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PIK3CA-Mutated Melanoma Cells Rely on Cooperative Signaling through mTORC1/2 for Sustained Proliferation

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SUMMARY

Malignant conversion of *BRAF*- or *NRAS*-mutated melanocytes into melanoma cells can be promoted by PI3'-lipid signaling. Using human *NRAS*- or *BRAF*-mutated melanoma cells that co-express mutationally activated *PIK3CA*, we explored the contribution of PI3'-lipid signaling to cell proliferation. Despite mutational activation of *PIK3CA*, melanoma cells were more sensitive to the biochemical and anti-proliferative effects of broader spectrum PI3K inhibitors than to an α -selective PI3K inhibitor. Combined pharmacological inhibition of MEK1/2 and PI3K signaling elicited more potent anti-proliferative effects and greater inhibition of the cell division cycle compared to single-agent inhibition of either pathway alone. Analysis of signaling downstream of MEK1/2 or PI3K revealed that these pathways cooperate to regulate cell proliferation through mTORC1-mediated effects on ribosomal protein S6 and 4E-BP1 phosphorylation in an AKT-dependent manner. Although PI3K inhibition resulted in cytostatic effects on xenografted *NRAS*^{Q61H}/*PIK3CA*^{H1047R} melanoma, combined inhibition of MEK1/2 plus PI3K elicited significant melanoma regression. This study provides insights as to how mutationally activated *PIK3CA* acts in concert with MEK1/2 signaling to cooperatively regulate mTORC1/2 to sustain *PIK3CA*-mutated melanoma proliferation.

Keywords

melanoma; PIK3CA; MEK1/2; mTORC1/2; BRAF

INTRODUCTION

BRAF or *NRAS* are the most commonly mutated proto-oncogenes in melanoma with ~50% of melanomas expressing mutationally activated *BRAF*^{V600E} and 25–30% expressing mutationally activated *NRAS* (Brose et al., 2002; Cancer Genome Atlas, 2015; Davies et al., 2002; Hodis et al., 2012; Holderfield et al., 2014). *BRAF* (>80%) and *NRAS* (~14%)

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mutations are also present at a high frequency in pigmented nevi; however these melanocytic lesions are generally benign, rarely progress to melanoma, and display hallmarks of a senescence-like growth arrest (Michaloglou et al., 2005; Mooi and Peepers, 2006; Pollock et al., 2003). Thus, *BRAF*^{V600E} or *NRAS*-mediated conversion of normal melanocytes to malignant melanoma cells requires additional cooperating events (Brose et al., 2002; Cancer Genome Atlas, 2015; Davies et al., 2002; Hodis et al., 2012; Shain et al., 2015; Shull et al., 2012).

Aberrant PI3'-lipid accumulation is a common mechanism of melanoma progression and there are several ways in which PI3'-lipid signaling may be elevated in melanoma (Cancer Genome Atlas, 2015; Chin et al., 1998; Goel et al., 2006; Haluska et al., 2006; Hodis et al., 2012; Shull et al., 2012; Vredeveld et al., 2012). These include silencing of the *PTEN*, *INPP4B*, or *PIB5PA* lipid phosphatases, mutational activation of *PIK3CA* or *RAC1*, which can activate PI3K α or PI3K β respectively, or mutational activation of the PI3'-lipid effector kinases *AKT1* or *AKT3* (Cancer Genome Atlas, 2015; Chi et al., 2015; Hodis et al., 2012; Perez-Lorenzo et al., 2013; Shull et al., 2012; Ying et al., 2003). Although *PTEN* silencing is common in melanoma, mutational activation of *PIK3CA* is rare, despite the ability of activated PIK3CA^{H1047R} to promote progression of BRAF^{V600E}-initiated melanomas in mouse models and the high frequency of the *PIK3CA* mutation in other types of cancer (Cancer Genome Atlas, 2015; Curtin et al., 2006; Deuker et al., 2015; Hodis et al., 2012; Marsh Durban et al., 2013; Omholt et al., 2006; Samuels and Velculescu, 2004). Since mutationally activated *NRAS* is reported to simultaneously activate both RAF- and PI3K-mediated signaling, silencing of *PTEN* or mutational activation of *PIK3CA* occurs rarely in *NRAS*-mutated melanomas (Cancer Genome Atlas, 2015; Curtin et al., 2006; Hodis et al., 2012; Kim et al., 2012; Shull et al., 2012; Tsao et al., 2004). Furthermore, both the TCGA and Broad Institute genome sequencing analyses of melanoma samples were utilized to examine the frequency of co-existing mutations in *PIK3CA* plus *BRAF* or *NRAS* in melanoma. The TCGA analysis revealed that, 13 out of 287 (5%) melanoma tumor samples sequenced displayed an alteration in *PIK3CA* (Cerami et al., 2012; Gao et al., 2013). Of these 13 alterations, two were copy number gains (gene amplifications), three were known driver mutations, seven were variants of unknown significance, and one was a homozygous deletion (Cerami et al., 2012; Gao et al., 2013). Similarly, the Broad Institute analysis of 121 melanoma specimens also revealed a *PIK3CA* mutation frequency of 5% (6 out of 121) (Cerami et al., 2012; Gao et al., 2013). Of the six mutations identified in *PIK3CA* from the Broad Institute analysis, five are ascribed as driver mutations and one is a variant of unknown significance (Cerami et al., 2012; Gao et al., 2013). The vast majority of *PIK3CA* mutations co-occurred with either a *BRAF* or a *NRAS* mutation, but this is not surprising since mutational alterations of *BRAF* or *NRAS* was detected at a frequency of 81% or 86% in melanoma samples from the TCGA and Broad Institute analyses, respectively (Cerami et al., 2012; Gao et al., 2013). Hence, these data indicate that melanoma with co-existing mutations in *PIK3CA* plus *BRAF* or *NRAS* represent a small, but relevant subset of melanomas.

In *BRAF*-mutated melanoma cell lines, BRAF^{V600E} and PI3'-lipid signaling cooperate to regulate melanoma proliferation through AKT-independent, mTORC1-dependent effects on protein synthesis (Dankort et al., 2009; Deuker et al., 2015; Marsh Durban et al., 2013; Silva et al., 2014). Therefore, to further assess the role of PI3K signaling in melanoma, we

employed *PIK3CA*-mutated cell lines derived from chemo-naïve metastatic melanoma patients (NZM40: *NRAS*^{Q61H}/*PIK3CA*^{H1047R}; NZM52: *BRAF*^{V600E}/*PIK3CA*^{H1047R}; and NZM91: *PIK3CA*^{E545K}) (Kim et al., 2012). These cell lines were used to examine the contribution of mutationally activated p110α PI3K to melanoma cell signaling, proliferation, and tumorigenesis by interrogating the pathway with a panel of pharmacological PI3K inhibitors. We observed that combined inhibition of MEK1/2 and PI3K signaling elicited more potent anti-proliferative effects and greater suppression of S-phase progression of the cell cycle compared with single-agent inhibition of either pathway alone. Similarly, pharmacological inhibition of mTORC1/2 also displayed substantial anti-proliferative effects on *PIK3CA*-mutated melanoma cells. Surprisingly, *BRAF*^{V600E}/*PIK3CA*^{H1047R} mutant melanoma cell proliferation was largely resistant to *BRAF*^{V600E} inhibition despite the profound inhibition of S-phase progression in these cells, which may be a consequence of reactivation of MAPK signaling. Thus, these data support the rationale that the maintenance of *PIK3CA*-mutated melanoma cells depends on the ability of MEK1/2 and PI3K signaling to cooperatively regulate mTORC1.

RESULTS

Cooperative regulation of cell proliferation by MEK1/2 and PI3K signaling in *PIK3CA*-mutated melanoma cells

To determine the dependency of *PIK3CA*-mutated melanoma cells on MAPK and PI3K signaling, we examined the effects of pharmacological blockade of MEK1/2 or the differential effects of structurally unrelated, mechanistically dissimilar PI3K inhibitors using human melanoma-derived cell lines that were generated from metastatic melanoma patients in New Zealand (NZM40, NZM52, and NZM91; Supplementary Table S1 and S2) (Kim et al., 2012). The NZM40, NZM52, and NZM91 cell lines were selected based solely on their reported *PIK3CA* mutational status: NZM40 and NZM52 cells express *PIK3CA*^{H1047R} and NZM91 cells express *PIK3CA*^{E545K}, both of which are gain-of-function forms of PI3'-kinase-α (Kim et al., 2012). In addition, NZM40 cells express *NRAS*^{Q61H} and the NZM52 cell line expresses *BRAF*^{V600E}, the latter a combination of genetic abnormalities that we have examined in genetically engineered mouse (GEM) models (Deuker et al., 2015; Kim et al., 2012). Hybridization-based target enrichment and sequencing of approximately 500 cancer genes confirmed mutational activation of *NRAS*, *BRAF*, or *PIK3CA* in the relevant cell lines, but failed to identify an oncogenic driver of RAS→RAF→MEK1/2→ERK1/2 MAP kinase signaling in NZM91 cells, including no evidence of bi-allelic loss of *NFI*.

To assess the effects of MEK1/2 or PI3K inhibition, NZM40, NZM52, and NZM91 cells were treated with inhibitors of MEK1/2 (5μM GDC-0973/MEKi¹), class I PI3K (5μM GDC-0941/PI3Ki¹; inhibits PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ), β-sparing PI3K (5μM GDC-0032/PI3Ki²; inhibits PI3Kα, PI3Kδ, and PI3Kγ) or PI3Kα (5μM BYL-719/PI3Kαi; selectively inhibits PI3Kα), either as single-agents or in combination, for 3–8 days, with viable cells fixed and stained using crystal violet (Fig. 1A). Single-agent inhibition of MEK1/2, PI3Kα, β-sparing PI3K, or class I PI3K led to suppression of proliferation of all three cell lines. Class I PI3K inhibition elicited the most potent anti-proliferative effects against NZM40 and NZM91 cells, whereas single-agent inhibition of class I PI3K or β-

sparing PI3K inhibited NZM52 proliferation to a similar extent. Of the various PI3K inhibitors tested, the PI3K α inhibitor displayed the least potent anti-proliferative effects against all three cell lines. Importantly, the combined inhibition of MEK1/2 plus class I PI3K, β -sparing PI3K, or PI3K α displayed enhanced anti-proliferative effects in all three cell lines compared with the corresponding single-agents.

To test whether mutationally activated *PIK3CA* contributes to S-phase progression, NZM cells were treated with inhibitors of MEK1/2 (5 μ M GDC-0973/MEKi¹), class I PI3K (5 μ M GDC-0941/PI3Ki¹), or PI3K α (5 μ M BYL-719/PI3K α i), either alone or in combination, for 48 hours with cells labeled with BrdU for the last 24 hours of the treatment and analyzed by flow cytometry (Fig. 1B). MEK1/2 inhibition significantly reduced BrdU incorporation in all three cell lines (NZM40: $p < 0.01$; NZM52 and NZM91: $p < 0.0005$) in a manner consistent with the decrease in proliferation observed following MEK1/2 inhibition (Fig. 1A). Perhaps surprisingly, treatment of NZM40 cells with either a PI3K α or class I PI3K inhibitor had only modest effects on BrdU incorporation, even though these inhibitors displayed potent anti-proliferative effects in these cells (Figs. 1A and B). By contrast, treatment of NZM91 cells with either a PI3K α or class I PI3K inhibitor significantly decreased BrdU incorporation (PI3K α : $p < 0.005$; class I PI3K: $p < 0.0001$) in accordance with the anti-proliferative effects of these inhibitors in this cell line (Fig. 1A and B). Strikingly, in NZM52 cells, PI3K α inhibition more potently suppressed S-phase progression compared to class I PI3K inhibition, whereas class I PI3K inhibition resulted in a greater inhibitory effect on NZM52 proliferation (Figs. 1A and B). Importantly, combined inhibition of MEK1/2 plus either PI3K α or class I PI3K inhibition led to a more profound reduction in BrdU incorporation (>86% decrease) compared with the corresponding single-agents in all three NZM cell lines, which was analogous to the anti-proliferative effects observed with these combined treatments (Figs. 1A and B). Hence, these data indicate that MEK1/2 and PI3'lipid signaling cooperate to regulate *PIK3CA*-mutated melanoma cell proliferation through S-phase of the cell division cycle.

To examine the consequences on intracellular signaling of single agent versus combined MEK1/2 or PI3K inhibition on the NZM melanoma cell lines, cells were treated with inhibitors of MEK1/2 (5 μ M GDC-0973/MEKi¹), class I PI3K (5 μ M GDC-0941/PI3Ki¹), β -sparing PI3K (5 μ M GDC-0032/PI3Ki²), or PI3K α (5 μ M BYL-719/PI3K α i), either alone or in combination, for 24 hours with extracts analyzed by immunoblotting (Fig. 1C). As expected, MEK1/2 inhibition led to decreased ERK1/2 phosphorylation (P-ERK1/2 Thr202/Tyr204) with little or no effect on AKT phosphorylation (P-AKT Ser473). Conversely, class I PI3K, β -sparing PI3K, or PI3K α inhibition resulted in reduced P-AKT and its direct downstream target, P-PRAS40 (Thr246) with largely no effect on P-ERK1/2. Strikingly, MEK1/2 inhibition had little or no effect on the phosphorylation of the mTOR Complex I (mTORC1) downstream effectors, ribosomal protein (rp) S6 (P-rpS6 Ser235/236 and Ser240/244) and 4E-BP1 (P-4EBP1 Thr37/46 and Ser65), despite inhibition of P-p70^{S6K} (Thr389), the upstream kinase of rpS6. The lack of efficacy of the MEK1/2 inhibitor to reduce P-rpS6 or P-4E-BP1 in NZM52 (BRAF^{V600E}, PIK3CA^{H1047R}) cells stands in contrast to the potent inhibitory effects of MEK1/2 inhibition on P-rpS6 and P-4E-BP1 in *BRAF*-mutated cells as previously reported (Silva et al., 2014). Single-agent inhibition of class I PI3K, β -sparing PI3K, or PI3K α led to decreased P-rpS6 and P-4E-BP1, but this

occurred in the absence of any changes in P-p70^{S6K} in NZM40 and NZM91 cells. Class I PI3K or β -sparing PI3K also reduced P-rpS6 and P-4E-BP1 without altering P-p70^{S6K} in NZM52 cells. However, inhibition of PI3K α had little or no effect on P-p70^{S6K}, P-rpS6, or P-4E-BP1 in NZM52 cells. Combined MEK1/2 plus class I PI3K, β -sparing PI3K, or PI3K α inhibition more potently suppressed P-rpS6 compared with the action of the various single-agents in all NZM cell lines. Although combined inhibition of MEK1/2 and class I PI3K, β -sparing PI3K, or PI3K α led to a greater reduction in P-4E-BP1 in NZM52 cells, these combined treatments were ineffective in further decreasing P-4E-BP1 in NZM40 or NZM91 cells compared to any of the three PI3K inhibitors. Taken together, these data suggest that MEK1/2 and PI3K signaling cooperate to regulate the mTORC1 signaling axis through the phosphorylation of rpS6 and 4E-BP1, which in turn may contribute to the proliferation of *PIK3CA*-mutated melanoma cells.

BRAF^{V600E} inhibitor resistance in BRAF^{V600E}/PIK3CA^{H1047R}-mutated melanoma cells

Previous analyses demonstrated the sensitivity of *BRAF*-mutated melanoma cells to BRAF^{V600E} inhibition, consequently we tested the effects of BRAF^{V600E} inhibition on NZM52 proliferation and signaling (Deuker et al., 2015; Marsh Durban et al., 2013; Silva et al., 2014). NZM52 cells were treated with inhibitors of BRAF^{V600E} (100nM LGX-818/BRAFi), class I PI3K (5 μ M GDC-0941/PI3Ki¹), or PI3K α (5 μ M BYL-719/PI3K α i), either alone or in combination, with viable cells stained using crystal violet (Fig. 2A). To our surprise, neither single-agent nor combined inhibition of BRAF^{V600E} or PI3K α displayed anti-proliferative activity against these cells. Class I PI3K inhibition significantly suppressed NZM52 proliferation while the combined inhibition of BRAF^{V600E} and class I PI3K did not further reduce proliferation compared to single-agent treatment. These results differ from previous analyses, in which combined inhibition of BRAF^{V600E} and class I PI3K more profoundly inhibited *BRAF*-mutated melanoma proliferation compared to their corresponding single-agents (Deuker et al., 2015; Silva et al., 2014).

To examine the effect of combined inhibition of BRAF^{V600E} and PI3K α on NZM52 intracellular signaling, NZM52 cells were treated with BRAF^{V600E} (100nM LGX-818/BRAFi) or PI3K α (5 μ M BYL-719/PI3K α i) inhibitors, either alone or in combination, for 24 hours with cell lysates analyzed by immunoblotting (Fig. 2B). Although BRAF^{V600E} inhibition led to decreased P-ERK1/2, there was substantial residual P-ERK1/2 after 24 hours of drug treatment in these cells. As observed previously, inhibition of BRAF^{V600E} had little or no effect on P-AKT, but suppressed P-p70^{S6K}. Inhibition of PI3K α reduced P-AKT and P-PRAS40 with little or no effect on P-p70^{S6K}. Single-agent or combined inhibition of BRAF^{V600E} or PI3K α had no discernible inhibitory effect on P-rpS6 or P-4E-BP1 in NZM52 cells.

To determine whether the residual P-ERK1/2 detected in NZM52 cells following inhibition of BRAF^{V600E} may reflect reactivation of the MAPK pathway, NZM52 cells were treated with the BRAF^{V600E} inhibitor (100nM LGX-818/BRAFi) for 4, 10, or 18 hours with extracts analyzed by immunoblotting (Fig. 2C). BRAF^{V600E} inhibition led to decreased P-ERK1/2 at 4 hours, but by 10 hours P-ERK1/2 levels began to increase with a substantial rebound in P-ERK1/2 at 18 hours. These data suggest that in NZM52 cells, inhibition of

BRAF^{V600E} initially suppresses BRAF^{V600E}→MEK1/2→ERK1/2 signaling, but eventually leads to a substantial rebound in P-ERK1/2, as previously observed in other contexts as in BRAF^{T1799A}-mutated colorectal or thyroid cancer cell lines (Montero-Conde et al., 2013; Sun et al., 2014). These data may also explain the lack of profound anti-proliferative activity of single-agent BRAF^{V600E} inhibition in NZM52 cells.

The striking inability of the BRAF^{V600E} inhibitor to suppress the proliferation of NZM52 cells and to sustain inhibition of P-ERK1/2 was somewhat surprising based on previous demonstrations of the sensitivity of BRAF-mutated melanoma cells to BRAF^{V600E} inhibitors (Deuker et al., 2015; Marsh Durban et al., 2013; Silva et al., 2014). Thus, we sought to test the effects of BRAF^{V600E} inhibition on S-phase progression of NZM52 cells. To that end, NZM52 cells were treated with a BRAF^{V600E} inhibitor (100nM LGX-818/BRAFi) for 48 hours with cells incubated with BrdU for the last 24 hours of drug treatment. S-phase progression was assessed by flow cytometric analysis of BrdU incorporation (Fig. 2D). BRAF^{V600E} inhibition significantly reduced BrdU incorporation by approximately 90%, despite the apparent inability of this inhibitor to exert anti-proliferative effects in these cells. These data indicate that BRAF^{V600E} signaling is required for S-phase progression of the cell division cycle in the NZM52 cells.

***PIK3CA*-mutated melanoma cells are dependent on both MEK1/2 and AKT→mTORC1 signaling for their sustained proliferation**

AKT is postulated to be a major downstream effector of PI3'-lipid signaling in cancer (Pal et al., 2010; She et al., 2010). Consequently, we examined the dependency of NZM cell proliferation and downstream signaling on AKT activity. To test the effect of single-agent or combined inhibition of MEK1/2 or AKT on cellular proliferation, NZM cells were treated with a MEK1/2 (1μM GSK1120212/MEKi²) or AKT (5μM GSK690693/AKTi) inhibitor, either alone or in combination, with viable cells stained using crystal violet (Fig. 3A). Single-agent inhibition of MEK1/2 or AKT led to significant decreased cell proliferation of NZM40 (MEKi²: p<0.0005; AKTi: p<0.005) and NZM91 (MEKi²: p<0.05; AKTi: p<0.05) cells. Conversely, inhibition of MEK1/2 or AKT displayed only modest anti-proliferative effects on NZM52 cells. Intriguingly, while MEK1/2 inhibition with GDC-0973 was previously shown to inhibit NZM52 proliferation, MEK1/2 inhibition with GSK1120212 failed to inhibit NZM52 proliferation to the same extent. However, the combined inhibition of MEK1/2 plus AKT more profoundly suppressed proliferation compared with the corresponding single-agents in all three NZM cell lines (Fig. 3A).

To examine the effect of combined inhibition of MEK1/2 and AKT on *PIK3CA*-mutated melanoma cell signaling, NZM cells were treated with single-agent or combined inhibition of MEK1/2 (1μM GSK1120212/MEKi²) or AKT (5μM GSK690693/AKTi) for 24 hours with lysates analyzed by immunoblotting (Fig. 3B). MEK1/2 inhibition led to a substantial decrease in P-ERK1/2 with negligible effects on P-rpS6 or P-4E-BP1 despite the reduction in P-p70^{S6K} in all three cell lines. By contrast, AKT inhibition greatly enhanced P-AKT, as predicted by this agent's mechanism of action, reduced P-PRAS40, and had little or no effect on P-p70^{S6K} or P-4E-BP1 (Heerding et al., 2008; Pal et al., 2010; Rhodes et al., 2008). Although AKT inhibition had negligible effects on P-rpS6 in NZM40 or NZM52

cells, AKT inhibition did reduce P-rpS6 in the NZM91 cell line. Combined MEK1/2 plus AKT inhibition significantly suppressed P-rpS6 in all three cell lines compared with single-agent inhibition of MEK1/2 or AKT, whereas P-4E-BP1 was not further decreased by combined MEK1/2 and AKT inhibition. Together, these data suggest that AKT cooperates with MEK1/2 signaling to regulate the proliferation of the *PIK3CA*-mutated NZM melanoma cells and to sustain phosphorylation of rpS6. Perhaps surprisingly, inhibition of MEK1/2 or AKT, either alone or in combination, had no discernible effect on the phosphorylation of 4E-BP1 in these NZM cell lines.

To assess a possible role for mTORC1 signaling on *PIK3CA*-mutated melanoma cell proliferation and the regulation of rpS6 and 4E-BP1 phosphorylation, NZM cells were treated with the mTORC1/2 inhibitor, PP242, (1 μ M or 5 μ M mTORi) with cells stained using crystal violet and extracts analyzed by immunoblotting (Figs. 4A and B). Inhibition of mTORC1/2 at either concentration significantly inhibited NZM cell proliferation as well as P-rpS6 and P-4E-BP1. Inhibition of mTORC1/2 at 5 μ M elicited more potent anti-proliferative effects and suppression of both P-rpS6 and P-4E-BP1 in all three cell lines compared to 1 μ M PP242. Consistent with this data, a mechanistically distinct mTORC1/2 inhibitor, GDC-0349, confirmed the anti-proliferative and signaling effects of PP242 (Supplementary Fig. S1A and B) (Pei et al., 2013). In addition, PP242 or GDC-0349 exhibited the greatest inhibitory effect on mTORC1/2 at 5 μ M as evident by the magnitude of the decrease in P-AKT (Fig. 4B and Supplementary Fig. S1B). Hence, the phosphorylation of rpS6 and 4E-BP1 in the *PIK3CA*-mutated NZM melanoma cells appears to be dependent on mTORC1 signaling.

In parallel, we assessed whether combined inhibition of MEK1/2 plus mTORC1/2 might provide a more robust inhibition of *PIK3CA*-mutated melanoma proliferation and signaling. To test the effects of single-agent versus combined MEK1/2 plus mTORC1/2 inhibition, NZM cells were treated with a MEK1/2 (1 μ M PD0325901/MEKi³) or mTORC1/2 (5 μ M PP242/mTORi) inhibitor, either alone or in combination, with viable cells stained using crystal violet and cell lysates analyzed by immunoblotting (Figs. 4C and D). Similar to the results with the other MEK1/2 inhibitors (GDC-0973 and GSK1120212), MEK1/2 inhibition significantly inhibited the proliferation of NZM40 (p<0.0001) and NZM91 (p<0.05) melanoma cells. However, PD0325901 had no discernible effect on NZM52 proliferation, which is consistent with the lack of anti-proliferative effects of GSK1120212 on these cells. Although mTORC1/2 inhibition potently suppressed NZM proliferation, MEK1/2 inhibition did not substantially enhance the anti-proliferative effects of mTORC1/2 inhibition. As previously demonstrated, MEK1/2 inhibition led to decreased P-p70^{S6K} and P-ERK1/2 with little or no effect on P-AKT, P-PRAS40, P-rpS6, or P-4E-BP1. Conversely, mTORC1/2 inhibition suppressed P-AKT, P-PRAS40, P-rpS6, and P-4E-BP1, but did not reduce P-ERK1/2 or P-p70^{S6K} levels. Combined MEK1/2 and mTORC1/2 inhibition exhibited cooperative effects on P-rpS6, whereas P-4E-BP1 was not further diminished compared with single-agent mTORC1/2 inhibition. Thus, the coordinate control of mTORC1 by MEK1/2 and PI3K signaling appears to be important for regulating the proliferation of NZM melanoma cell proliferation through effects on rpS6 and 4E-BP1 phosphorylation.

Combination MEK1/2 plus β -sparing PI3K inhibition inhibits tumorigenesis of xenografted NRAS^{Q61H}/PIK3CA^{H1047R}-mutated cells

It has recently been demonstrated that, compared to single-agent blockade, combined inhibition of MEK1/2 and PI3K α signaling significantly enhanced the durability of the response of BRAF^{V600E}/PIK3CA^{H1047R}-driven mouse melanomas to pathway targeted inhibition (Deuker et al., 2015). These results, combined with the anti-proliferative activity of combined MEK1/2 plus either the PI3K α (BYL-719) or β -sparing PI3K (GDC-0032) inhibitors on NZM40 cells *in vitro*, prompted us to determine whether MEK1/2 and either PI3K α or β -sparing PI3K inhibition could inhibit tumorigenesis following implantation of NZM40 (NRAS^{Q61H}/PIK3CA^{H1047R}) cells into immunocompromised mice. NZM40 cells were injected subcutaneously into nude mice and, three weeks after injection when tumors had grown to approximately 600mm³ (Fig. 5A) or 230mm³ (Fig. 5B), mice were randomized to receive vehicle, GDC-0973 (2mg/kg or 4mg/kg, once a day/MEKi¹), BYL-719 (50mg/kg twice a day/PI3K α i), GDC-0032 (6mg/kg, once a day/PI3Ki²), or the combination of GDC-0973 plus either BYL-719 or GDC-0032 treatment (Figs. 5A and 5B respectively). It should be noted that mice dosed at 4mg/kg with GDC-0973 in combination with the β -sparing PI3K inhibitor, GDC-0032, led to severe toxicity issues in the NZM40 xenografted mice. Therefore, in order to dose the mice in combination with the MEK1/2 and β -sparing PI3K inhibitors without eliciting substantial toxicities, mice were treated with 2mg/kg of the MEK1/2 inhibitor. Target inhibition of the various signaling pathways was assessed by immunoblot analyses of tumor cell lysates (Supplementary Fig. S2A–D). In brief, combined inhibition of MEK1/2 and either PI3K α or β -sparing PI3K led to a greater decrease in P-AKT, P-PRAS40, P-p70^{S6K}, P-rpS6 and P-4E-BP1 compared with the corresponding single-agents.

Consistent with previously published reports, single-agent MEK1/2 inhibition failed to elicit melanoma regression, and only modestly attenuated tumor growth (Figs. 5A and B) (Kwong et al., 2012; Posch et al., 2013). The lack of efficacy of single-agent treatment with the MEK1/2 inhibitor may be due to insufficient target coverage of aberrant NRAS^{Q61H}→RAF→MEK1/2→ERK1/2 signaling at these concentrations, which was further substantiated by the inability of the MEK1/2 inhibitor to fully suppress P-ERK1/2 levels (Supplementary Fig. S2A and S2B). Treatment of melanoma bearing mice with either the PI3K α or β -sparing PI3K inhibitors elicited significant tumor growth inhibition compared to MEK1/2 monotherapy (PI3K α : $p < 0.0001$; PI3Ki²: $p < 0.005$), but this effect was largely cytostatic and did not promote tumor regression. Importantly, the combination of MEK1/2 plus β -sparing PI3K inhibitor resulted in more potent tumor regression than achieved with either MEK1/2 or β -sparing PI3K monotherapy (Figs. 5B and 5D). By contrast, combined MEK1/2 plus PI3K α inhibition induced more profound tumor growth inhibition as compared to MEK1/2 monotherapy ($p < 0.0001$). However, the combination of MEK1/2 plus the PI3K α inhibitor did not significantly enhance the anti-tumor effects of the PI3K α inhibitor (Fig. 5A). The analysis of the best overall responses revealed that only 1 of 7 mice treated with the combination of MEK1/2 and PI3K α inhibition displayed greater than 30% melanoma regression, which scores as a partial response by modified response evaluation criteria in solid tumors (RECIST) 1.1 guidelines (Fig. 5C) (Eisenhauer et al.,

2009). By contrast, 4 out of 6 mice treated with the combination of MEK1/2 plus β -sparing PI3K inhibitors achieved a partial response by modified RECIST guidelines (Fig. 5D).

DISCUSSION

Malignant transformation of melanocytes is thought to be promoted by the accrual of genetic alterations in key signaling pathways that cooperate in the initiation and progression of melanoma cells (Chin et al., 1998; Chin et al., 1997; Dhomen et al., 2009; Goel et al., 2006; Gray-Schopfer et al., 2005; Haluska et al., 2006; Hodis et al., 2012; Vredeveld et al., 2012). Although mutational activation of *BRAF* can promote benign proliferation of melanocytes resulting in nevus formation, cooperating alterations in additional pathways are required for progression to melanoma (Cancer Genome Atlas, 2015; Dankort et al., 2009; Deuker et al., 2015; Goel et al., 2006; Haluska et al., 2006; Hodis et al., 2012; Marsh Durban et al., 2013; Vredeveld et al., 2012). Thus, the components of the PI3K signaling pathway, which controls PI3'-lipid accumulation, is frequently mutated in melanoma cells. PI3'-lipid accumulation in melanoma cells can be promoted by silencing of the *PTEN* tumor suppressor or through mutational activation of *PIK3CA*, or *RAC1*, the last of which is reported to activate PI3K β (Cancer Genome Atlas, 2015; Curtin et al., 2006; Fritsch et al., 2013; Hodis et al., 2012; Kim et al., 2012; Omholt et al., 2006; Samuels and Velculescu, 2004; Shull et al., 2012). However in melanoma, as in other cancers, a critical question is the extent to which mutational alterations of components of PI3'-lipid signaling predicts for therapeutic responses to inhibitors of PI3'-kinases or its direct downstream effectors such as the AKT, serum- and glucocorticoid-inducible protein kinase (SGK), or other effectors of PI3'-lipid signaling (Cancer Genome Atlas, 2015; Hodis et al., 2012; She et al., 2010; Tessier and Woodgett, 2006; Vanhaesebroeck et al., 2012; Vasudevan et al., 2009). In genetically engineered mouse models, BRAF^{V600E} cooperates with either *PTEN* silencing or mutational activation of *PIK3CA* for melanomagenesis, however, pharmacological inhibition of PI3'-lipid accumulation in these cells fails to elicit melanoma regression resulting in largely cytostatic effects (Dankort et al., 2009; Deuker et al., 2015; Marsh Durban et al., 2013). However, inhibitors of PI3K forestalled the onset of resistance to agents that target BRAF^{V600E} signaling in these GEM models (Deuker et al., 2015).

In this study, using *PIK3CA*-mutated metastatic melanoma cell lines, we showed that such cells were sensitive to pharmacological inhibition of PI3K signaling. Moreover, these cells were also sensitive to combined blockade of both MEK1/2 and PI3K signaling. This suggests that *PIK3CA*-mutated melanoma cell lines have a dependency on both MEK1/2 and PI3K signaling for their maintenance. Despite containing a *PIK3CA* mutation, both PI3'-lipid signaling and NZM cell proliferation was more sensitive to more broad spectrum PI3K inhibitors than to an α -selective PI3K inhibitor. These data suggest that, at least *in vitro*, more than one isoform of PI3K is contributing to the PI3'-lipid accumulation in these cells.

The importance of BRAF^{V600E} signaling in melanoma maintenance is emphasized by the clinical success of BRAF^{V600E} inhibitors to elicit potent tumor regression in *BRAF*-mutated melanoma patients with advanced disease (Chapman et al., 2011; Flaherty et al., 2010). Moreover, both *in vitro* and *in vivo*, BRAF^{V600E}/PI3K α driven mouse melanomas and their

derivative cell lines were sensitive to pathway-targeted blockade of either BRAF^{V600E} or PIK3CA^{H1047R} (Deuker et al., 2015; Marsh Durban et al., 2013). Although N52 (BRAF^{V600E}/PIK3CA^{H1047R}) cells were sensitive to MEK1/2 inhibition, it was surprising that these cells appeared to be largely resistant to BRAF^{V600E} inhibition despite the substantial effect of BRAF^{V600E} inhibition to suppress S-phase progression in these cells. The lack of anti-proliferative effects of BRAF^{V600E} inhibition may be explained by the apparent reactivation of MEK1/2→ERK1/2 signaling at later time points following drug addition. Moreover, we cannot exclude the possibility that these cells may also be activating alternative signaling mechanisms downstream of ERK1/2 in a manner that was not revealed by our targeted next-generation genomic sequencing of these cells.

We have previously reported that several human *BRAF*-mutated melanoma cell lines are largely insensitive to either allosteric or conventional ATP-competitive inhibitors of AKT (Marsh Durban et al., 2013; Silva et al., 2014). Here we report that single-agent inhibition of AKT had only modest effects on the proliferation of the *PIK3CA*-mutated N52 melanoma cell lines, but cooperated with MEK1/2 inhibition for robust suppression of N52 cell proliferation. This is consistent with the sensitivity of GEM models of BRAF^{V600E}/PIK3CA^{H1047R} mouse melanomas and cell lines to AKT inhibition and its ability to effectively suppress growth (Marsh Durban et al., 2013). Inhibition of the PI3K→AKT signaling axis is predicted to inhibit the mTORC1 downstream signaling effectors p70^{S6K}, rpS6, and 4E-BP1 (Zoncu et al., 2011). Our data revealed that phosphorylation of p70^{S6K} appears to be largely under the control MEK1/2 signaling in the N52 cell lines, as we have previously observed in other melanoma cells, whereas rpS6 phosphorylation appears to be under the coordinate control of both the MEK1/2 and PI3K signaling axes (Silva et al., 2014). By contrast, the phosphorylation of 4E-BP1, a regulator of cap-dependent translation initiation, was sensitive to PI3K inhibition, but largely resistant to the inhibition of either MEK1/2 or AKT signaling. This is consistent with recent data indicating the existence of a PDK1→SGK signaling axis that regulates mTORC1 activity independently of AKT and engenders resistance to PI3K inhibitors (Castel et al., 2016). In previous work, we and others have inferred the importance of the coordinate control of mTORC1 by both MAPK and PI3K signaling as being essential for melanoma cell proliferation. Our data demonstrates that the N52 melanoma cells were sensitive to mTORC1/2 inhibition as evident by the profound decrease in rpS6 and 4E-BP1 phosphorylation and cell proliferation. However, the effect of mTORC1/2 inhibition was not potentiated by pharmacological inhibition of MEK1/2 signaling. These data further reinforce the importance of mTORC1/2 as a target for suppression of melanoma cell proliferation (Gowrishankar et al., 2012; Silva et al., 2014).

Despite the anti-proliferative effects of either the β -sparing PI3K or PI3K α inhibitor on the proliferation of N52 (NRAS^{Q61H}/PIK3CA^{H1047R}) melanoma cells *in vitro*, these agents elicited largely cytostatic effects on N52 xenografted tumors. Although combined inhibition of MEK1/2 plus β -sparing PI3K displayed significant cooperation in reducing tumor growth compared to the corresponding single agents, the ability of this combination to elicit tumor regression was quite modest and transient. The inability of this combined treatment to promote tumor regression may be reflected in the fact that we did not observe evidence of N52 cell death *in vitro*, even though the combined blockade of MEK1/2 plus PI3K signaling potently inhibited DNA synthesis. It is possible that the dosing regimen(s)

did not provide adequate target coverage of $\text{NRAS}^{\text{Q61H}} \rightarrow \text{RAF} \rightarrow \text{MEK1/2} \rightarrow \text{ERK1/2}$ or PI3'-lipid signaling in these tumors, and therefore, we are unable to propose that these data provide a compelling rationale for the deployment of PI3K inhibitors for the treatment of those rare patients whose melanomas express mutationally activated *PIK3CA*.

MATERIALS AND METHODS

Cell Culture and Drug Treatments

Human melanoma cell lines NZM40, NZM52, and NZM91 were maintained as previously described (Supplementary Table S1) (Kim et al., 2012). Confirmation of the various gene mutations in *BRAF*, *NRAS*, or *PIK3CA* was performed by the UCSF Clinical Cancer Genomics Laboratory using next-generation genomic sequencing, in which the mutational status of approximately 500 cancer-relevant genes was assessed (<http://cancer.ucsf.edu/intranet/ccgl>). Pathway-targeted pharmacological inhibitors of intracellular signaling were obtained from various colleagues in the private sector or from commercial sources (Barrett et al., 2008; Feldman et al., 2009; Folkes et al., 2008; Furet et al., 2013; Gilmartin et al., 2011; Heerding et al., 2008; Ndubaku et al., 2013; Pei et al., 2013; Rice et al., 2012; Stuart DD, 2012). Drug concentrations used for each treatment are listed in Supplementary Table S2.

Cell Proliferation Assays

Melanoma cell proliferation was assessed by seeding 5×10^4 cells in 6-well plates or 2×10^4 cells in 12-well plates. Cells were cultured in the absence or presence of the various pharmacological agents for 4–21 days, at which time viable cells were fixed and stained using crystal violet. To quantify cellular proliferation, crystal violet stained cells were solubilized in 33% acetic acid with absorbance measured at 562nm.

Transit of cells through S-phase was assessed by measurement of bromodeoxyuridine (BrdU) incorporation and DNA content using the FITC BrdU Flow Kit (BD Pharmingen) according to the manufacturer's instructions. Cells seeded at a density of $1\text{--}3 \times 10^5$ cells/well in 6-well plates were treated with the various pharmacological agents for 48 hours and then labeled with 10 μ M BrdU for the remaining 24 hours of the drug treatment. BrdU labeled cells were fixed and stained with FITC-conjugated anti-BrdU antibody and 7-aminoactinomycin D (7-AAD) and then analyzed by two-color flow cytometry. Quantification of BrdU positive cells was performed using FlowJo v.10.0.8 software.

Immunoblot Analysis

Cell lysates were prepared for immunoblot analysis as previously described (Silva et al., 2014; Silva and McMahon, 2014). Membranes were probed with the primary antibodies as described in Supplementary Table S3. Antigen-antibody complexes were detected using fluorescent goat anti-Rabbit or anti-Mouse IRDye 800 or goat anti-Mouse IRDye 680 secondary antibodies (LI-COR Biosciences) and visualized using a LI-COR Odyssey Classic infrared imaging system. Immunoblot data was analyzed using the Odyssey application software v3.0.30 software (LI-COR Biosciences) (Silva and McMahon, 2014).

Xenograft Tumor Studies in Immunocompromised Mice

The University of California, San Francisco Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal procedures. Four to six week old athymic nude female mice were subcutaneously injected with 5×10^6 to 1×10^7 NZM40 human melanoma cells suspended in Matrigel. In the case of GDC-0973 plus GDC-0032 inhibitor study, drug treatment was initiated when the mean tumor volume exceeded approximately 230 mm^3 . For the GDC-0973 plus BYL-719 inhibitor study, drug treatment was initiated when the tumor volume was approximately 600 mm^3 . At this point, mice were divided among the treatment arms to give an equal distribution of tumor volume. Mice were assigned to receive vehicle; BYL-719 (50mg/kg, b.i.d.) formulated in 0.5% carboxymethylcellulose/0.5% Tween-80; GDC-0973 (2mg/kg for GDC-0973/GDC-0032 treatment or 4 mg/kg for GDC-0973/BYL-719 treatment, q.d.) or GDC-0032 (6mg/kg, q.d.) both formulated in 0.5% methylcellulose/0.2% Tween-80; either as single agents or in combination and administered via oral gavage 5 days per week. Melanoma growth was measured weekly using digital calipers with relative tumor volume (RTV) estimated using the ellipsoid volume formula as described previously (Marsh Durban et al., 2013). Animals were euthanized based on a body conditioning score (BCS ≥ 2) as described (Ullman-Cullere and Foltz, 1999) or when the tumor volume exceeded 2 cm^3 , whichever occurred first. At necropsy, tissue was snap-frozen in liquid nitrogen and homogenized in RIPA buffer using the Tissue Lyser II (Qiagen). Immunoblot analyses were performed as previously described (Silva et al., 2014; Silva and McMahon, 2014).

Statistical Analysis

All quantitative data are represented as mean $\pm$ SEM of at least three or more independent experiments. GraphPad Prism 6 statistical software was used to determine *P* values by performing paired, two-tailed *t* tests or one-way or two-way ANOVA analyses as indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

Although the components of the PI3K pathway are frequently mutated in melanoma cells, the contribution of mutationally activated *PIK3CA* to the cooperative mechanisms of MAPK and PI3K signaling remains elusive. Here, we demonstrate that *PIK3CA*-mutated melanoma cell proliferation has a dependency on both MEK1/2 and PI3K signaling, but were more sensitive to a more broad range PI3K inhibitor compared to an α -selective PI3K inhibitor. Importantly, these data suggest that mTORC1 is a key moderator of cooperating MAPK and PI3K activity for the maintenance of *PIK3CA*-mutated melanoma cells.

B

C

Fig. 1. Sensitivity of *PIK3CA*-mutated melanoma cells to single-agent or combined inhibition of MEK1/2 and class I PI3K, β -sparing PI3K, or PI3K α .

A. NZM40 (NRAS^{Q61H}/PIK3CA^{H1047R}), NZM52 (BRAF^{V600E}/PIK3CA^{H1047R}), and NZM91 (PIK3CA^{E545K}) cells were cultured in the continuous presence of MEK1/2 (5 μ M GDC-0973/MEKi¹), class I PI3K (5 μ M GDC-0941/PI3Ki¹), β -sparing PI3K (5 μ M GDC-0032/PI3Ki²), or PI3K α (5 μ M BYL-719/PI3K α i) inhibitors, either alone or in combination, for 3–8 days with viable cells fixed and stained using crystal violet (above). Quantification of crystal violet staining was assessed by solubilizing the fixed dye and measuring absorbance at $\lambda = 562$ nm (below). Results are expressed as a fold-change of the DMSO control and presented as mean $\pm$ SEM of at least three or more independent experiments. Paired, two-tailed *t* tests were performed to determine *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

B. NZM cells were treated with inhibitors of MEK1/2 (1 μ M GDC-0973/MEKi¹), class I PI3K (5 μ M GDC-0941/PI3Ki¹), or PI3K α (5 μ M BYL-719/PI3K α i), either alone or in combination, for 48 hours and pulsed with 10 μ M BrdU for the remaining 24 hours of drug treatment with BrdU positive cells quantified by flow cytometry. Data are represented as a fold-change of BrdU positive cells of the DMSO control and presented as mean $\pm$ SEM of at

least three or more independent experiments. One-way ANOVA analyses were performed to determine *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001).

C. All three NZM cell lines were treated with inhibitors of MEK1/2 (5μM GDC-0973/MEKi¹), class I PI3K (5μM GDC-0941/PI3Ki¹), β-sparing PI3K (5μM GDC-0032/PI3Ki²), or PI3Kα (5μM BYL-719/PI3Kαi), either alone or in combination, for 24 hours with lysates analyzed by immunoblotting with the indicated antibodies.

A

B

C

D

Fig. 2. Resistance of BRAF^{V600E}/PIK3CA^{H1047R} mutant melanoma cells to BRAF^{V600E} inhibition

A. NZM52 (BRAF^{V600E}/PIK3CA^{H1047R}) cells were treated in the continuous presence of BRAF^{V600E} (100nM LGX-818/BRAFi), class I PI3K (5μM GDC-0941/PI3Ki¹), or PI3Kα (5μM BYL-719/PI3Kαi) inhibitors, either alone or in combination, for approximately 20 days with viable cells fixed and stained using crystal violet (above). Quantification of crystal violet staining was assessed by solubilizing the fixed dye and measuring absorbance at λ = 562nm (below). Results are expressed as a fold-change of the DMSO control and presented as mean ± SEM of four independent experiments. Paired, two-tailed *t* tests were performed to determine *P* values (*, *P* < 0.05).

B. and C. NZM52 cells were treated with inhibitors of BRAF^{V600E} (100nM LGX-818/BRAFi) or PI3Kα (5μM BYL-719/PI3Kαi), either alone or in combination, for 24 hours (B) or LGX-818 for 4, 10, or 18 hours (C). Cell lysates were analyzed by immunoblotting with the indicated antibodies.

D. NZM52 cells were treated with 100nM LGX-818 (BRAFi) for 48 hours and pulsed with 10 μ M BrdU for the remaining 24 hours of drug treatment with BrdU positive cells quantified by flow cytometry. Data are represented as a fold-change of BrdU positive cells of the DMSO control and presented as mean $\pm$ SEM of three independent experiments. One-way ANOVA analysis was performed to determine *P*value (***, *P* < 0.001).

B

Fig. 3. Cooperation of MEK1/2 and AKT signaling contributes to the regulation of PIK3CA-mutated melanoma cell proliferation and rpS6 phosphorylation

A. NKM40 (NRAS^{Q61H}/PIK3CA^{H1047R}), NKM52 (BRAF^{V600E}/PIK3CA^{H1047R}), and NKM91 (PIK3CA^{E545K}) cell lines were treated with inhibitors of MEK1/2 (1μM GSK1120212/MEKi2) or AKT (5μM GSK690693/AKTi), either alone or in combination, for 4–12 days with viable cells fixed and stained using crystal violet (above). Quantification of crystal violet staining was assessed by solubilizing the fixed dye and measuring absorbance at $\lambda = 562\text{nm}$ (below). Data are expressed as a fold-change of the DMSO control and presented as mean $\pm$ SEM of three independent experiments. Paired, two-tailed *t* tests were performed to determine *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

B. All three NKM cell lines were treated with single-agent or combined inhibition of MEK1/2 (1μM GSK1120212/MEKi2) or AKT (5μM GSK690693/AKTi) for 24 hours with cell lysates analyzed by immunoblotting with the indicated antibodies.

B

C

D

Fig. 4. mTORC1-dependent effects on *PIK3CA*-mutated melanoma cell proliferation and signaling

A. NNM40 (NRAS^{Q61H}/PIK3CA^{H1047R}), NNM52 (BRAF^{V600E}/PIK3CA^{H1047R}), and NNM91 (PIK3CA^{E545K}) cells were treated with either 1μM or 5μM of the mTORC1/2 inhibitor, PP242 (mTORi), for 4–12 days with viable cells fixed and stained using crystal violet (above). Quantification of crystal violet staining was assessed by solubilizing the fixed dye and measuring absorbance at $\lambda = 562\text{nm}$ (below). Results are expressed as a fold-change of the DMSO control and presented as mean $\pm$ SEM of three independent experiments. Paired, two-tailed *t* tests were performed to determine *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001).

B. All three NNM cell lines were treated with either 1μM or 5μM PP242 (mTORi) for 24 hours with extracts analyzed by immunoblotting with the indicated antibodies.

C. NNM40, NNM52, and NNM91 cells were treated with single-agent or combined inhibition of MEK1/2 (1μM PD0325901/MEKi³) or mTORC1/2 (5μM PP242/mTORi) for 4–11 days with viable cells fixed and stained using crystal violet. Crystal violet staining was

quantified as described above. Results are expressed as a fold-change of the DMSO control and presented as mean $\pm$ SEM of three independent experiments. Paired, two-tailed *t* tests were performed to determine *P* values (*, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005; ****, *P* < 0.0001).

D. NZM cells were treated with inhibitors of MEK1/2 (1 μ M PD0325901/MEKi³) or mTORC1/2 (5 μ M PP242/mTORi), either alone or in combination, for 24 hours with extracts analyzed by immunoblotting with the indicated antibodies.

A

B

C

Fig. 5. Combined inhibition of MEK1/2 and β -sparing PI3K promotes reduction of NRAS^{Q61H}/PIK3CA^{H1047R} xenograft tumors

A. & B. Melanoma tumors were initiated by subcutaneously injecting NZM40 cells into nude mice. Following randomization, mice were treated with vehicle, GDC-0973 (4 mg/kg (A) or 2 mg/kg (B), once a day/MEKi¹), BYL-719 (50 mg/kg, twice a day/PI3Ki¹), GDC-0032 (6 mg/kg, once a day/PI3Ki²), or the combination of the agents. Melanoma growth or regression was measured weekly using digital calipers over the course of drug treatment. Tumor sizes are displayed as the average percentage change in tumor size from the start of treatment with error bars representing SEM. Two-way ANOVA analyses were performed to determine *P* values (***, *P* < 0.0005; ****, *P* < 0.0001).

C. & D. Waterfall plots of the best tumor response for each mouse that received vehicle or drug treatment in A or B. Data are represented as the percent change in tumor size from the start of the treatment (y-axis) with negative values indicating tumor shrinkage.