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Combined targeting of MEK and PI3K/mTOR effector pathways is necessary to effectively inhibit NRAS mutant melanoma in vitro and in vivo

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Activating mutations in the neuroblastoma rat sarcoma viral oncogene homolog (NRAS) gene are common genetic events in malignant melanoma being found in 15–25% of cases. NRAS is thought to activate both mitogen activated protein kinase (MAPK) and PI3K signaling in melanoma cells. We studied the influence of different components on the MAP/extracellular signal-regulated (ERK) kinase (MEK) and PI3K/mammalian target of rapamycin (mTOR)-signaling cascade in NRAS mutant melanoma cells. In general, these cells were more sensitive to MEK inhibition compared with inhibition in the PI3K/mTOR cascade. Combined targeting of MEK and PI3K was superior to MEK and $mTOR_{1,2}$ inhibition in all NRAS mutant melanoma cell lines tested, suggesting that PI3K signaling is more important for cell survival in NRAS mutant melanoma when MEK is inhibited. However, targeting of $PI3K/mTOR_{1,2}$ in combination with MEK inhibitors is necessary to effectively abolish growth of NRAS mutant melanoma cells in vitro and regress xenografted NRAS mutant melanoma. Furthermore, we showed that MEK and $PI3K/mTOR_{1,2}$ inhibition is synergistic. Expression analysis confirms that combined MEK and $PI3K/mTOR_{1,2}$ inhibition predominantly influences genes in the rat sarcoma (RAS) pathway and growth factor receptor pathways, which signal through MEK/ERK and PI3K/mTOR, respectively. Our results suggest that combined targeting of the MEK/ERK and PI3K/mTOR pathways has antitumor activity and might serve as a therapeutic option in the treatment of NRAS mutant melanoma, for which there are currently no effective therapies.

Oncogenic mutations in codons 12, 13, or 61 of the rat sarcoma (RAS) family of small GTPases, Kirsten rat sarcoma viral oncogene homolog (KRAS), Harvey rat sarcoma viral oncogene homolog (HRAS), and neuroblastoma RAS viral oncogene homolog (NRAS) occur in approximately one-third of all human cancers with NRAS mutations found in about 15–20% of melanomas (1–7). Mutated RAS proteins activate signaling pathways that promote the cell division cycle and cell growth and suppress apoptosis. Small interfering RNA (siRNA)-mediated depletion of NRAS in melanoma cell lines inhibits proliferation and renders cells sensitive to chemotherapy, making mutant NRAS and its signaling effectors relevant targets for melanoma therapy (8, 9). Efforts at developing therapeutics that inhibit mutant RAS directly have so far not been successful. The high affinity of RAS for GTP and the high concentrations of GTP intracellularly has meant that the identification of small molecules, which selectively prevent accumulation of RAS-GTP, has not been possible (10). Targeting mutant NRAS with siRNA is still limited to preclinical models because of the significant challenge in delivering antisense oligonucleotides in vivo. The response of NRAS mutant melanoma and other melanomas to various chemotherapeutic regiments has been very scarce with only 6% of patients responding (11). Alternatively, farnesyltransferase inhibitors (FTIs) were thought to inhibit RAS activation by blocking farnesylation, a key posttranslational modification step of RAS that is essential for RAS function. One FTI, R115777 (also known as tipifarnib), was evaluated in a single-agent, single-arm phase II trial in patients with metastatic melanoma. The lack of responses among the first 14 patients led to the early closure of the trial. A paucity of efficacy has also been observed for this approach in other RAS-mutated malignancies. Recently, an oral mitogen activated protein (MAP)/extracellular signal-regulated (ERK) kinase (MEK) inhibitor (MEK162) was tested in patients with metastatic melanoma harboring murine sarcoma viral oncogene homolog B1 (BRAF) or NRAS mutations with encouraging results (12). In this study, we evaluate in detail NRAS mutant primary melanomas, melanoma metastases, and 10 human NRAS mutant melanoma cell lines. The expression and role of MEK/ERK and PI3K/mammalian target of rapamycin (mTOR) phospho-proteins in viability, growth, and therapeutics of NRAS mutant melanoma tumors are assessed. Our data show that combined targeting of MEK and $PI3K/mTOR_{1,2}$ is necessary to regress NRAS mutant melanoma, thus opening the possibility of a beneficial treatment strategy.

Results

NRAS Mutant Melanoma Activates the MEK/ERK, the PI3K/mTOR Pathway, or both. Levels of phospho-ERK, p-MEK phospho-murine thymoma viral oncogene homolog 1 (p-AKT), phospho-S6 ribosomal protein (p-S6), and phosphatase and tensin homolog (PTEN) were measured in 14 primary melanomas and 18 metastases from 32 patient specimens of NRAS-mutated melanoma. Sample information, including mutation status, is provided in Table S1. Protein levels were measured by immunohistochemistry (IHC) and evaluated as the average rating of staining intensity by four independent reviewers on a scale from 3 = +++ positive to 0 = negative. Analysis of inter- and intrarater reliability can be found in Fig. S1. Across all patients, tumors displayed the strongest staining for p-ERK (1.34 ± 0.14) and p-S6 (1.16 ± 0.12). Comparing the staining patterns between patients, scoring results were divided into quartiles. Negative staining was defined as a score ≤0.75 (quartile 1), and positive staining was defined as a score >0.75 (quartile 2, 3, 4). Positive staining for p-ERK or p-S6 or both was found in 95.24% of samples stained, with 42.86% of patients positive for both proteins, 28.57% positive only for p-MEK, and 23.81% positive only for p-S6 (Fig. 1). In 28.6% of patient samples, p-ERK/p-MEK staining was negative. This was predominantly the case in melanoma metastasis although it was not statistically significant; 57.9% of slides expressed PTEN, with an average staining

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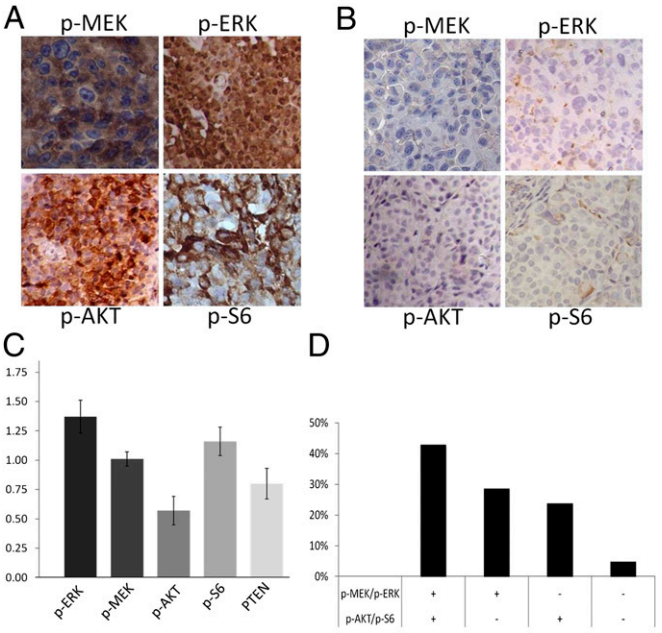

Fig. 1. (*A*) Representative +++ positive staining for p-MEK, p-ERK, p-AKT, and p-S6. (*B*) Representative negative staining for p-MEK, p-ERK, p-AKT, and p-S6. (*C*) Staining intensity of proteins in the MEK/ERK and PI3K/mTOR cascade across 32 different NRAS mutant melanomas. Error bars indicate the SD for the intensity scores from four independent raters. (*D*) Percentage of patients with positive and negative staining for p-MEK/p-ERK, p-AKT/p-S6, or both.

intensity of 0.8 ± 0.13, and 38.9% of slides expressed p-ATK with an average staining intensity of 0.57 ± 0.12.

NRAS Mutant Melanoma Cell Lines Are More Sensitive to Inhibition in the MEK/ERK than in the PI3K/mTOR Cascade. Ten NRAS mutant melanoma cell lines—3 of which harbored mutations in G12, 1 in G13, and 6 in Q61—were tested for growth inhibition in response to inhibitors in the MEK/ERK and the PI3K/mTOR pathways. Three cell lines presented with additional, low-activating BRAF mutations (G469E, D549G, D594N) (Table S2) (13). However, none of the three cell lines displayed a decrease in cell viability or changes in signaling cascades in response to a BRAF inhibitor. Moreover, siRNA directed against mutant NRAS(G13D) decreased cell viability in MaMel30I cells bearing the NRAS (G13D) mutation, demonstrating dependence on NRAS signaling (Fig. S2).

All cell lines were more sensitive to MEK inhibitors (MEKi) (JTP-74057, PD325901) than to inhibitors in the PI3K/mTOR cascade. At equimolar concentrations, the MEKi JTP-74057 displayed greater growth inhibition compared with the MEKi PD325901 [mean fold difference at 50% growth inhibition (GI50) = 4.6 ± 2.8]. Growth inhibition with single inhibitors against PI3K (GDC-0941), AKT (GSK690693), $mTOR_1$ (rapamycin), and $mTOR_{1,2}$ (PP242) was weak. Of those inhibitors, $mTORi_{1,2}$ and PI3Ki were the strongest. AKTi and rapamycin did not reduce cell viability with concentrations used in this study. Incubation with inhibitors against $PI3K/mTOR_{1,2}$, the most potent inhibitors of viability in the PI3K/mTOR cascade, decreased cell growth in all cells tested (Fig. 2). Values GI50 are displayed in Tables S3 and S4.

Immunoblot analyses showed a dose-dependent reduction of p-ERK with MEKi in all cell lines and a reduction in p-S6 levels in MM485, MM415, and Sk-Mel-2 cells. MEKi further induced p-AKT expression in MM485, MM415, WM1366, WM3629, and WM3670 cells. $PI3K/mTORi_{1,2}$ reduced phosphoprotein levels of AKT and S6 in a dose-dependent manner and resulted in slight induction of p-ERK expression in MM485, MM415, WM1366, WM3629, WM3670, and MaMel30I cells (Fig. 2 and Fig. S3).

Combination of MEK and $PI3K/mTOR_{1,2}$ Inhibitors Is Most Effective in Reducing Cell Viability in NRAS Mutant Melanoma. We further examined the growth inhibitory effect of different combinations of selective inhibitors. Combining a MEKi with a PI3Ki, $mTORi_1$, $mTORi_{1,2}$, or AKTi resulted in greater growth inhibition than with a MEKi alone in all cell lines. The combination of MEKi+PI3Ki was superior to combinations of MEKi with $mTORi_1$, $mTORi_{1,2}$, or AKTi. However, the strongest decrease in cell viability was seen with MEKi+$PI3K/mTORi_{1,2}$ (Fig. 3 and Fig. S3).

As expected, immunoblot analyses revealed "paradoxical" AKT hyperphosphorylation when cells were treated with the AKTi. This previously described effect is not due to enhancement of upstream signals to compensate for AKT signal loss, but rather is related to occupation of the AKT nucleotide-binding pocket by the AKT inhibitor (14,15). We further found that MEKi+$mTORi_{1,2}$ reduced levels of p-S6 more potently than MEKi+PI3Ki. Interestingly, the latter has a stronger effect in the reduction of viability in NRAS cell lines. Only the combination of MEKi and $PI3K/mTORi_{1,2}$ showed complete suppression of p-ERK, p-AKT, and p-S6 in D04, MaMel30I, MM485, WM3670, WM3060, and WM1366 and almost complete suppression in Sk-Mel-2 and WM3629 cells (Fig. 3 and Fig. S3).

Combined Inhibition of MEK/ERK and PI3K/mTOR Pathways Induces Apoptosis in NRAS Mutant Melanoma Cells. To evaluate if growth inhibition induced with single or combination treatment is due to apoptosis, we determined the number of cells with phosphatidyl serine exposed outside the cell membrane by labeling with an Annexin V-FITC antibody. Cells with loss of membrane integrity, reflecting the latest stage of cell death, were identified by propidium iodide (PI) staining. Results confirmed that the reduction of cell count in viability assays is a result of apoptosis (16). MEK or $PI3K/mTOR_{1,2}$ inhibition alone was not sufficient in inducing apoptosis to the same extent as the combination treatment in NRAS mutant cell lines after 24 h of incubation. Immunoblot analyses for cleaved caspase 3 and luminescent assays for caspase 3 and 7 activity further support the induction of apoptosis by the combination of MEKi+$PI3K/mTORi_{1,2}$ (Fig. 4 and Fig. S4).

Oral Dosing with the Combination of MEK and $PI3K/mTOR_{1,2}$ Inhibitors Results in Tumor Size Reduction in a Nude Mouse Xenograft Model. Concluding from our in vitro data, we hypothesized that the preferential reduction in cell growth and induction of apoptosis with the MEKi+$PI3K/mTORi_{1,2}$ in NRAS mutant cells might also be effective in inhibiting tumor growth and inducing tumor reduction in vivo. To evaluate this, we tested a MEKi, a PI3Ki, a $PI3K/mTORi_{1,2}$, and their combinations in a nude mouse NRAS mutant xenograft tumor model. NRAS mutant melanoma cell lines D04, MaMel30I, MM485, Sk-Mel-2, and WM3629 gave rise to tumors within 4–6 wk after injection, and treatment was established when tumors reached a volume of about 100 mm^3 (Fig. 5, arrow). Single-drug treatment with a PI3Ki alone did not alter tumor growth (Fig. S5). Treatment with either MEKi or $PI3K/mTORi_{1,2}$ slightly decreased or stabilized tumors in all cell lines. The combination MEKi+$PI3K/mTORi_{1,2}$ resulted in significant tumor regression after 5 d of treatment ($P < 0.05$: comparison of the combination to either MEKi or $PI3K/mTORi_{1,2}$ alone). We observed one complete response in a mouse bearing tumors with cell line MaMel30I. Weight loss, periorificial redness, and dry skin was noted in mice treated with MEKi+$PI3K/mTORi_{1,2}$. Target inhibition was assessed by immunoblotting of tumor tissue. Treatment with the MEKi alone reduced p-MEK and p-ERK expression. The $PI3K/mTORi_{1,2}$ completely inhibited p-AKT expression whereas levels of p-MEK and p-ERK were unchanged. Phospho S6 was suppressed with the $PI3K/mTORi_{1,2}$ and reduced with the MEKi. In contrast, the combination treatment potently suppressed p-MEK, p-ERK, p-AKT, and p-S6 expression in D04 and WM3629 tumors (Fig. 6) and markedly reduced phosphoprotein levels in MM485, MaMel30I, and Sk-Mel-2 tumors (Fig. S6). Apoptosis was confirmed by elevated

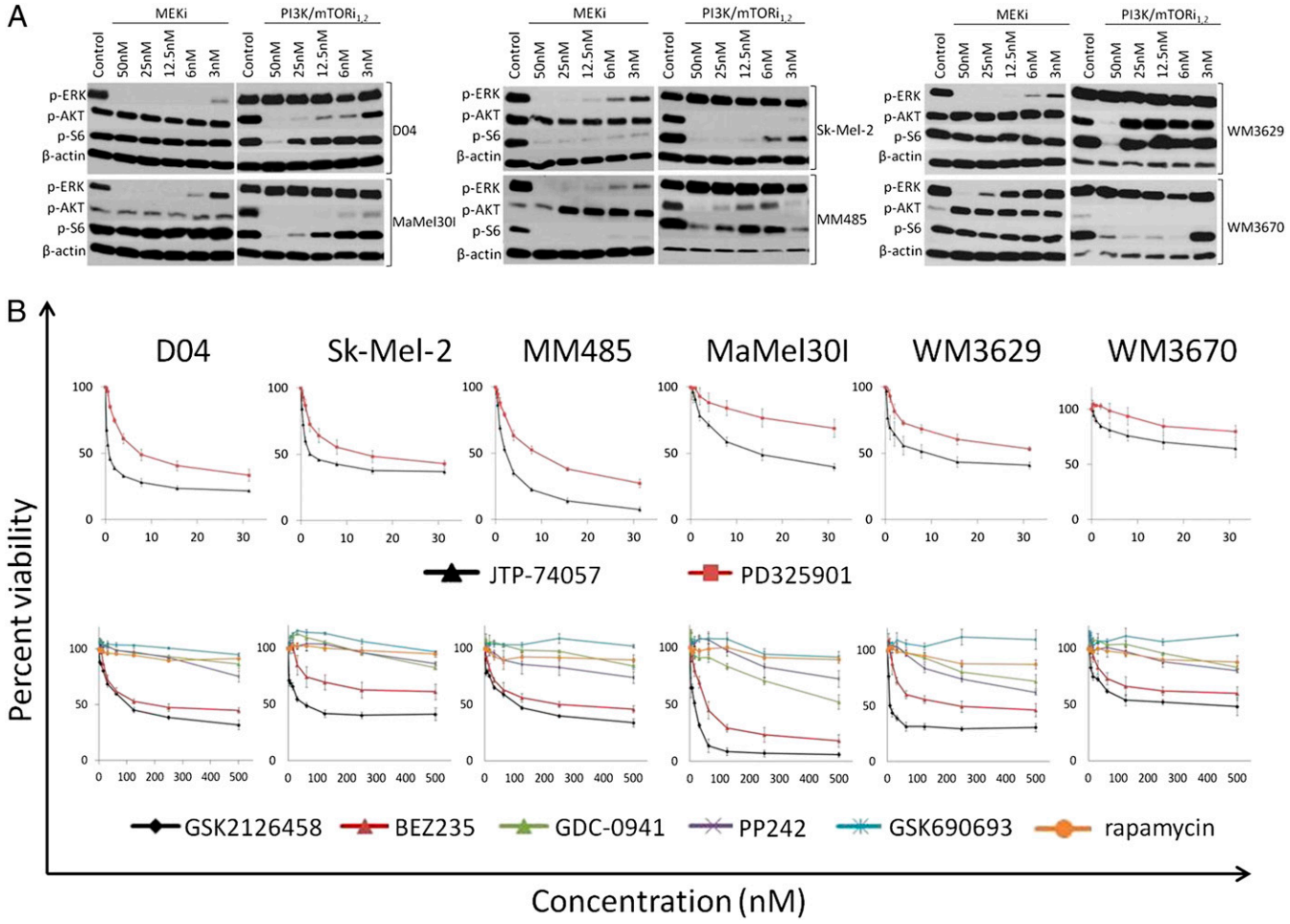

Fig. 2. (*A*) Immunoblot analyses for effector proteins of the MEK/ERK and PI3K/mTOR pathway in six NRAS mutant melanoma cell lines. Dose-dependent reduction of p-ERK by the MEKi as well as p-AKT and p-S6 by the PI3K/mTORi$_{1,2}$. Induction of p-AKT by the MEKi in MM485, WM3629, and WM3670 cells. (MEKi: JTP-74057). Slight induction of p-ERK in MM485, Sk-Mel-2, and MaMel30I cells by PI3K/mTOR$_{1,2}$ inhibition (PI3K/mTORi$_{1,2}$: GSK2126458). (*B*) Growth response curves for six NRAS mutant melanoma cell lines treated with MEK inhibitors (*Upper*) or inhibitors in the PI3K/mTOR cascade (*Lower*). MEK inhibitors are more potent in reducing cell viability than inhibitors in the PI3K/mTOR cascade with PI3K/mTORi$_{1,2}$ being more potent than selective PI3Ki, mTORi$_{1,2}$, mTOR$_1$, or AKTi. (Assay results are the mean of three replicates ± SD.)

levels of cleaved caspase 3 in tumors treated with the combination therapy.

MEK and PI3K/mTOR$_{1,2}$ Inhibition Is Synergistic in NRAS Mutant Cell Lines. We investigated whether the inhibitory effect seen was additive or synergistic. Different ratios for combinations of the MEKi+PI3K/mTORi$_{1,2}$ were assayed for synergism. NRAS mutant cell lines were synergistically inhibited with several different ratios [combination index (CI) <0.7], but all cell lines were synergistically inhibited by a molar ratio of MEKi:PI3K/mTORi$_{1,2}$ = 1:16 (JTP-74057:GSK2126458) (Fig. S4 and Table S5). When we used a different MEKi and PI3K/mTORi$_{1,2}$ such as PD325901 and BEZ235, we also found synergistic growth inhibition in MM485, MaMel30I, MaMel27II, WM3060, and WM1366 cells and additive effects in all remaining cell lines. However, these inhibitors required higher total concentrations for equipotent growth inhibition in all cells tested. Tables S3 and S4 display CI values for all human NRAS mutant cell lines treated with two different MEKi and two PI3K/mTORi$_{1,2}$, including the dose reduction index, which indicates how much the dose of each drug in a synergistic combination may be reduced at a given effect level, compared with the doses of each drug alone (17). We further found that the combination of the MEKi with the selective PI3Ki was synergistic in MaMel30I, MM485, MM415, WM1366, and WM3629 cells. Selective MEKi+ mTORi$_{1,2}$ was synergistic in MM485, MM415, and WM3629 cells.

Human Kinome Analysis of NRAS Mutant Cell Lines Reveals That Metabolic and Receptor Tyrosine Kinase Pathways Are the Most Affected by the Combination Treatment. Since protein kinases play a major role in signaling networks controlling cell growth, we investigated kinases that are mostly affected by the synergistic combination of MEKi+PI3K/mTORi$_{1,2}$. D04 and MaMel30I cells were exposed to vehicle (control), the MEKi, the PI3K/mTORi$_{1,2}$, or the combination of both drugs for 12 h. Total RNA was extracted and processed for the TaqMan OpenArray Human Kinome Panel. A total of 828 kinases and kinase-related genes were assessed with 126 probes for the cell line D04 (15.2% of total assays on the Kinome Array chip) and with 102 probes for the cell line MaMel30I (12.3% of total assays on the Kinome Array chip) displaying an at least twofold change in expression compared with the vehicle control (Dataset S1). The number of genes that showed a greater than twofold change confirmed that the effect of the combination treatment on gene expression in D04 and MaMel30I cells was greater than with the MEKi or PI3K/mTORi$_{1,2}$ alone (Fig. 7). We found that proteins involved in receptor signaling pathways, such as EGF-, VGF-, PDGF-, and FGF-signaling pathways and metabolic pathways such as the

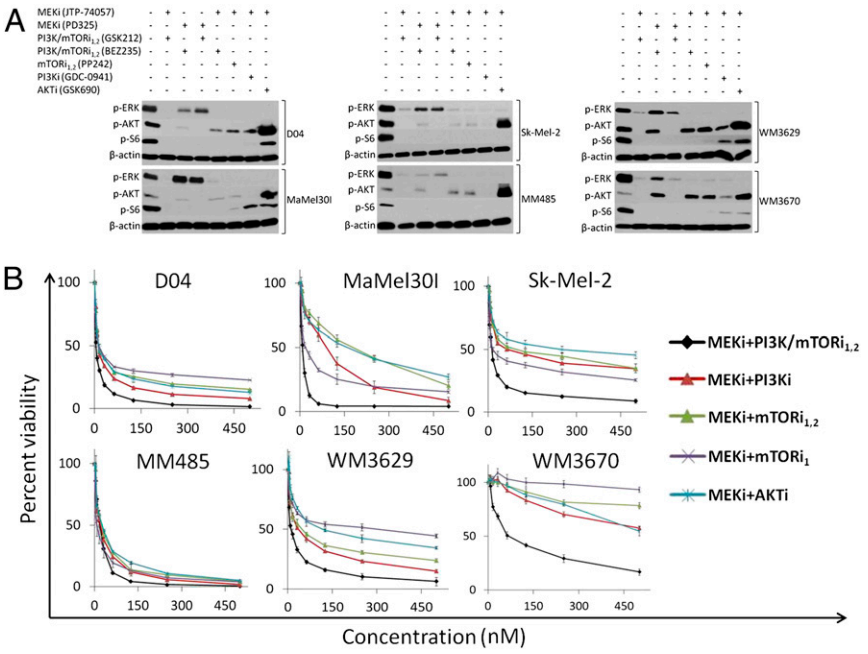

Fig. 3. (*A*) Immunoblot analyses for six NRAS mutant cell lines incubated with different combinations of inhibitors. The combination of the MEKi+PI3K/mTORi$_{1,2}$ completely suppressed p-ERK, p-AKT, and p-S6 levels in D04, MaMel30I, WM3670, and MM485 cells. MEKi+PI3Ki was more potent in reducing p-AKT, but had lower impact on p-S6 levels than MEKi+mTORi$_{1,2}$. [MEKi = 10 nM (JTP-74057, PD325901) inhibitors in the PI3K/mTOR pathway = 160 nM (PI3K/mTORi$_{1,2}$: GSK2126458, BEZ235; PI3Ki: GDC-0941; mTORi$_{1,2}$: PP242; mTORi$_1$: rapamycin; AKTi: GSK690693]. (*B*) Growth response curves for six NRAS mutant melanoma cell lines with the inhibitor combinations indicated. MEKi+PI3K/mTORi$_{1,2}$ was most effective in reducing viability in all cell lines followed by the combination of MEKi+PI3Ki in 9 of 10 cell lines. (Assay results are the mean of three replicates ± SD.)

insulin-signaling cascade, were the most affected by the combination (Fig. S7). As an example, we noted down-regulation from PFKFB4, a gene that acts to fine-tune the process by which cells convert glucose into energy. The combination also affected genes involved in cell division such as DBF4, CDC7, MAP2K6, FLT1, and NME1, supporting the substantial decrease in cyclin D1 seen by MEKi+PI3KimTORi$_{12}$ (Fig. 7). Furthermore, the combination caused the up-regulation of tumor suppressor genes such as RB1CC1 and STK11 and proapoptosis genes such as CABC1, MAP3K10, DAPK3, and MAP3K9 (Fig. S8).

Discussion

About 18–20% of all melanomas harbor oncogenic mutations in the NRAS gene, resulting in its inability to hydrolyze GTP (18, 19). The accumulation of active, GTP-bound NRAS leads to anchorage-independent signaling through several downstream cascades critical for cell proliferation, migration, and survival, thus contributing to the malignant phenotype of NRAS mutant melanoma (18, 20). The MEK/ERK and the PI3K/mTOR cascade are prototypic survival pathways that have been implicated in tumorogenesis of NRAS mutant melanoma (9, 21). Emerging preclinical evidence suggests that combined targeting of these two pathways with selective small-molecule inhibitors might be effective in treating malignancies with preexistent or acquired RAS mutations (22–25). In this study, we demonstrate that MEKi combined with PI3K/mTOR$_{1,2}$ inhibition is able to synergistically reduce cell viability in vitro and decrease tumor size in vivo in a large panel of human NRAS mutant melanoma cells.

It has recently been demonstrated that cotargeting of the MEK/ERK and PI3K/mTOR pathways with MEKi+AKTi and MEKi+mTORi$_1$ is effective in BRAF mutant cells with acquired resistance to BRAF inhibitors due to activating mutations in NRAS (26, 27). Although these combinations also decreased cell growth in our panel of 10 human NRAS mutant melanoma cell lines, we observed that it was less effective in decreasing cell viability than MEKi+PI3K/mTORi$_{1,2}$. Another study showed in vitro growth inhibition of GNAQ/GNA11 mutant cells with the combination of MEKi+PI3K/mTORi$_{1,2}$. Although these cells share partial similarities in their phosphorylation cascade with NRAS cell lines, distinct differences in their signaling cascade are evident. Most importantly, loss of mutant GNAQ or GNA11 has no significant effect on p-AKT levels. In contrast, NRAS mutant melanoma cells signal strongly through AKT, pointing to the fact that, although different cell lineages may share sensitivity to therapeutics, differences in the signaling network and in vivo response might be found (9, 16).

Other studies based on data derived from mouse melanocytes from an inducible NRAS transgenic mouse model identified CDK4 as a coextinction target with MEK (28). We treated our

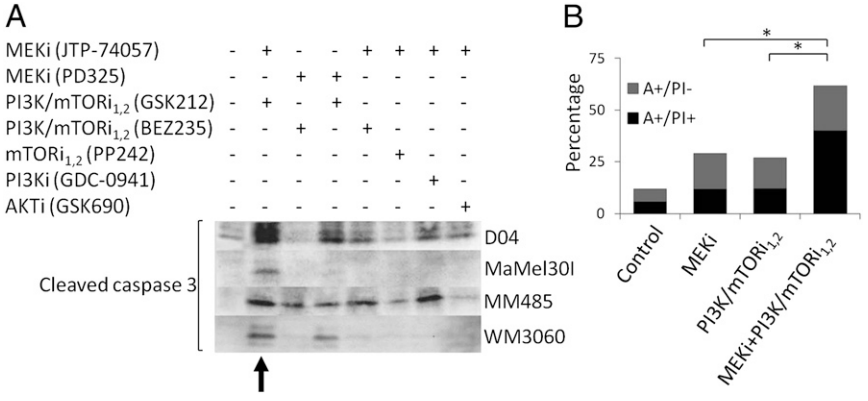

Fig. 4. (*A*) Induction of cleaved caspase 3 by the combination MEKi+PI3K/mTORi$_{1,2}$ (arrow) in D04, MaMel30I, MM485, and WM3060 cells. (*B*) Flow cytometric analysis to determine the number of apoptotic cells. Six NRAS mutant cell lines were treated with DMSO (control), a MEKi, a PI3K/mTORi$_{1,2}$, or the combination of both for 24 h. Column charts display the average of Annexin V+/PI+ (apoptotic) cells and Annexin V+/PI– (preapoptotic) cells for all cell lines treated with the indicated conditions. (MEKi: JTP-74057 = 10 nM; PI3K/mTORi$_{1,2}$: GSK2126458 = 160 nM; *$P < 0.05$; $n = 2$.)

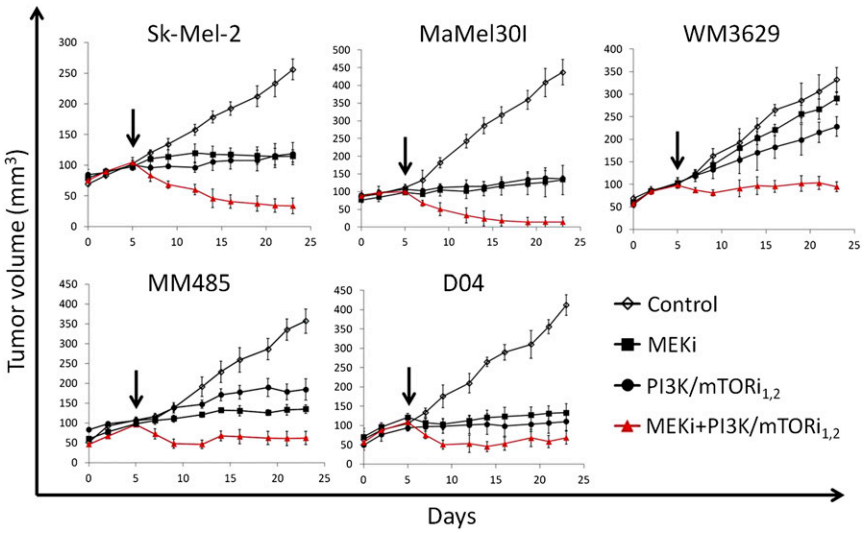

Fig. 5. Treatment of established tumors in a nude mouse xenograft model of NRAS mutant melanoma with tumor size reduction after initiation of treatment (arrow) with the combination of a MEKi (1.5 $mg \cdot kg^{-1} \cdot d^{-1}$) and PI3K/mTORi$_{1,2}$ (1.5 $mg \cdot kg^{-1} \cdot d^{-1}$), but not with either MEKi or PI3K/mTORi$_{1,2}$ alone. (cell lines: Sk-Mel-2, MaMel30I, WM3629, MM485, D04; MEKi: JTP-74057; PI3K/mTORi$_{1,2}$: GSK2126458) Results are the mean tumor volume at indicated time points ± SD; $n = 4$ mice per group.

panel of 10 human NRAS mutant melanoma cell lines with a MEKi, CDK4/6i, or the combination of both at different concentrations and ratios. In our hands, the observed effect with the combination treatment was antagonistic in all cell lines at a ratio of MEKi:CDKi$_{4,6}$ = 1:16. When different ratios were used in cell line MM415, no significant difference was noted between the MEKi alone and the combination of MEKi+CDKi$_{4,6}$ (Fig. S9). However, these results need to be interpreted with caution as other ratios and concentrations could still be synergistic. We also did not test MEKi+CDKi$_{4,6}$ in vivo.

This study uses a very large set of human NRAS mutant melanoma cell lines, demonstrating in vitro and in vivo, that combined targeting of MEK and PI3K/mTOR$_{1,2}$ inhibits cell growth and induces apoptosis leading to tumor size reduction.

In our first set of experiments, we evaluated the expression of phosphorylated effector proteins in the MEK/ERK and the PI3K/mTOR pathway in primary and metastatic NRAS mutant melanomas of 32 different patients by IHC. We found that over 95% of the samples expressed phosphorylated effector proteins in either the MEK/ERK or the PI3K/mTOR pathway, adding evidence to previous studies that found that these pathways are active in NRAS mutant melanoma (9, 29). Over half of the slides showed expression of phosphoproteins in both pathways. Staining intensity was significantly higher for the MEK/ERK than for the PI3K/mTOR pathway (MEK/ERK 1.2 ± 0.2 PI3K/mTOR 0.86 ± 0.3 $P = 0.015$), suggesting a predominance of this pathway in NRAS mutant melanomas stained. Recent studies demonstrate that cells with activating mutations in the RAS pathway respond to MEK inhibitors; however, in melanoma, this is especially true for RAF mutant tumors with no other activating alternations (12, 30, 31). To test whether NRAS mutant melanoma cell lines are sensitive to MEK inhibition, we characterized their response to two different MEK inhibitors. Treatment reduced cell growth in all cell lines, but only D04 cells were particularly sensitive to MEK inhibition (Fig. 2 and Tables S3 and S4). This is in agreement with findings from other groups and might be explained by the recruitment of additional prosurvival downstream effector pathways by mutant NRAS such as the PI3K/mTOR pathway (29, 30, 32, 33). Indeed, previous studies found that binding of RAS to PI3Kp100α is required for RAS-driven tumorigenesis and that mTOR$_2$ is essential for transformation as well as for the vitality of melanoma cells (32, 34). However, targeting the PI3K/mTOR pathway in isolation only moderately reduced cell viability with PI3K/mTOR$_{1,2}$ inhibitors being the most potent. In this study, dual pathway activation and cross talk are supported by IHC results from patient specimens and immunoblot analyses that show dose-dependent induction of p-AKT in response to MEKi and induction of p-ERK by a PI3K/mTORi$_{1,2}$ (Figs. 1 and 2). This suggests that activation of the MEK/ERK and the PI3K/mTOR pathways by mutant NRAS confers relative resistance to drugs targeting one pathway in isolation (29, 35, 36).

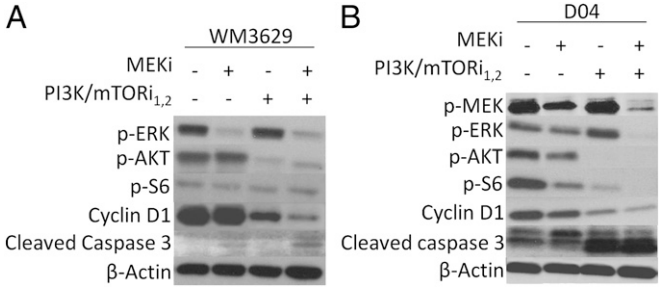

Fig. 6. Immunoblot analyses of tumors from mice treated with the conditions indicated. (*A*) The combination MEKi+PI3K/mTORi$_{1,2}$ markedly reduced phospho-protein levels in WM3629 tumors and (*B*) completely abolished expression of p-ERK, p-AKT, and p-S6 in D04 tumors. (*A*) Dramatic decrease of cyclin D1 levels in WM3629 and (*B*) a strong induction of cleaved caspase 3 was seen in D04 tumors.

Combining a MEKi with inhibitors of PI3K, mTOR complexes, or AKT provides additional inhibitory activity compared with MEKi alone. Residual protein levels of p-ERK, p-AKT, and p-S6 show incomplete inhibition, which might explain cell survival (37, 38). In contrast, MEKi+PI3K/mTORi$_{1,2}$ revealed complete suppression of p-ERK, p-AKT, and p-S6 levels in most cell lines and was most potent in reducing cell viability in all cell lines tested in this study (Fig. 3). Furthermore, we demonstrate tumor size reduction in vivo with five different human NRAS mutant melanoma cell lines. Targets that might account for the synergistic effect were assessed by kinome expression profiling, which revealed that MEKi+PI3K/mTORi$_{1,2}$ affected proteins involved in metabolic and receptor signaling pathways as well as proapoptotic and tumor suppressor proteins (Fig. 7).

Our study offers a unique comparative insight into the differences among the effect of various single and combined inhibitors of the MEK/ERK and PI3K/mTOR cascades. Taking together the preclinical data presented in this work, MEKi+PI3K/mTORi$_{1,2}$ is the most effective combination to inhibit NRAS mutant melanoma cells in vitro and in vivo and might serve as a therapeutic option in the treatment of NRAS mutant melanoma, for which there are currently no effective therapies.

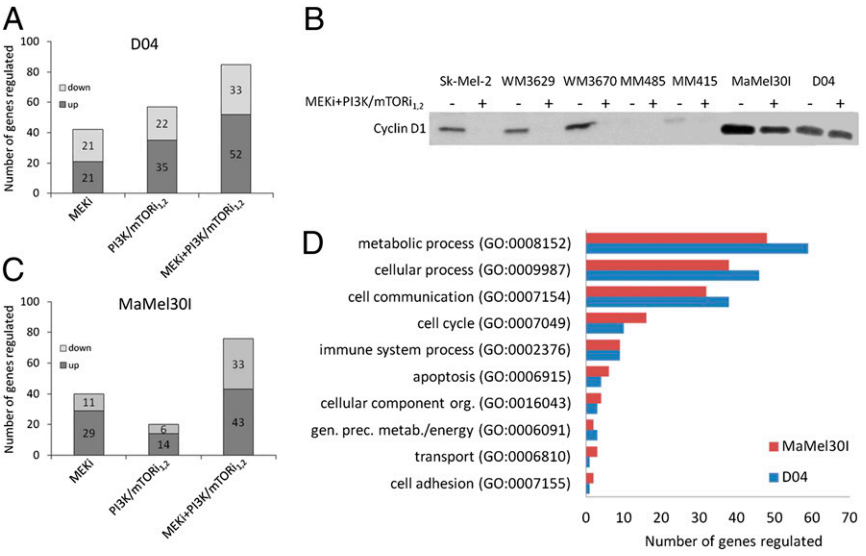

Fig. 7. (*A* and *C*) Number of genes equal to or more than twofold up- and down-regulated in the three treatment groups compared with the vehicle control for cell lines D04 and MaMel30I. (*B*) The combination MEKi+PI3K/mTORi$_{1,2}$ completely abolished cyclin D1 levels in Sk-Mel-2, WM3629, WM3670, and MM415 cells. (*D*) Biological processes regulated by MEKi+PI3K/mTORi$_{1,2}$. Bars represent the number of genes regulated by the combination treatment. gen., generation; metab., metabolism; prec., precursor.

Materials and Methods

All inhibitors (JTP-74057, PD325901, GSK2126458, BEZ235, GDC-0941, PP242, rapamycin, GSK690693, and PD0332991) were purchased from Selleck Chemicals and ChemieTek. Schematic interaction sites for the specific inhibitors are displayed in Fig. S10. Cell culturing and growth inhibition experiments as well as immunohistochemistry, immunoblotting, xenograft studies, and kinome array experiments have been performed as previously described. Detailed information can also be found in *SI Materials and Methods*.

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