Introduction

RAS–RAF mitogen-activated protein kinase (MAPK) signaling cascade plays a central role in the regulation of cell proliferation and survival, whereas the deregulation of this pathway frequently occurs in human cancers (1–3). As mutations in RAS or BRAF occur in more than 30% of human cancers, these proteins are very attractive therapeutic targets in many cancer types. Among them, BRAF mutations occur in about 7% of human cancers, with highest prevalence in melanomas (66%) and thyroid (35–70%) tumors (4, 5). Interestingly, 80% of all BRAF mutations are found on a single substitution of glutamic acid for valine (V600E) within the kinase domain (4). Compared with BRAF, mutations in the 2 RAF isoforms, ARAF and CRAF, are rarely found in human cancers, which is likely due to lower basal kinase activities (6, 7). All 3 RAF isoforms (KRAS, NRAS, and HRAS) are found mutationaly activated in 30% of all human cancers, with highest prevalence in pancreas (90%), colon (50%), thyroid (50%), and lung (30%) cancers (1, 2). Although the RAS oncogene has been studied for more than 3 decades, there is no drug on the market that sufficiently inhibits RAS, despite extensive efforts to inhibit activated RAS with low molecular weight inhibitors (3, 8). As an alternative therapeutic strategy, RAF and MAP/ERK kinase (MEK) inhibitors have been developed to inhibit the pathway downstream of RAS, and only 1 RAF inhibitor Sorafineb has been approved by Food and Drug Administration. However, clinical responses from these reagents are not as effective or durable as expected, drug resistance frequently occurs in tumors treated with RAF or MEK inhibitors. As an alternative therapeutic strategy, protein kinase D3 (PRKD3) could enhance cell killing of RAF and MEK inhibitors across multiple melanoma cell lines of various genotypes and sensitivities to RAF265. PRKD3 blockade cooperated with RAF265 to prevent reactivation of the MAPK signaling pathway, interrupt cell cycle progression, trigger apoptosis, and inhibit colony formation growth. Our findings offer initial proof-of-concept that PRKD3 is a valid target to overcome drug resistance being encountered widely in the clinic with RAF or MEK inhibitors. Cancer Res; 71(12); 4280–91. ©2011 AACR.
can interact with more than 20 effectors including RAF, phosphatidylinositol 3-kinases (PI3K), RAC, RAL, and phospholipase C epsilon to regulate cell proliferation, survival, and differentiation (2). There are multiple feedback loops known to activate downstream of RAS which are differentially regulated depending on the genetic background and tumor lineage being studied (23, 24). These feedback loops can lead to compensatory activation of parallel survival pathways upon drug treatments, and tumor cells are flexible at utilizing the signal pathways for growth and transformation, resulting in rapid drug resistance. Clinical reports show that activation of the MAPK pathway was induced via S6K–PI3K–RAS signaling in tumor samples from patients treated with RAD001, an inhibitor of PI3K pathway (17). In neuroendocrine tumor cells, inhibitor of RAF strongly induced AKT phosphorylation reflecting an activation of PI3K pathway, and inhibitors of PI3K pathways induced ERK phosphorylation indicating MAPK pathway activation (25). Dual targeting of both PI3K and MAPK signaling pathways showed more potent antitumor effect than single treatment alone (17, 21, 25).

Despite the intensive research efforts on RAS signaling, our understanding of its regulation is still limited. To identify potential modulators of RAS signaling pathways and to uncover the molecular mechanisms underlying resistance to RAF or MEK inhibitors, we conducted a siRNA screen in combination with a RAF inhibitor (RAF265) to identify genes and/or pathways that sensitize to RAF265 treatment in a BRAF (V600E) mutant melanoma cell line (A2058) which is insensitive to RAF265-induced cell death. By using this approach, we identified protein kinase D3 (PRKD3) that when knocked down could enhance cell killing by RAF265 in A2058. PRKD3 is 1 of 3 members in the protein kinase D (PKD) family which includes PRKD1 and PRKD2. The physiologic functions of PRKD3 are not well understood. Similar to PRKD1 and PRKD2, PRKD3 has been shown to function in protein trafficking and as a histone deacetylase kinase (26, 27). One report showed that PRKD3 enhanced CCK-mediated pancreatic amylase secretion via MEK–ERK–RSK signaling and this process was activated by GI hormone (28). Another report showed that PRKD3 expression levels were elevated in human prostate cancers compared with normal tissues; PRKD3 overexpression activated AKT and ERK in prostate cancer cell lines and promoted cell growth and survival (29). Essential role of PRKD3 in prostate cancer cells is likely due to regulation of MAPK signaling (29). By using A2058 and A375 (RAF265 sensitive cell line) as melanoma cellular models, we showed that PRKD3 inhibition cooperates with RAF265 to prevent the reactivation of MAPK signaling pathway, induce PARP cleavage and caspase activity, interrupt cell-cycle progression, and inhibit colony formation. Finally, we showed that the PRKD3 inhibition sensitizes with multiple RAF and MEK inhibitors in a panel of melanoma cell lines, suggesting that PRKD3 functionally interacts with the MAPK signaling pathway. Thus, PRKD3 provides a potential cancer target to develop effective therapeutic strategies to overcome or prevent drug resistance from RAF or MEK inhibitors.

Materials and Methods

Kinome siRNA synthetic lethal screen and data analysis

The kinome siRNA SMARTpool library directed against 779 kinases was purchased from Dharmacon (catalogue no. G003500). RNAi screens were prepared with 2 compound doses for 12 plates in A2058 cells (6 plates, in duplicate). Briefly, 4 μL of 206 nmol/L siRNAs in serum-free medium were stamped into each well, 0.03 μL of DharmaFECT 1 in 4 μL serum-free medium was mixed and added into each well followed by 30-minute incubation at room temperature to form a lipid/siRNA complex. Then 1,500 cells in 25 μL complete medium were loaded on top of siRNA–lipid complex. Final concentration of siRNA for each reaction was 25 nmol/L. The cells were incubated at 37°C with 5% CO₂. At 24 hours after siRNA transfection, 5 μL of RAF265 was added into each well to make the final concentration of RAF265 at 0.4 μmol/L. Cell viability was analyzed at 72 hours post-RAF265 treatment by using CellTiter-Glo (CTG) Assay (Promega), and data were acquired by using an Envision (PerkinElmer). From the primary screen, the CTG value was normalized by 1-dimensional (1D) normalization scheme based on plate median of screened 384-well plates. The formula for calculating the 1D-normalized value (denoted as x₁D) is x₁D = log x₁D where x₁D = CTG/median plate CTG. The normalized Z score (NZ) was calculated for every x₁D. The formula used for calculating NZ₁D is NZ₁D = [x₁D − median (x₁D)]/MAD (x₁D). MAD (x₁D) is the median absolute deviation of x₁D and is equal to 1.4826 × median [ |x₁D − median (x₁D)| ].

Additional Materials and Methods are shown in the Supplementary Data.

Results

A kinome synthetic lethal siRNA screen with RAF265 in A2058 melanoma cells

RAF265 is an orally bioavailable small molecule that is known to inhibit CRAF, wild-type BRAF, mutant BRAF (V600E), and VEGF receptor 2 (VEGFR-2) and is currently in phase I clinical trials for advanced melanoma (30, 31). As other RAF or MEK inhibitors, RAF265 showed less efficacy in tumors with RAS mutations compared with tumors with a BRAF mutation (31). RAF265 also shows resistance in some tumors bearing the BRAF V600E mutation such as the melanoma cell line A2058. By using A2058 as the cellular model, we conducted a synthetic lethal siRNA screen to identify genetic sensitizers of RAF265. The siRNA SMARTpool kinome library of 779 kinases was used for the screen. We chose to run our screen in the presence of 0.4 μmol/L RAF265 where 20% growth inhibition was achieved, leaving a suitable window for additional, siRNA-enhanced cellular toxicity. As positive technical controls, we used siRNAs targeting Polo-like kinases 1 (PLK1), which is a regulator of mitosis and, when depleted, results in cell lethality (32). RAF265 siRNAs were used as the biological positive controls and luciferase (LUC) siRNAs were used as the negative controls. Duplicates were used for each siRNA and NZ were calculated. As shown in Figure 1A and B, control LUC siRNA
centered at NZ of 0 and PLK1 siRNAs have a NZ ranging from −17 to −28, indicating a robust signal to noise in the screens. We considered a primary hit, a gene whose knockdown has a minimal effect on cell growth (NZ < 1.5) in the absence of RAF265 but enhances cell killing (NZ > 4.5) in the presence of a low dose of RAF265 (Fig. 1C). Twelve primary hits that met our predefined criteria were identified including MGC5601, C9orf96, ALPK1, CDC7, PRKD3, PDGFRB, PAPSS2, PIK3R4, RAGE, RPS6KB2, DAPK1, and MAPK11 (Fig. 1C). To further evaluate the sensitizers, we generated dose curves of RAF265 for all the hits with SMARTpool siRNAs and observed significant IC_{50} shifts with 4 hits: ALPK1, MAPK11, PRKD3, and PIK3R4 (Fig. 1D). The mRNA knockdown efficiencies of the 4 target genes were confirmed by quantitative real-time PCR (RT-PCR; Fig. 1E).

**PRKD3 inhibition sensitizes RAF265 to kill A2058 melanoma cells**

To prioritize hits to follow up and to minimize off-target effects when using a SMARTpool siRNA approach, we tested individual siRNAs of the 4 hits for sensitization with RAF265. On the basis of the mRNA knockdown levels of the target genes, we selected 2 potent siRNAs from the 4 individual siRNAs for ALPK1, MAPK11, PRKD3, and PIK3R4 (left panels, Fig. 2A–D). Among them, PRKD3 was the only gene in which multiple independent siRNAs resulted in a significant IC_{50} shift for RAF265 and distinguished itself as the best sensitizer identified from this study (Fig. 2D). To confirm the sensitization observed between PRKD3 siRNAs and RAF265, we established 3 independent stable cell lines with PRKD3 knockdown (15). We observed increased phospho-CRAF (Ser338) after RAF265 treatments (Fig. 3B), which has been shown that both RAF and MEK inhibitors tested alone were unable to induce a sustained inhibition of pERK at 72 hours after compound treatment. In contrast, we were able to restore pERK inhibition in combination with PRKD3 siRNAs. In addition, pMEK and pMEK levels were found to be reduced either by PRKD3 siRNA treatment or by RAF265 treatment alone and further reduced after PRKD3 siRNAs in combination with RAF265 (Fig. 3B).

Because elevated CRAF protein levels contributed to resistance to RAF inhibition in a subset of BRAF mutant tumor cells, we tested whether CRAF levels were altered by PRKD3 knockdown (15). We observed increased phospho-CRAF (Ser338) after RAF265 treatments (Fig. 3B), which has been reported for other RAF inhibitors (33). However, PRKD3 siRNA mediated knockdown had no effect on pCRAF or CRAF levels, indicating that PRKD3 affects pERK levels in a CRAF-independent manner. We also used genetic approaches to validate the sensitization between PRKD3 inhibition and BRAF but not CRAF. PRKD3 siRNAs in combination with BRAF siRNA treatments resulted in a greater growth inhibition compared with any of the single treatment (Fig. 3C). However, neither the combination of PRKD3 and CRAF siRNA treatments nor the combination of BRAF and CRAF siRNAs showed potent growth inhibition (Fig. 3C). The knockdown efficiencies of PRKD3, BRAF, and CRAF siRNAs were validated by Western blots (Fig. 3C).

To further understand the mechanism of PRKD3 knockdown–induced RAF265 sensitization, we analyzed apoptosis markers, PARP cleavage and caspase activity. RAF265 alone was not able to induce detectable PARP cleavage or caspase activity in A2058 cells (Figs. 3B and 4A). Similar observations were reported for MEK inhibitor AZD6244 which was shown...
Figure 1. Primary synthetic lethal siRNA screens with RAF265 in A2058 cells. A, SMARTpool siRNA screens without RAF265 or B, in the presence of 0.4 μmol/L RAF265. The Y- and X-axes refer to the NZ replicate 1 and 2, respectively. C, the primary hits selected from the screen. The Y- and X-axes refer to the averaged NZ in the presence of 0 and 0.4 μmol/L of RAF265, respectively. The primary hits are labeled by their gene symbols. For A–C, LUC, BRAF, and PLK1 siRNA controls are shown in black stars, diamonds, and triangles, respectively. All experimental SMARTpool siRNAs are shown in grey dots. D, dose curves of RAF265 for selected primary hits in A2058. E, relative RNA expression for selected primary hits as measured by RT-PCR. Error bars are shown and indicate SD calculated from 8 independent experiments.
Figure 2. Validation assays for 4 primary hits identified from siRNA screens in A2058 cells. Relative RNA expression and dose curves of RAF265 after transfection with siRNAs against ALPK1 (A), MAPK11 (B), PIK3R4 (C), and PRKD3 (D) are shown. E, RAF265 dose curves for 3 stable cell lines expressing PRKD3 shRNAs with (left) or without (right) DOX exposure are shown. Western blots show PRKD3 protein levels with and without DOX induction in 3 stable cell lines. The numbers below the image blots indicate the quantification of signal intensities normalized against tubulin. Arrow indicates the PRKD3 protein at the predicted molecular weight, an asterix (*) refers to a nonspecific band. Error bars indicate the SDs for 4 replicates at each condition.
to induce cell-cycle arrest and growth inhibition rather than apoptosis in melanoma cell lines and xenograft models (34). PRKD3 siRNA in combination with RAF265 treatments induced significant PARP cleavage and increased caspase activities, indicating PRKD3 knockdown sensitized RAF265 to induce apoptosis (Figs. 3B and 4A).

Previous report showed that cyclin D1, a cell-cycle regulator for G1 phase entry, was reduced after RAF265 treatments (31).

Figure 3. PRKD3 inhibition prevents reactivation of pERK and pAKT in A2058 cells. A, Western blots showing protein levels at 2 hours after RAF265 treatment, with and without PRKD3_01 siRNA transfections. B, Western blots showing protein levels at 72 hours after RAF265 treatment, with and without PRKD3_01 siRNA transfections. Tubulin levels served as a loading control. The numbers below the image blots indicate the quantification of signal intensities normalized against tubulin. The signal intensity of 0 µmol/L compound in the absence of PRKD3 knockdown was set as reference 1.0. C, cell viability measured 72 hours after cotransfection with BRAF and PRKD3 siRNAs, CRAF and PRKD3 siRNAs, and BRAF and CRAF siRNAs. Western blots showing protein levels of PRKD3, BRAF, and CRAF after siRNA transfections against PRKD3, BRAF, and CRAF, respectively. Arrow indicates the PRKD3 protein at the predicted molecular weight, an asterix (*) refers to a nonspecific band. Error bars indicate SDs calculated from 4 independent experiments.
Cyclin D1 was analyzed in A2058 cells in the presence or absence of PRKD3 siRNAs. Consistent with previous reports, RAF265 treatments resulted in a reduction of cyclin D1, and the reduction was dose dependent (Fig. 3B). PRKD3 siRNA alone did not affect cyclin D1 expression, but PRKD3 siRNA in combination with RAF265 enhanced the reduction of cyclin D1, suggesting an interruption of cell-cycle progression (Fig. 3B). Consistent with this observation, cell-cycle profiles showed that PRKD3 siRNAs or 0.5 µmol/L RAF265 alone partially blocked G2–M progression, but PRKD3 siRNAs in combination with 0.5 µmol/L RAF265 completely blocked G2–M progression, result in a 0% of cells in G2–M (Fig. 4B, Supplementary Fig. S3). It was shown that PRKD3 was phosphorylated at its activation loops during mitosis, suggesting that PRKD3 activity is regulated in a cell-cycle dependent manner (35). Although PRKD3 siRNAs or 0.5 µmol/L RAF265 alone resulted in a reduction of G0–G1 cell numbers, we did not observe cooperative effects in reducing G0–G1 cell numbers (Fig. 4B, Supplementary Fig. S3). It is possible that the cooperative effects of PRKD3 inhibition and RAF265 in reduction of cyclin D1 is a consequence from blocking of G2–M entry.

Finally, the sensitization of PRKD3 knockdown and RAF265 was tested in a colony formation growth assay. PRKD3 siRNAs or RAF265 (0.5 and 2 µmol/L) alone partially inhibited the colony formation of A2058 cells, but PRKD3...
siRNAs in combination with 0.5 μmol/L or 2 μmol/L RAF265 completely inhibit A2058 cell growth in colonies (Fig. 4C). Taken together, we have shown that PRKD3 knockdown mediates RAF265 sensitization to prevent the reactivation of MAPK signaling, induce apoptosis markers, reduce cell-cycle progression, and induce tumor cell growth inhibition at high density in plastic and low density colony formation assays in A2058 cells.

**PRKD3 inhibition sensitizes RAF265 to kill the A375 melanoma cells**

We analyzed a RAF265 sensitive melanoma cell line A375, which also harbors the BRAF (V600E) mutation. A375 cells are sensitive to RAF265 with an IC_{50} of 0.3 μmol/L as shown by control LUC siRNAs transfected cells (Fig. 5A). After PRKD3 siRNA transfection, the IC_{50} for RAF265 was shifted from 0.3 μmol/L to 0.16 μmol/L for both PRKD3 siRNAs (Fig. 5A). Next, colony formation assays were done. PRKD3 siRNAs or RAF265 (0.05 μmol) alone only partially inhibited A375 cell growth in colony formation, but PRKD3 siRNAs in combination with 0.05 μmol/L RAF265 completely inhibited cell growth in colony formation (Fig. 5B).

Similar to A2058 cells, pERK was reactivated after 72 hours of RAF265 treatment and reduced after the combination with PRKD3 knockdown (Fig. 5D). pMEK and MEK levels were reduced after RAF265 treatment, and further reduced after combination with PRKD3 knockdown in A375 cells (Fig. 5D). RAF265 resulted in a reduction of cyclin D1, and the reduction was dose dependent (Fig. 5C and D). PRKD3 siRNAs alone did not affect cyclin D1 expression, but PRKD3 siRNA in combination with RAF265 enhance the reduction of cyclin D1 (Fig. 5D). PRKD3 siRNA transfection or RAF265 treatments alone in A375 were not sufficient to induce detectable PARP cleavage, whereas PRKD3 siRNA transfection in combination with RAF265 treatments resulted in significant induction of PARP cleavage (Fig. 5C and D). We did not see changes on total CRAF after RAF265 treatment in A375 cells (Fig. 5D). Instead, we observed decreased phospho-CRAF (Ser338) after RAF265 treatments (Fig. 5D), it is likely that CRAF is an efficacy target of RAF265 in the A375 cell line. PRKD3 siRNA mediated knockdown had no effect on pCRAF or CRAF levels, indicating PRKD3 affects pERK levels in a CRAF-independent manner. In A375, PRKD3 siRNAs in combination with BRAF siRNA treatments resulted in a greater growth inhibition compared with any of the single treatments (Fig. 5E). However, neither the combination of PRKD3 and CRAF siRNA treatments nor the combination of BRAF and CRAF siRNAs showed additive growth inhibition (Fig. 5E). The knockdown efficiencies of PRKD3, BRAF, and CRAF siRNAs were validated by Western blots (Fig. 5E). The effects on levels of pERK, pMEK, cyclin D1, and PARP cleavage, as well as colony formation, after PRKD3 knockdown in combination with RAF265 are similar in A375 and A2058 cells, indicating this mechanism is shared by different tumor cells from the same lineage or with the same genetic background BRAF (V600E). These data also suggest that single agent sensitivity to RAF265 is not a requirement for combination PRKD3 knockdown-induced cell killing.

**PRKD3 inhibition sensitizes with RAF and MEK inhibitors across multiple melanoma cells**

To better understand whether the sensitization between PRKD3 inhibition and RAF265 in A2058 and A375 cells is shared with other RAF or MEK inhibitors across cell lines of various lineage and genetic background, we tested additional RAF and MEK inhibitors in a panel of 12 cell lines (Fig. 6 and Supplementary Fig. S4). In addition to RAF265, we analyzed another RAF inhibitor PLX4032, and 2 MEK inhibitors, U0126 and PD0325901. We tested 12 cell lines including 6 melanoma cell lines carrying BRAF (V600E) mutation (RPMI7951, IGR39, A2058, A375, SKMEL5, and WM115), 4 cell lines bearing KRAS mutations of various lineages (PANC1, A549, SW620, and DU145), 1 nonmelanoma cell line with BRAF (V600E) mutation (SKHEP1, liver cancer), and 1 cell line which is wild type for both BRAF and RAS (G402, kidney cancer; Fig. 6).

For both RAF inhibitors RAF265 and PLX4032, 6 of 6 melanoma cell lines tested showed sensitization with both PRKD3 siRNAs (Fig. 6). Among the lines tested, 3 cell lines RPMI7951, IGR39, and A2058 were resistant to RAF265 and PLX4032 (Fig. 6A and C) when 2 μmol/L of compound was used in combination with PRKD3 siRNAs (Fig. 6B and D). For 3 sensitive cell lines A375, SKMEL5, and WM115, 0.3 μmol/L of compound was used in the sensitization assays (Fig. 6B and D). Among the cell lines tested, the sensitization between PRKD3 knockdown and RAF inhibitors was only observed in the melanoma cell lines tested, with only 1 exception in which PRKD3 siRNAs sensitized with RAF265 in colon cancer cell line SW620. It could be that additional genetic lesions outside of the MAPK signaling pathway are necessary for PRKD3 sensitization in nonmelanoma lineages.

For both MEK inhibitors, the sensitization was mainly observed with melanoma cell lines (Supplementary Fig. S4). For MEK inhibitor U0126, sensitization with both PRKD3 siRNAs was observed in 5 of 6 melanoma cell lines showed (Supplementary Fig. S4B), with 1 exception that no sensitization was found with SKMEL5. For MEK inhibitor PD0325901, 4 of 6 melanoma cell lines exhibited sensitization with both PRKD3 siRNAs, but sensitization was not found with melanoma cells IGR39 and WM115 (Supplementary Fig. S4D). In addition, PRKD3 siRNAs sensitized with PD0325901 in 2 nonmelanoma cell lines, SW620 and G402. Collectively, we showed that PRKD3 inhibition sensitizes with RAF and MEK inhibitors in multiple melanoma cell lines. Therefore, it seems that the sensitization between PRKD3 inhibition and RAF or MEK inhibitors is in melanoma lineage selective.

**Discussion**

Primary and acquired resistance to RAF and MEK inhibitors has been linked to reactivation of the MAPK pathway and PI3K pathway. Rebound of pERK levels has been observed for RAF inhibitor such as PLX4032 and MEK inhibitor like PD0325901 (12, 15, 36). Compensatory activation AKT has been reported both for the RAF inhibitor RAF265 and MEK inhibitor AZD6244 (AstraZeneca; refs. 20, 25, 37, 38). Here, we show that inhibition of PRKD3 sensitizes with RAF265 in a resistant melanoma cell line (A2058) by preventing rebound of
pERK and pAKT. Our data support a compound-induced resistance mechanism in which the reactivation of the MAPK pathway and PI3K pathway act coordinately to promote cell survival when RAF and/or MEK are inhibited. We observed that pAKT was reduced when PRKD3 was knocked down together with RAF265 in A2058 cells, but not in A375, a difference that could be attributable to the presence of the PTEN deletion in the A2058 cells (20).

The mechanism of how PRKD3 interacts with pERK and pAKT remains to be clarified. PKCe has been shown to
Figure 6. PRKD3 inhibition sensitizes RAF inhibitor RAF265 and PLX4032 in multiple melanoma cells. A, GI50s for RAF265 in multiple cell lines (GI50 refers to the concentration of compound that inhibits growth of cells by 50%) not reached up to 20 µmol/L. B, relative cell viabilities after RAF265 treatments, with or without PRKD3 siRNA transfections, are shown for multiple cell lines. C, GI50s for PLX4032 across multiple cell lines. D, relative cell viabilities after PLX4032 treatments, with or without PRKD3 siRNA transfections, are shown for each cell line. Cell viabilities are normalized to 0 µmol/L compound under each condition. Error bars indicate SDs calculated from 6 independent experiments, an asterisk (*) refers to a cell line showing sensitization with both PRKD3 siRNAs compared with the control LUC siRNA condition (P < 0.01).
phosphorylate and regulate PRKD3 activity in prostate cancer cells (29). We tested whether PKCβ knockdown can phenocopy the PRKD3 knockdown in sensitizing RAF inhibitor. However, we did not observe any sensitization effects (data not shown). Among the 3 PKD proteins, PRKD1 is the most extensively studied and has been implicated in a broad range of cellular process and can be activated by a variety of regulatory peptides (39–41). It has been shown that PRKD1 phosphorylates RIN1 and releases it from competing with RAF for binding to RAS, thus resulting in an activation of the RAF–MEK–ERK pathway (42). It will be interesting to investigate whether PRKD3 shares any function with PRKD1 such as RIN1 phosphorylation. We have tested whether PKD1 or RIN1 siRNAs can sensitize RAF265 in killing A2058. We did not observe any sensitization effects (data not shown). In melanoma cells, PRKD2 protein expression was undetectable and thus it was not tested in our sensitization studies. Even though PRKD members share some cellular functions, they can be differentially expressed and exert different functions (28). Further investigation of PRKD3 specific cellular function(s) will be required to better understand how PRKD3 ablation augments RAF265 activity in melanoma. One limitation of our screen is that RAF265 can inhibit multiple RAF isofoms, a variety kinases, and VEGFR (43). Similar to Sorafenib, the efficacy RAF265 in vivo may not be due exclusively to the activity on BRAF (V600E) (44). Thus, the sensitization seen with PRKD3 knockdown and RAF265 could be because of a combinatorial effect(s) with other targets in addition to RAF isofoms and VEGFR. The lack of selectivity of RAF265 complicates the interpretation of PRKD3 knockdown sensitization and confounds the ability to clearly define a mechanism of action at this time.

This study is the first demonstration that PRKD3 inhibition can sensitize with RAF or MEK inhibitors in BRAF (V600E) melanoma cells. Our current data support a model in which PRKD3 could prevent reactivation of MAPK signaling caused by RAF or MEK inhibitors and sensitize with these inhibitors to kill resistant tumor cells. PRKD3 is a potentially druggable kinase because there are known inhibitors of this class of enzymes (44, 45). It is not clear whether the kinase activity or a potential scaffold activity of PRKD3 is required for sensitization with the RAF or MEK inhibitor(s). Although our understanding of how PRKD3 modulates pERK and pAKT in response to RAF265 requires further study, PRKD3 provides a synthetic lethal target opportunity to develop effective combination therapy to overcome drug-induced resistance caused by RAF or MEK inhibitors.

Disclosure of Potential Conflicts of Interest

M. Labow and L.A. Gaither are shareholders of Novartis. The other authors disclosed no potential conflicts of interest.

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Protein Kinase D3 Sensitizes RAF Inhibitor RAF265

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Protein Kinase D3 Sensitizes RAF Inhibitor RAF265 in Melanoma Cells by Preventing Reactivation of MAPK Signaling

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