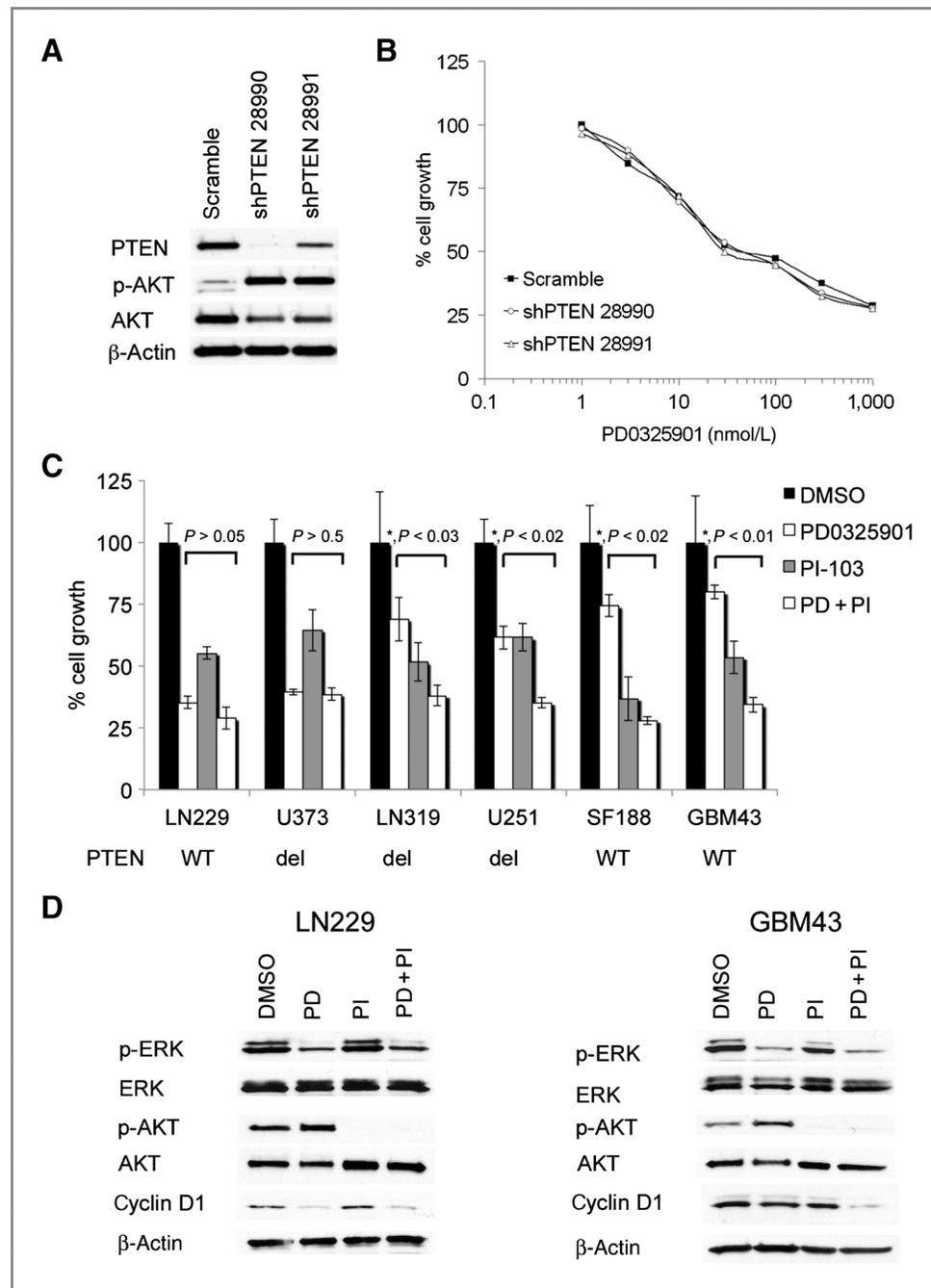


Figure 6. Inhibition of PI3K/mTOR signaling enhances sensitivity to MEK inhibition in a subset of NF1-deficient GBM cells. A, LN229 GBM cells expressing scramble or PTEN shRNAs (28990 or 28991) were subjected to immunoblot analysis using the indicated antibodies. B, LN229 cells expressing scramble or PTEN shRNAs (28990 or 28991) were treated in triplicate with PD0325901 for 5 days and analyzed for cell growth by Alamar Blue assay. C, NF1-deficient GBM cells were treated with dimethyl sulfoxide (DMSO), 100 nmol/L PD0325901, 500 nmol/L PI-103, or both drugs in combination for 5 days, and cell growth

was determined by counting viable cells. Values were derived from 3 independent experiments. Bar, SEM. *P* value determined by Student *t* test. D, LN229 or GBM43 cells were treated as in C for 24 hours and subjected to immunoblot analysis using the indicated antibodies.

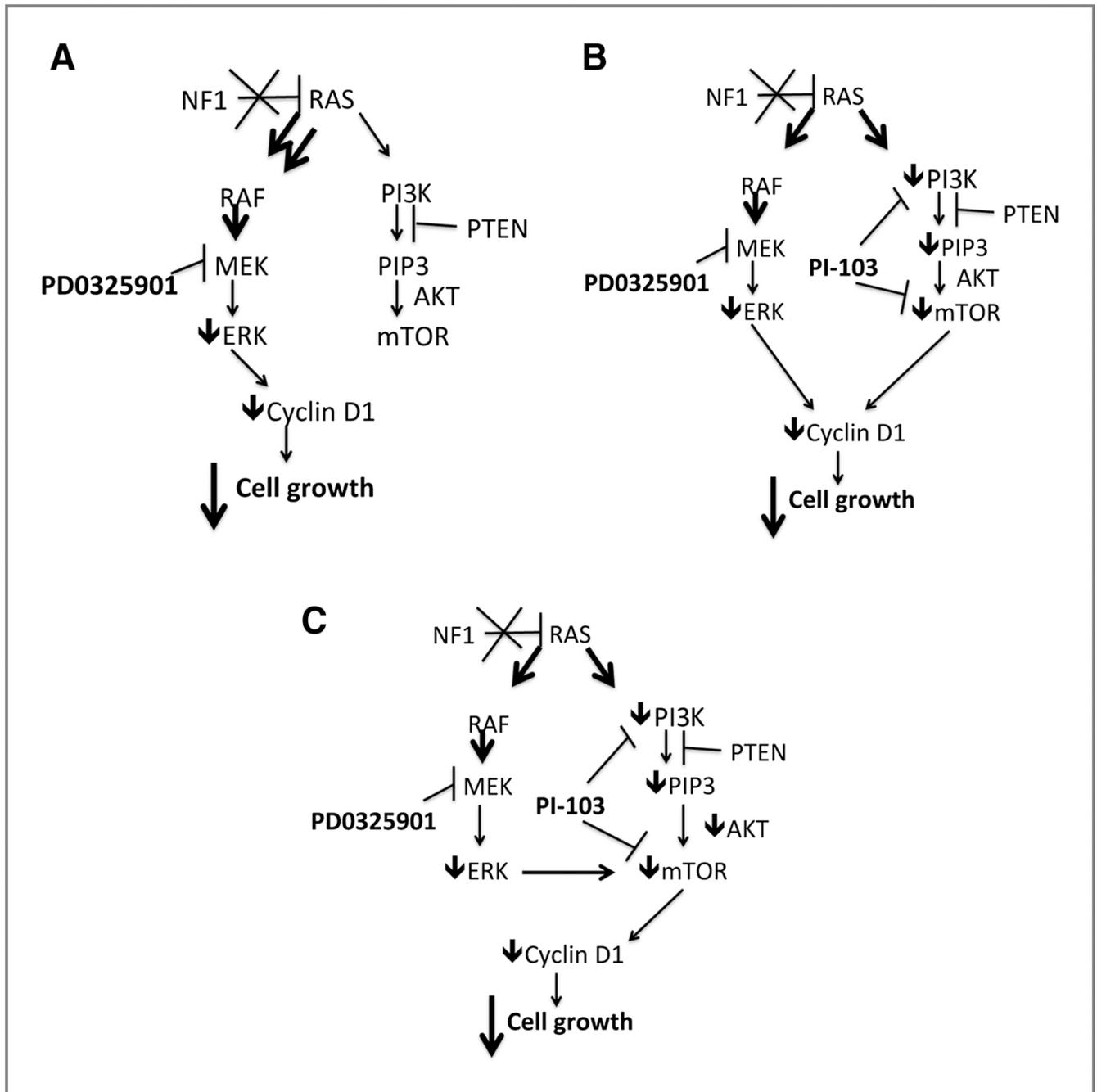


Figure 7. Models of Ras signaling in NF1-deficient GBM cell lines. A, NF1-deficient, MEK inhibitor-sensitive GBM signal through the RAF/MEK/ERK pathway. MEK/ERK inhibition by PD0325901 alone decreases cyclin D1 levels and cell growth. B, NF1-deficient, MEK inhibitor-resistant GBM signal through both RAF/MEK/ERK and PI3K/mTOR pathways. Both PD0325901 and PI-103 are required to decrease cyclin D1 levels. C, NF1-deficient, MEK inhibitor-resistant GBM signal through both the RAF/MEK/ERK and PI3K/mTOR

pathways but ERK cross-talks with the mTOR pathway. PD0325901 and PI-103 may be required to decrease mTOR-regulated translation of cyclin D1.