Published in final edited form as:

Mol Carcinog. 2020 January ; 59(1): 5–14. doi:10.1002/mc.23123.

The role of Polo-like kinase 3 in the response of BRAF-mutant cells to targeted anti-cancer therapies.

Mahamat Babagana¹, Julia V. Kichina¹, Hannah Slabodkin¹, Sydney Johnson¹, Alexei Maslov¹, Lorin Brown¹, Kristopher Attwood², Mikhail A. Nikiforov³, Eugene S. Kandel^{1,*}

¹Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY.

²Department of Biostatistics & Bioinformatics, Roswell Park Comprehensive Cancer Center, Buffalo, NY.

³Department of Cancer Biology, Wake Forest University Baptist Medical Center, Winston-Salem, NC.

Abstract

The activation of oncogenic MAP kinase cascade via mutations in BRAF is often observed in human melanomas. Targeted inhibitors of BRAF (BRAFi), alone or as a part of a combination therapy, offer a significant benefit to such patients. Unfortunately, some cases are initially non-responsive to these drugs, while others become refractory in the course of treatment, underscoring the need to understand and mitigate the underlying resistance mechanisms. We report that interference with Polo-like kinase 3 (PLK3) reduces the tolerance of BRAF-mutant melanoma cells to BRAFi, while increased PLK3 expression has the opposite effect. Accordingly, PLK3 expression correlates with tolerance to BRAFi in a panel of BRAF-mutant cell lines and is elevated in a subset of recurring BRAFi-resistant melanomas. In PLK3-expressing cells, R406, a kinase inhibitor whose targets include PLK3, recapitulates the sensitizing effects of genetic PLK3 inhibitors. The findings support a role for PLK3 as a predictor of BRAFi efficacy and suggest suppression of PLK3 as a way to improve the efficacy of targeted therapy.

Keywords

BRAF; PLK3; vemurafenib; R406; cobimetinib

Introduction.

Aberrant activation of the MAP kinase cascade is a common oncogenic driver of human cancer. In melanoma, the most frequent abnormality behind this phenomenon is an activating mutation in BRAF. Pharmacological targeting of this pathway is an important part of the recent revolution in the care of metastatic melanoma. BRAF inhibitors, vemurafenib and dabrafenib, as well as the MEK inhibitors, trametinib and cobimetinib, dramatically

*Corresponding Author: Eugene S. Kandel, Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Elm & Carlton St., Buffalo, NY. USA, Phone: 1-716-845-3530, Fax: 1-716-845-3944, eugene.kandel@roswellpark.org.

Data Availability Statement.

Data available on request from the authors.

improve previously dismal survival and therapeutic response rates in this disease. In BRAF-mutant metastatic melanoma, BRAF inhibitors alone or combined with inhibitors of MEK achieve objective response in 40–60%^{1–3} and 60–70%⁴ of cases respectively. Unfortunately, this still leaves a sizable subset of tumors refractory to this intervention. Furthermore, complete responses to these drugs are rare, and most cases experience emergence of a drug-resistant disease during treatment. The situation is even worse in non-melanoma BRAF-mutant cancers, where BRAF inhibitors, typically, are less effective^{5,6}. The low frequency of complete responses and the existence of intrinsic and adaptive resistance to MAPK pathway inhibitors underscores the need to identify the molecular determinants of resistance to these compounds, along with the strategies to increase drug efficacy.

PLK3 is a member of the polo-like kinase family. Despite structural similarity, its function is often contrasted with those of other PLKs⁷. PLK3 has been implicated in multiple pathways regulating cell cycle progression and stress response^{8–14}. Some earlier reports suggested that PLK3 is a tumor suppressor¹⁵, but this remains controversial, as later studies failed to reproduce those observations^{11,16}. According to its expression pattern, PLK3 is classified with immediate-early genes¹⁷, a group of genes that are rapidly activated by mitogenic factors and includes numerous proto-oncogenes¹⁸. Interestingly, PLK3 interacts and cooperates with MEK at the onset of cell division¹³, while inhibition of PLK3 sensitizes cells to ER stress⁹ and cisplatin¹⁹. Expression of PLK3 is elevated in ovarian and breast cancers^{20,21}, as well as in melanoma²², indicating that the tumor-suppressive role of PLK3, if any, is not universal.

In this study, we report that PLK3 modulates sensitivity of BRAF-mutated cells to inhibitors of MAP kinase cascade: elevated levels of PLK3 increase resistance, while suppression of this enzyme sensitizes to treatment. These results, obtained on cultured cells and xenografts, agree with the data on the correlation between PLK3 levels and vemurafenib resistance in melanoma cell lines and on the high incidence of increased PLK3 expression in recurring drug-resistant disease. Our findings suggest that PLK3 is a marker of melanoma resistance to the inhibitors of MAP kinase cascade and a target for increasing the efficacy of these drugs.

Materials and Methods

Cell lines and reagents

A375 and SK-MEL28 cells were obtained from the ATCC and maintained in high-glucose Dulbecco's Modified Eagle Medium and Minimum Essential Medium, respectively. B-CPAP cells were a gift from Dr. Katerina Gurova and were maintained in RPMI1640. All culture media were supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal bovine serum. All cultures were kept at 37°C in the presence of 5% CO₂.

The cells were free of mycoplasma (as assessed by MycoAlert kit from Lonza; LT07–318) and contaminating replication-competent retroviruses^{23,24}.

Vemurafenib was obtained from LC Laboratories (#918504–65-1). R406 (#S2194), cobimetinib (#S8041), and AZD6244 (#S1008) were obtained from Selleck Chemicals. DMSO was purchased from Sigma Aldrich.

PLK3-deficient A375 derivatives.

A construct for a gRNA targeting PLK3 (GACAGGAAACCGGCGGCAGG) was supplied by GenScript Biotech Corp. in pLentiCRISPRv2 vector, which also expresses Cas9²⁵. This construct was transiently transfected into A375 cell line. Three days later, single-cell clones were established, expanded and tested for PLK3 expression by Western blotting. Two clones, A375#10 and A375#15, which showed undetectable levels of PLK3, were chosen for further studies.

Vectors and viral transductions

Human PLK3-KD and PLK3-PBD (EGFP fusions from Addgene plasmids #23266 and #23267) were cloned in a lentiviral vector pLM-CMV-neo (a gift of Dr. P. Chumakov) to make pLM-CMV-PLK3-KD-neo and pLM-CMV-PLK3-PBD-neo respectively. The EGFP part of pLM-CMV-PLK3-KD-neo was replaced with the C-terminal half of PLK3 to generate PLK3-expressing vector pLM-CMV-PLK3-WT. Alternatively, pJP1520-PLK3 (DNASU plasmid ID HsCD00037942), was used to express complete human PLK3 in some experiments. For the constitutive RNAi experiments, shRNA#1 or #2 against PLK3 (Dharmacon V2LHS_172853 and V2LHS_172854, respectively) or a non-silencing control “Non-Sil” (Dharmacon RHS4346) were used. For inducible RNAi, PLK3 shRNA (TRE-shPLK3 #1-#3; Dharmacon V3THS_393300, V3THS_393301, V3THS_393302) were used. Lentiviral transduction was performed as described elsewhere²⁶.

Proliferation and Drug Assays

Drug response curves were carried out with the indicated cell lines in their respective culture media. Drug treatments began 24 hours after seeding. Cells were cultured for 5 days and fixed with 100% methanol. Cell numbers were compared by the methylene blue staining/extraction method as described elsewhere²⁷. IC50 values for each cell line were computed using GraphPad Prism 6 (GraphPad Software, Inc.).

Cell proliferation was compared using an EdU incorporation assay. Cells were pulsed with EdU (10uM) for 1 hour, fixed with 3.7% paraformaldehyde for 15 min at room temperature and subsequently washed with phosphate-buffered saline. Incubation in Triton X-100 (0.5%) for 20 min was used to permeabilize cells. Subsequently, cells were stained using DAPI and the Click-iT™ Alexa Fluor 488 imaging kit (C10086; ThermoFisher Scientific) according to the manufacturer's protocol. For each cell line/condition, 5 random fields of view were assessed. The images were converted to binary format in ImageJ software.

Antibodies and Immunoblotting

RIPA buffer (#899900; Thermo Scientific) supplemented with protease/phosphatase inhibitors (#5872S; Cell Signaling) and EDTA solution (0.5mM [final]) was used to lyse cells. Membranes were probed overnight in 5% BSA at 4°C with gentle rocking with antibodies at manufacturers' recommended dilutions. PLK3 (#4896), pMEK1/2^{S217/221}

(#9121S) and MEK1/2 (#4694S) antibodies were obtained from Cell Signaling. pERK^{Y204} (#SC-7383), ERK1/2 (#SC-292838), beta-actin (SC-47778) and tubulin (#SC-8035) antibodies were obtained from Santa Cruz Biotechnology. GAPDH (#AM4300) antibody was obtained from Ambion. GFP antibody (#ab290) was obtained from Abcam.

Quantitative RT-PCR

RNA was extracted using Trizol reagent per manufacturer's recommendations (Invitrogen #15596-018). First strand synthesis was performed using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen # 18064014). The relative PLK3 expression was assessed via quantitative RT-PCR (primers 5'-3'-Fwd-CCTTGCGCGGACCTGAG and 5'-3'-Rev-AGGATCTTCTCGCGCTGATG). Human GAPDH (primers 5'-3'-Fwd-ACCACCCTGTTGCTGTAGCCAA and 5'-3'-Rev-GTCTCCTCTGACTTCAACAGCG) was used as an internal reference control. QPCR reactions were carried out in technical replicas of 4. The cycle threshold data was converted to change-fold in expression by the "delta delta Ct" method²⁸.

Expression Dataset Analysis

PLK3 expressional data for 37 melanoma cell lines was obtained from the Cancer Cell Line Encyclopedia (CCLE) using the Integrative Genomic Viewer (portals.broadinstitute.org/ccle/home). The pharmacological profiling dataset (CCLE_NP24.2009_Drug_data_2015.02.24) deposited in the CCLE (portals.broadinstitute.org/ccle/home) was used to obtain PLX4720 effectiveness.

The data on PLK3 expression in patient tumors before BRAF inhibitor treatment and in the recurring tumors was obtained from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/; GEO dataset GSE50509) and analyzed using the GEO2R tool (www.ncbi.nlm.nih.gov/geo/geo2r/).

Drug Cooperativity Analysis

Cooperativity between drugs was evaluated using Compusyn software (ComboSyn, Inc.). It relies on the median-effect method of Chou–Talalay, which is founded on the "combination index" theorem^{29,30}, to define synergy. The calculation of a "Combination Index" (CI) compares the observed effect and a calculated additive effect. CI values of 1, >1, and <1 indicate additivity, antagonism, and synergism, respectively.

Mouse Xenografts

A375-Clone#15 (A375-Cl#15) cells harboring either the PLK3-expression construct ("PLK3") or the corresponding empty vector control ("Control") were subcutaneously injected into SCID mice. Tumors were measured daily using calipers and the volume calculated as before³¹.

When the tumors reached approximately 100mm³, the mice started receiving daily IP injections of vemurafenib (15mg/ml). The maximal fraction, by which a treated tumor decreased in size relatively to its volume at the start of the treatment, was determined and

plotted as a “box and whiskers” graph for each group. 6 SCID male mice were used for each group.

Results

The analysis of gene expression and pharmacological profiling data deposited in the Cancer Cell Line Encyclopedia³² reveals a significant ($p=0.004$) negative correlation between the efficacy of a BRAFi (PLX4720³³, a close analogue of vemurafenib) and PLK3 expression in 37 BRAF mutant melanoma cell lines (Fig. 1A). Interestingly, in a vemurafenib-sensitive BRAF-mutant melanoma cells line, A375, expression of PLK3 protein profoundly decreases following vemurafenib treatment (Fig. 1B). The latter agrees with the behavior of PLK3 mRNA in a previously described dataset (GEO dataset GSE42872; www.ncbi.nlm.nih.gov/geo/) generated under similar treatment conditions³⁴. We hypothesized that suppression of PLK3 expression is an important factor in BRAFi activity.

To test whether PLK3 suppression increases BRAFi efficacy, we transduced the BRAF mutant SK-MEL-28 cell line either with shRNAs targeting PLK3 or with the respective control vector, and challenged these cells with vemurafenib. We observed that PLK3 suppression increased vemurafenib sensitivity relative to the control cells (Fig. 1C–D). A greater effect was noted in SK-MEL-28-shPLK3 #1 cells ($IC_{50}=51\text{nM}$, 95%CI 46–56nM) compared to SK-MEL-28-shPLK3 #2 ($IC_{50}=72\text{nM}$, 95%CI 52–95nM) in agreement with the relative potency of the shRNAs in knocking down their intended target (Suppl. Fig. 1A). Knockdown of PLK3 also sensitized A375 cells to vemurafenib and PLX4720 (Suppl. Fig. 1B–E). Furthermore, using a series of regulated (“Tet-on”) shPLK3 expression constructs to reduce PLK3 expression, we observed doxycycline-dependent sensitization of SK-MEL-28 cells to vemurafenib, which roughly corresponded to the efficacy of individual shRNAs (Fig. 1E and Suppl. Fig. 1F). Doxycycline-regulated PLK3 knockdown sensitized A375 cells as well (Suppl. Fig. 1G).

Accordingly, upon vemurafenib treatment, the cells with reduced levels of PLK3 retained a significantly ($p = 1.2\text{E-}05$) lower capacity for DNA replication, as evidenced by decreased incorporation of a nucleotide analogue EdU (5-ethynyl-2-deoxyuridine) (Fig. 1F–G).

We also investigated the effect of elevated PLK3 using ectopic expression. Ectopic expression of PLK3, unlike the endogenous one, persists under BRAFi treatment (Fig. 2A), suggesting that the latter predominantly suppresses PLK3 expression at the RNA, but not the protein level (compare to Fig. 1B). Importantly, transduction with a PLK3-expressing vector in SK-MEL-28 and A375 elevated the resistance of these cells to vemurafenib (Fig. 2B–E).

PLK3 contains a conserved Polo Box Domain (PBD), which, among other things, affects the localization of the protein; and a kinase domain (KD), which contains the enzymatic activity. Expression of PLK3-KD, devoid of the PBD, behaves as an active form of PLK3, at least, in some circumstances^{15,35}. We relied on GFP-fused versions of PLK3-KD and PLK3-PBD, which were previously described by others¹⁵ and which were readily expressed in A375 cells (Suppl. Fig. 1A). Interestingly, ectopic expression of PLK3-KD, but not of PLK3-PBD, protected A375 cells from a BRAFi (Suppl. Fig. 2B–C).

In addition to BRAFi, MEK inhibitors (MEKi) are effective against BRAF-mutated melanomas, and the two classes of inhibitors are commonly combined for improved outcomes^{4,36}. We explored the effect of PLK3 overexpression on the response of SK-MEL-28 to a MEKi cobimetinib (aka GDC-0973), and observed that the overexpressing cells were more tolerant of the drug than their vector-bearing counterparts (Fig. 2F). Overexpression of PLK3 in A375 also yielded protection from another MEKi, selumetinib (aka AZD6244) (Suppl. Fig. 2D–E). Once again, the effect was seen upon expression of PLK3-KD, but not PLK3-PBD (Suppl. Fig. 2F).

To investigate whether our observations could translate to in vivo studies, we subcutaneously injected mice with a clonal derivative of the A375 cell line (clone A375#15) transduced with either a PLK3-expressing construct or the corresponding vector control. Of note, unmodified A375#15 cells express negligible levels of endogenous PLK3 (Suppl. Fig. 2G and data not shown). Upon reaching ~100mm³, the xenografts were treated with a modest dose of vemurafenib (15mg/ml intraperitoneally daily) and monitored for volumetric changes. The maximal response for each tumor was defined as the maximal fraction of the original volume, by which the tumor shrunk at any point of the treatment. Ectopic expression of PLK3 greatly reduced the maximal response, as compared to the vector-bearing control group (Fig. 2G). The notion that elevated PLK3 expression is associated with higher tolerance to BRAFi is in agreement with the data on the changes in PLK3 mRNA levels in tumors progressing under BRAFi therapy (Fig. 2H): >70% of these cases have shown an increase in PLK3 expression. Notably, not only are the cases with increased PLK3 expression more prevalent, but also the magnitude of change in this cohort is significantly higher ($p<0.01$ by two-sided Mann-Whitney test).

The efficacy of MAPK cascade inhibitors is correlated with their ability to prevent activating phosphorylation of ERKs (ERK1 and 2, in particular) by MEKs. Accordingly, knockdown of PLK3 in A375 and SK-MEL-28 cells in the presence of vemurafenib led to further reduction in the amounts of active (phosphorylated) ERK (Fig. 3A–B). Similar results were seen in A375-TRE-shPLK3 cells treated with vemurafenib (Fig. 3C). Interestingly, this was not accompanied by a further decrease in phosphorylation of MEK. In fact, we have consistently observed higher amounts of phosphorylated MEK in PLK3-knockdown cells under these conditions (Fig. 3A–C). Consequently, in the control cells vemurafenib treatment similarly reduced pMEK and pERK, but in the cells with PLK3 knockdown pERK was relatively more vulnerable, as indicated by the changes in the pERK/pMEK ratios upon treatment (Fig. 3E–F). Consistent with these findings, in the presence of vemurafenib, PLK3-overexpressing A375 cells exhibited higher levels of active ERK, but similar levels of active MEK, when compared to their vector-transduced counterparts (Fig. 3D).

Taken together, these data indicate that PLK3 affects the correlation between stimulatory phosphorylation of serines 217/221 on MEK and the phosphorylation status of immediate MEK target sites on ERKs. In this manner, PLK3 determines what amount of upstream (coming through MEK) signaling is required to keep a certain level of ERK activity: high PLK3 activity helps keep ERKs active even when BRAF activity is reduced by BRAFi, while the lack of PLK3 makes it easier to achieve suppression of ERKs with a partial

inhibition of BRAF. In this regard, PLK3 resembles PAK1³⁷, another known modulator of BRAFi- and MEKi-tolerance²⁷.

Our observations predict that pharmacological targeting of PLK3 may improve the efficacy of MAPK kinase inhibition. We are unaware of a highly-specific inhibitor of PLK3, possibly because the kinase was previously considered a tumor suppressor and, hence, an undesirable target⁷. However, R406, a compound first introduced as a SYK inhibitor³⁸, has a much higher affinity towards PLK3 than towards other polo-like kinases or SYK³⁹. Importantly, melanoma cells lack SYK and cannot tolerate expression of this kinase⁴⁰. Therefore, we decided to investigate the ability of R406 to cooperate with vemurafenib in A375 and SK-MEL-28 cells.

The cells were treated with a moderate dose of vemurafenib, with or without addition of an otherwise non-toxic dose of R406. Both A375 (Fig. 4A) and SK-MEL-28 (Suppl. Fig. 3A) cells treated with the drug combination demonstrated higher sensitivity. The analysis of cell survival using the Compusyn program, which relies on the Chou-Talalay method²⁹, confirmed drug synergism, as attested by combination index values lower than 1 for both A375 (Table 1) and SK-MEL-28 (Suppl. Fig. 3B).

To investigate whether PLK3 inhibition could also improve BRAFi efficacy in BRAF-mutated cancers of non-melanoma origin, we challenged B-CPAP, a BRAFV600E mutant thyroid cancer cell line, with vemurafenib in the presence or absence of R406. In agreement with prior reports⁴¹, B-CPAP were highly resistant to BRAF inhibition, with vemurafenib achieving less suppression in B-CPAP at 100 nM than in A375 at 20 nM (compare Fig. 4B and Fig. 4A). Interestingly, challenging B-CPAP with vemurafenib in the presence of R406, which had only a weak effect alone, demonstrated synergistic inhibition (Fig. 4B & Table 2). These results indicate that addition of R406 to BRAF inhibitors may increase the efficacy of the latter in otherwise resistant cases.

In order to explore PLK3-dependence of the sensitizing effect of R406, we used clonal derivatives of A375 (A375#10 and A375#15) that express negligible levels of endogenous PLK3 (Suppl. Fig. 2E and data not shown). As expected, both A375#15 (Fig. 4C) and A375#10 (Suppl. Figure 4) gained resistance upon ectopic expression of PLK3. Importantly, vemurafenib-R406 combination was more potent than either drug alone against the PLK3-expressing derivatives of these cells, but was no more effective than vemurafenib alone in the control vector-transduced A375#15 (Fig. 4C) and A375#10 (Suppl. Fig. 4).

We also observed that, in the presence of vemurafenib, R406 treatment of A375 cells reduced the levels of phosphorylated ERK, but not MEK (Fig. 4D), resembling the effect of genetic suppression of PLK3 (Fig. 3). Considering that R406 offers no additional sensitization in the absence of PLK3, that R406 overcomes PLK3-mediated resistance, and that the biochemical consequences of R406 treatment in the presence of vemurafenib resemble those of PLK3 shRNAs, we concluded that in our experimental conditions PLK3 is the relevant target of R406-induced sensitization to BRAFi.

Discussion.

Overall, our observations point to PLK3 as an important mediator of BRAFi response. Inhibition of PLK3 sensitized cells to BRAFi, which was seen with different shRNAs and shRNA-expression systems and in different cell lines with at least two BRAFi (vemurafenib and PLX4720). Although the risks of shRNA off-target effects are well-known⁴², the concordance between multiple different shRNAs strongly argues that the observed effect is PLK3-specific. Furthermore, this effect was recapitulated using a putative PLK3 chemical inhibitor, and was mirrored by a protective effect of PLK3 overexpression. This assessment is consistent with the data on PLK3 expression in recurring tumors and with the general correlation between PLK3 levels and BRAFi resistance in cell lines.

Our findings bring up important issues and areas for future research. We believe that this work adds to the body of evidence that positions PLK3 as a legitimate target for cancer therapy. Nevertheless, PLK3 remains relatively poorly studied, and the consequences of its systemic inhibition are worth further investigation. Promisingly, PLK3 knockout animals are viable, with normal fecundity and development^{11,16}. Furthermore, R406, which inhibits PLK3 among other targets, is an active form of fostamatinib (aka R788), which is currently undergoing multiple clinical trials (information accessible at clinicaltrials.gov), albeit not for the indications discussed herein.

In the context of melanoma, BRAFi are commonly combined with MEKi. Predictably, PLK3, which protects from BRAFi and MEKi individually, also provides protection from BRAFi/MEKi combination (Suppl. Fig.2H). It remains to be seen whether in practical settings PLK3 inhibition would be best used as an additive to a BRAFi/MEKi combination or as a replacement for MEKi. Importantly, in other cancers, BRAFi are actively investigated as single agents or in combinations that lack MEKi (information accessible at clinicaltrials.gov). The observation that PLK3 expression is regulated by the MAPK pathway, while PLK3 itself contributes to the pathway activity adds to the list of cancer-relevant pathways where positive feedback loops can be established by deregulation of components (e.g.^{43,44}).

Importantly, inhibition of PLK3 appears to reduce the correlation between the activation of MEK and the activation of ERK, with lower levels of phosphorylated ERK observed under the same or even higher levels of phosphorylated MEK (Fig.3 and Fig.4D). Of note, many reported mechanisms of resistance to BRAFi still depend on MEK-ERK interaction. For example, enhanced dimerization of BRAF, which makes the latter less susceptible to some BRAFi⁴⁵, maintains higher MEK activity in treated cells. Furthermore, the so-called paradoxical activation of the MAPK cascade^{46,47}, which BRAFi induce in cells lacking BRAF mutations and which can contribute to secondary cancers in treated patients⁴⁸, includes MEK-dependent activation of ERKs. It is tempting to speculate that inhibition of PLK3 may be effective in overcoming such resistance mechanisms and preventing such side effects. A new generation of “paradox breaking” BRAFi suppress MEK-ERK signaling in BRAF-mutant cells without activating it in cells with wild type BRAF⁴⁹, yet the resistance to these compounds is also associated with increased ERK phosphorylation⁵⁰. Thus, it would be interesting to examine whether the potency of such agents can be improved by

concomitant PLK3 inhibition. Furthermore, MAP kinase cascade contributes to some pathophysiological conditions besides cancer^{51,52}. Thus, it would be interesting to explore the merits of PLK3 inhibition in various additional contexts.

The lack of a highly selective PLK3 inhibitor remains a concern. Perhaps, R406 could provide structural insights into the design of such a molecule. On the other hand, PLK1 is essential for the survival of melanoma cells⁵³ and is a well-established target in cancer⁵⁴. Hence, an agent targeting both PLK1 and PLK3 might present an acceptable compromise on the issue of specificity.

Finally, the exact mechanism connecting PLK3 to ERK activation is yet to be firmly established. Formation of a co-regulated tripartite PLK3/MEK/ERK complex, which was previously described by others¹³, provides a plausible mechanistic explanation for our findings. Under such a scenario, PLK3 stabilizes the MEK-ERK interaction, which allows for efficient phosphorylation of ERK even if the concentration of active MEK is relatively low. Future studies would be needed to establish the accuracy of this model and to exclude the contribution of other conceivable mechanisms, such as regulation of an ERK-specific phosphatase by PLK3. If the hypothesis about the role of the PLK3/MEK/ERK complex is further validated, disrupting the association of these proteins may prove to be a more specific and safe therapeutic strategy than direct targeting of the PLK3 enzymatic activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

The participation of HS, SJ and LB was made possible through Science Research Program at Buffalo City Honors School.

Financial Support:

The work was supported by grants from the Roswell Park Alliance Foundation, Jennifer Linscott Tietgen Family Foundation and the National Institutes of Health (R03 CA205178 and R21 CA137708) to ESK. The use of Roswell Park Cancer Institute's shared resources is supported through the National Cancer Institute (NCI) grant P30 CA016056.

References.

1. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. The New England journal of medicine. 2011;364(26):2507–2516. [PubMed: 21639808]
2. Hauschild A, Grob JJ, Demidov LV, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet. 2012;380(9839):358–365. [PubMed: 22735384]
3. Ascierto PA, Minor D, Ribas A, et al. Phase II trial (BREAK-2) of the BRAF inhibitor dabrafenib (GSK2118436) in patients with metastatic melanoma. J Clin Oncol. 2013;31(26):3205–3211. [PubMed: 23918947]
4. Long GV, Stroyakovskiy D, Gogas H, et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. The New England journal of medicine. 2014;371(20):1877–1888. [PubMed: 25265492]

5. Hyman DM, Puzanov I, Subbiah V, et al. Vemurafenib in Multiple Nonmelanoma Cancers with BRAF V600 Mutations. *The New England journal of medicine*. 2015;373(8):726–736. [PubMed: 26287849]
6. Kopetz S, Desai J, Chan E, et al. Phase II Pilot Study of Vemurafenib in Patients With Metastatic BRAF-Mutated Colorectal Cancer. *J Clin Oncol*. 2015;33(34):4032–4038. [PubMed: 26460303]
7. Helmke C, Becker S, Strebhardt K. The role of Plk3 in oncogenesis. *Oncogene*. 2016;35(2):135–147. [PubMed: 25915845]
8. Xie S, Wu H, Wang Q, et al. Plk3 functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. *The Journal of biological chemistry*. 2001;276(46):43305–43312. [PubMed: 11551930]
9. Mamrosh JL, Lee JM, Wagner M, et al. Nuclear receptor LRH-1/NR5A2 is required and targetable for liver endoplasmic reticulum stress resolution. *eLife*. 2014;3:e01694.
10. Ouyang B, Li W, Pan H, Meadows J, Hoffmann I, Dai W. The physical association and phosphorylation of Cdc25C protein phosphatase by Prk. *Oncogene*. 1999;18(44):6029–6036. [PubMed: 10557092]
11. Myer DL, Robbins SB, Yin M, et al. Absence of polo-like kinase 3 in mice stabilizes Cdc25A after DNA damage but is not sufficient to produce tumors. *Mutation research*. 2011;714(1–2):1–10. [PubMed: 21376736]
12. Lopez-Sanchez I, Sanz-Garcia M, Lazo PA. Plk3 interacts with and specifically phosphorylates VRK1 in Ser342, a downstream target in a pathway that induces Golgi fragmentation. *Molecular and cellular biology*. 2009;29(5):1189–1201. [PubMed: 19103756]
13. Xie S, Wang Q, Ruan Q, et al. MEK1-induced Golgi dynamics during cell cycle progression is partly mediated by Polo-like kinase-3. *Oncogene*. 2004;23(21):3822–3829. [PubMed: 15021912]
14. Helmke C, Raab M, Rodel F, et al. Ligand stimulation of CD95 induces activation of Plk3 followed by phosphorylation of caspase-8. *Cell research*. 2016;26(8):914–934. [PubMed: 27325299]
15. Yang Y, Bai J, Shen R, et al. Polo-like kinase 3 functions as a tumor suppressor and is a negative regulator of hypoxia-inducible factor-1 alpha under hypoxic conditions. *Cancer Res*. 2008;68(11):4077–4085. [PubMed: 18519666]
16. Michel DR, Mun KS, Ho CC, Stambrook PJ. Cytoskeletal architecture and cell motility remain unperturbed in mouse embryonic fibroblasts from Plk3 knockout mice. *Experimental biology and medicine*. 2016;241(6):603–610. [PubMed: 26843517]
17. Donohue PJ, Alberts GF, Guo Y, Winkles JA. Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. *The Journal of biological chemistry*. 1995;270(17):10351–10357. [PubMed: 7730342]
18. Bahrami S, Drablos F. Gene regulation in the immediate-early response process. *Advances in biological regulation*. 2016;62:37–49. [PubMed: 27220739]
19. Sang M, Ando K, Okoshi R, et al. Plk3 inhibits pro-apoptotic activity of p73 through physical interaction and phosphorylation. *Genes to cells : devoted to molecular & cellular mechanisms*. 2009;14(7):775–788. [PubMed: 19490146]
20. Weichert W, Kristiansen G, Winzer KJ, et al. Polo-like kinase isoforms in breast cancer: expression patterns and prognostic implications. *Virchows Archiv : an international journal of pathology*. 2005;446(4):442–450. [PubMed: 15785925]
21. Weichert W, Denkert C, Schmidt M, et al. Polo-like kinase isoform expression is a prognostic factor in ovarian carcinoma. *British journal of cancer*. 2004;90(4):815–821. [PubMed: 14970859]
22. Xu D, Wang Q, Jiang Y, et al. Roles of Polo-like kinase 3 in suppressing tumor angiogenesis. *Experimental hematology & oncology*. 2012;1(1):5. [PubMed: 23210979]
23. Dong B, Silverman RH, Kandel ES. A natural human retrovirus efficiently complements vectors based on murine leukemia virus. *PLoS One*. 2008;3(9):e3144.
24. Yang J, Battacharya P, Singhal R, Kandel ES. Xenotropic murine leukemia virus-related virus (XMRV) in prostate cancer cells likely represents a laboratory artifact. *Oncotarget*. 2011;2(5):358–362. [PubMed: 21642749]
25. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nature methods*. 2014;11(8):783–784. [PubMed: 25075903]

26. Singhal R, Deng X, Chenchik AA, Kandel ES. Long-distance effects of insertional mutagenesis. *PLoS One*. 2011;6(1):e15832.
27. Babagana M, Johnson S, Slabodkin H, Bshara W, Morrison C, Kandel ES. P21-activated kinase 1 regulates resistance to BRAF inhibition in human cancer cells. *Molecular carcinogenesis*. 2017;56(5):1515–1525. [PubMed: 28052407]
28. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols*. 2008;3(6):1101–1108. [PubMed: 18546601]
29. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*. 2010;70(2):440–446. [PubMed: 20068163]
30. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Advances in enzyme regulation*. 1984;22:27–55. [PubMed: 6382953]
31. Somanath PR, Vijai J, Kichina JV, Byzova T, Kandel ES. The role of PAK-1 in activation of MAP kinase cascade and oncogenic transformation by Akt. *Oncogene*. 2009;28(25):2365–2369. [PubMed: 19421139]
32. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483(7391):603–607. [PubMed: 22460905]
33. Tsai J, Lee JT, Wang W, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(8):3041–3046. [PubMed: 18287029]
34. Parmenter TJ, Kleinschmidt M, Kinross KM, et al. Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis. *Cancer discovery*. 2014;4(4):423–433. [PubMed: 24469106]
35. Jiang N, Wang X, Jhanwar-Uniyal M, Darzynkiewicz Z, Dai W. Polo box domain of Plk3 functions as a centrosome localization signal, overexpression of which causes mitotic arrest, cytokinesis defects, and apoptosis. *The Journal of biological chemistry*. 2006;281(15):10577–10582. [PubMed: 16478733]
36. Smalley KS, Eroglu Z, Sondak VK. Combination Therapies for Melanoma: A New Standard of Care? *American journal of clinical dermatology*. 2016;17(2):99–105. [PubMed: 26860106]
37. Eblen ST, Slack JK, Weber MJ, Catling AD. Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Molecular and cellular biology*. 2002;22(17):6023–6033. [PubMed: 12167697]
38. Braselmann S, Taylor V, Zhao H, et al. R406, an orally available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune complex-mediated inflammation. *The Journal of pharmacology and experimental therapeutics*. 2006;319(3):998–1008. [PubMed: 16946104]
39. Davis MI, Hunt JP, Herrgard S, et al. Comprehensive analysis of kinase inhibitor selectivity. *Nature biotechnology*. 2011;29(11):1046–1051.
40. Baillet O, Fenouille N, Abbe P, et al. Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest. *Cancer Res*. 2009;69(7):2748–2756. [PubMed: 19293188]
41. Vanden Borre P, Gunda V, McFadden DG, et al. Combined BRAF(V600E)- and SRC-inhibition induces apoptosis, evokes an immune response and reduces tumor growth in an immunocompetent orthotopic mouse model of anaplastic thyroid cancer. *Oncotarget*. 2014;5(12):3996–4010. [PubMed: 24994118]
42. Gartel AL, Kandel ES. RNA interference in cancer. *Biomolecular engineering*. 2006;23(1):17–34. [PubMed: 16466964]
43. Kandel ES, Lu T, Wan Y, Agarwal MK, Jackson MW, Stark GR. Mutagenesis by reversible promoter insertion to study the activation of NF-kappaB. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(18):6425–6430. [PubMed: 15851657]
44. Liu J, Hong J, Ahn KS, et al. ERK-dependent IL-6 positive feedback loop mediates resistance against a combined treatment using danusertib and BKM120 in Burkitt lymphoma cell lines. *Leukemia & lymphoma*. 2019:1–9.
45. Karoulia Z, Wu Y, Ahmed TA, et al. An Integrated Model of RAF Inhibitor Action Predicts Inhibitor Activity against Oncogenic BRAF Signaling. *Cancer cell*. 2016;30(3):501–503.

46. Cox AD, Der CJ. The raf inhibitor paradox: unexpected consequences of targeted drugs. *Cancer cell*. 2010;17(3):221–223. [PubMed: 20227037]
47. Cox AD, Der CJ. The RAF inhibitor paradox revisited. *Cancer cell*. 2012;21(2):147–149. [PubMed: 22340588]
48. Gibney GT, Messina JL, Fedorenko IV, Sondak VK, Smalley KS. Paradoxical oncogenesis--the long-term effects of BRAF inhibition in melanoma. *Nature reviews Clinical oncology*. 2013;10(7):390–399.
49. Zhang C, Spevak W, Zhang Y, et al. RAF inhibitors that evade paradoxical MAPK pathway activation. *Nature*. 2015;526(7574):583–586. [PubMed: 26466569]
50. Hartsough EJ, Kugel CH 3rd, Vido MJ, et al. Response and Resistance to Paradox-Breaking BRAF Inhibitor in Melanomas In Vivo and Ex Vivo. *Molecular cancer therapeutics*. 2018;17(1):84–95. [PubMed: 29133617]
51. Zynda ER, Maloy MH, Kandel ES. The role of PAK1 in the sensitivity of kidney epithelial cells to ischemia-like conditions. *Cell cycle*. 2019;18(5):596–604. [PubMed: 30724698]
52. Namura S, Iihara K, Takami S, et al. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98(20):11569–11574. [PubMed: 11504919]
53. Schmit TL, Zhong W, Setaluri V, Spiegelman VS, Ahmad N. Targeted depletion of Polo-like kinase (Plk) 1 through lentiviral shRNA or a small-molecule inhibitor causes mitotic catastrophe and induction of apoptosis in human melanoma cells. *The Journal of investigative dermatology*. 2009;129(12):2843–2853. [PubMed: 19554017]
54. Gutteridge RE, Ndiaye MA, Liu X, Ahmad N. Plk1 Inhibitors in Cancer Therapy: From Laboratory to Clinics. *Molecular cancer therapeutics*. 2016;15(7):1427–1435. [PubMed: 27330107]

Figure 1. PLK3 Knockdown Increases the Efficacy of BRAFi in BRAF Mutated Melanoma.

A) PLK3 expression vs PLX4720 activity area (“effectiveness”) was plotted for 37 melanoma cell lines. Expression data was obtained from the Cancer Cell Line Encyclopedia (CCLE) using the Integrative Genomic Viewer. A pharmacological profiling dataset deposited in the CCLE was used to obtain PLX4720 effectiveness. **B)** A375 cells were treated with vemurafenib (40nM) for 24 and 48 hours and analyzed via immunoblotting with anti-PLK3 and -GAPDH antibodies. **C)** SK-MEL-28 cells were transduced with a lentiviral vector expressing shRNA#1 or #2 against PLK3 or a non-silencing control “Non-Sil” as

described previously²⁶. Cells were treated with the indicated doses of vemurafenib for 5 days. Remaining cell numbers were scored by methylene blue staining and extraction method, as described before²⁷. IC50 values were calculated using GraphPad Prism software and plotted relative to the vector control. The error bars represent the upper and lower 95% confidence intervals. **D)** Dose-response curves for the experiment described in panel C. The error bars represent the standard deviation of quadruplicates. **E)** SK-MEL-28 were transduced with tetracycline-inducible shPLK3 expression constructs (TRE-shPLK3 #1-#3) or a non-silencing control. The cells were cultured with (induced) or without (uninduced) 100ng/ml of doxycycline for 48 hours prior to a 5 day vemurafenib (40nM) or DMSO treatment. The remaining cells were scored by methylene blue staining and extraction method, and the data were normalized to corresponding uninduced controls. Error bars show standard deviations of quadruplicates. **F)** A375 harboring shPLK3#1 or a non-silencing shRNA were treated with vemurafenib (40nM) for 48 hours and pulsed with EdU (10uM) for 1 hour. The cells were fixed and stained using DAPI and the Click-it Alexa Fluor 488 imaging kit. EdU and DAPI images were converted to binary in ImageJ software. A representative field of view is shown. **G)** For each culture treated as in F, the fraction of EdU-positive cells was scored in 5 random fields of view. The values for vemurafenib-treated cells are shown relatively to the respective DMSO treated controls. The error bars represent the standard deviation of the 5 fields. “*” indicates a significant difference ($p<0.05$) between indicated values.

Figure 2. PLK3 Overexpression Conveys Resistance to Inhibitors of BRAF and MEK.

A) A375 cells expressing wild type PLK3 (“PLK3”) or an empty vector control (“control”) were treated with DMSO or vemurafenib (24 or 48 hours) and analyzed via immunoblotting. The lysates were probed for PLK3 or tubulin. **B)** SK-MEL-28 cells harboring a PLK3-expression construct (“PLK3”) or the corresponding empty vector control (“control”) were treated with the indicated doses of vemurafenib for 5 days and analyzed as in Fig. 1C. **C)** IC_{50} values for cells described in panel B were calculated using GraphPad Prism software and plotted relative to the vector control. The error bars represent the upper and lower 95%

confidence intervals. **D-E**) A375 cells were engineered, treated and analyzed as described for SK-MEL-28 in panels B and C. **F**) SK-MEL-28 described in panel B were treated with cobimetinib (10nM) or DMSO for 5 days. The remaining cells were scored as in Fig. 1C. Values are plotted relative to those of the vector control populations. **G**) A375#15 cells harboring either the PLK3-expression construct ("PLK3") or the corresponding empty vector control ("Control") were subcutaneously injected into SCID mice. When the tumors reached approximately 100mm³, the mice started receiving daily IP injections of vemurafenib (15mg/ml). The maximal fraction, by which a treated tumor decreased in size relatively to its volume at the start of the treatment, was determined and plotted as a "box and whiskers" graph for each group. **H**) The Log Fold Change (LogFC) for PLK3 expression in BRAF inhibitor (BRAFi) resistant patient tumors was assessed by comparing PLK3 levels each patient's tumor before BRAF inhibitor treatment to those in the recurring tumors. The values for individual patients are plotted in a descending order. "*" indicates a significant difference ($p < 0.05$) between indicated values.

Figure 3. PLK3 Differentially Affects ERK and MEK Status.

A) SK-MEL-28 cells transduced with PLK3 shRNA#1 (“shPLK3”) or a non-silencing control shRNA (“ns”) were treated with 40nM of vemurafenib (“VEM”) or vehicle alone (“DMSO”) for 48 hours and lysed. Protein extracts were probed via immunoblotting with antibodies for pERK^{Y204}, ERK1/2, pMEK1/2^{S217/221}, MEK1/2, and tubulin. **B)** A375-shPLK3 #1 (“shPLK3”) or -non-silencing control (“ns”) cells were treated as in panel A and probed for pERK Y204, pMEK1/2^{S217/221}, and tubulin. **C)** A375 harboring tetracycline-regulated shPLK3 #1 (“TRE-shPLK3”) or a non-silencing control (“Control”) were cultured

with doxycycline (100ng/ml) for 48 hours prior to vemurafenib (40nM) or DMSO treatment. Drug treatments were continued for 48 hours in the presence of doxycycline. Lysates were probed for pERK Y204, pMEK1/2^{S217/221}, and GAPDH. **D)** A375 cells transduced with PLK3 (“PLK3”) or the control vector (“control”) were treated with vemurafenib (60nM) for 48 and 24 hours and analyzed via immunoblotting as in panel C. **E)** In three experiments performed as in panel A, the intensity of pMEK and pERK signals (normalized to that of the loading control) was quantified using ImageJ software. To estimate the remaining fraction of pMEK and pERK in each experiment, the values in treated cells were divided for those in the untreated ones. The ratios of the remaining pERK and pMEK fractions were calculated for each cell line and are shown as averages of three experiments with standard deviations represented by the error bars. **F)** The ratios of the remaining pERK and pMEK fractions from three experiments performed as in panel C were analyzed and graphed as described for panel E. “*” indicates a significant difference ($p<0.05$) between indicated values.

Figure 4. R406 Synergizes with Vemurafenib in Cancer Cells.

A) A375 cells were treated with 20nM of vemurafenib, 500nM of R406, or a combination of both for 5 days and analyzed as in Fig. 1C. **B)** B-CPAP cells were treated with vemurafenib (100nM), R406 (250nM), or a combination of both drugs for 5 days and analyzed as in Fig. 1C. **C)** A375#15 cells transduced with a PLK3 expression construct ("PLK3") or the corresponding empty vector control ("Vector") were treated with vemurafenib (40nM), R406 (50nM), or both for 5 days. Cells were analyzed as described in Fig. 1C. **D)** A375 cells were treated with 40nM of vemurafenib, 50nM of R406, or both for 48 hours. Subsequently, the cells were probed via immunoblotting for pERK^{Y204} and pMEK^{S217/221}. The signals were normalized to the loading control (tubulin) and graphed relatively to those in respected untreated samples. "*" indicates a significant difference ($p < 0.05$) between indicated values.

Table 1.

The combinational index values (CI) for A375 cells were calculated for the indicated doses of vemurafenib and R406 using Compusyn software (ComboSyn, Inc) as described in Materials and Methods.

A375 cells, CI values		VEM, nM		
		20	30	40
R406, nM	250	0.57±0.06	0.71±0.04	0.85 ± 0.05
	500	0.58±0.09	0.67±0.07	0.81 ± 0.06

Table 2.

The combinational index values (CI) for B-CPAP cells were calculated for the indicated drug combinations as in Table 1.

B-CPAP cells, CI values		VEM, nM		
		50	100	250
R406, nM	250	0.25±0.06	0.18±0.09	0.16±0.06
	500	0.31±0.06	0.29±0.05	0.29±0.07