Phosphoproteomics of MAPK Inhibition in BRAF-Mutated Cells and a Role for the Lethal Synergism of Dual BRAF and CK2 Inhibition

Robert Parker¹, Roderick Clifton-Bligh², and Mark P. Molloy¹

Abstract
Activating mutations in the MAPK pathway are prevalent drivers of several cancers. The chief consequence of these mutations is a hyperactive ERK1/2 MAPK able to promote cell proliferation, producing a critical hallmark of metastatic disease. The biochemistry of the ERK pathway is well characterized; however, how the pathway achieves different outcomes in the face of genetic aberrations of cancer and subsequent treatment with chemical inhibitors is not clear. To investigate this, we used mass spectrometry to complete a global phosphoproteomic analysis of a BRAFV600E thyroid cancer cell line (SW1736) after treatment with the mutation-selective inhibitor vemurafenib (PLX4032) and MEK1/2 inhibitor selumetinib (AZD6244). We identified thousands of phosphorylation events orchestrated in BRAFV600E cells and performed kinase landscape analysis to identify putative kinases regulated in response to MAPK blockade. The abundance of phosphopeptides containing consensus motifs for acidophilic kinases increased after short-term inhibition with these compounds. We showed that coinhibition of the pleiotropic acidophilic protein kinase CK2 (CK2) and BRAFV600E synergistically reduced proliferation in patient-derived melanomas and thyroid cancer cells harboring the BRAF lesion. We investigated this mechanism and show a role for CK2 in controlling AKT activation that was not reliant on changes to PTEN or PDK1 phosphorylation. These findings highlight a role for CK2 blockade in potentiating the antiproliferative effects of BRAF and MEK inhibition in BRAF cancers.

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Introduction
Aberrant activity of protein kinases drives many of the hallmarks of cancer and also participates in the development of resistance to current treatments (1). In many cancers, dysregulation of the MAPK pathway is associated with poor prognosis, which results from activating mutations in genes encoding cytosolic signaling proteins (e.g., BRAF and RAS) or receptor tyrosine kinases (e.g., EGFR and RET). The BRAF gene is found mutated in approximately 1 of 4 of all anaplastic thyroid carcinomas (ATC), and 40% to 60% of papillary thyroid carcinomas (PTC; refs. 2–4). BRAF is commonly mutated by a single transversion (T1799A) that codes for a missense protein (V600E). Biochemically, BRAFV600E mimics phosphorylation of the T598 and S601 residues producing a constitutively active kinase able to hyper-phosphorylate MAPK kinases MEK1/2, driving cell transformation through unrestrained ERK1/2 activation. The discovery of specific activating mutations has led to the clinical development of mutation-specific, small-molecule kinase inhibitors. In melanoma, one such compound, vemurafenib (PLX4032), has shown high efficacy in BRAFV600E-positive patients (5), and the logical application of this approach in thyroid cancer is under investigation. However, as with many targeted therapies, acquired resistance to treatment is common, and thus successful application of targeted therapies will benefit from more sophisticated understanding of the events controlled by oncogenic mutations and the molecular responses that result from inhibiting these enzymes.

The impact of pharmacologic blocking of BRAFV600E by selective inhibition in thyroid cancer has been demonstrated in vivo. Inhibition reduces cell proliferation in PTC/ATC mouse tumor xenograft (6) and recently vemurafenib was shown to suppress tumor growth in BRAFV600E human ATC (7). At a molecular level, BRAFV600E mediates activation of the NF-κβ transcription factor, epigenetic reprogramming through methylation, and/or expression of genes such as TIMP3, HMG2, metalloproteases, and other structural extracellular matrix genes that can promote proliferation and cell invasion (8–10). NF-κβ-driven expression of TIMP1 is also implicated in BRAFV600E thyroid cancers, able to...
activate the PI3K/AKT pathway sustaining cell proliferation, with one possible consequence being the over activation of the mTOR pathway (8, 11). Gene expression, methylation, and molecular studies have revealed several key processes regulated by BRAF in thyroid cancer, but currently little is known about dysregulated posttranslational control of protein signaling. In particular, little is known about how selective inhibition of BRAFV600E alters the output of cell signaling processes. Data outlining the signaling events regulated by BRAFV600E, and how cells respond to small-molecule-based inhibition, are vital to designing combination therapy programs that are effective and combat the development of acquired resistance.

Lack of knowledge of phosphorylated sites within proteins and identification of upstream regulating kinases are major limiting factors in understanding what pathways are activated in cancer. Currently, several reliable techniques for phosphopeptide enrichment and characterization using mass spectrometry have been developed and promise to add significantly to this knowledge base (12, 13). Temporal characterization of the phosphoproteome cellular landscape provides a direct readout of kinase substrates and can be used to develop hypotheses about likely active kinases under those conditions. Here, we used established techniques to profile the phosphoproteome of a drug-sensitive BRAFV600E-positive ATC cell line (SW-1736), and monitor the quantitative response of approximately 2,000 phosphosites after selective blocking of BRAFV600E and MEK1/2 with the clinically tested small-molecule inhibitors vemurafenib (PLX4032) and selumetinib (AZD6244). We used thyroid cancer cell lines with defined mutations in BRAF and RET to demonstrate mutation-specific response in cell growth, cell cycle, and phosphorylation of known and novel effectors. Motif analysis of the flanking linear amino acid sequences of regulated phosphosites revealed several regulatory kinases that function in BRAFV600E signaling and in response to inhibition. Although ERK1/2 and cyclin dependent kinase (CDK1/2) substrates were expected in downregulated sites after selective blocking of BRAFV600E and MEK1/2 with the clinically tested small-molecule inhibitors vemurafenib (PLX4032) and selumetinib (AZD6244), we observed increases in some substrates consistent with the activity of acidophilic kinase(s). We demonstrated that combinatorial inhibition of BRAF and the acidophilic kinase CK2 in thyroid cancer and patient-derived melanoma cells is synergistically lethal and associated with decreased AKT signaling.

Materials and Methods

Cell culture, siRNA, and protein preparation

Thyroid cell lines were tested for authenticity by short tandem repeat profiling according to the ANSI/ATCC ASN-0002-2011 standards. Melanomas established from primary tissue were genotyped previously (14). All cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) bovine serum (Life Technologies) at 37°C in a humidified atmosphere of 5% CO2, and grown to 80% to 90% confluence. Cells were lysed as described in (12) with minor modifications (see Supplementary Methods) and protein amounts determined. A total of 1 to 25 pmol of siRNA-targeting transcripts of the human CK2α (Cell Signaling) and nontarget control siRNA were used to knock down CK2 expression in melanoma cells. siRNA was delivered using Lipofectamine RNAiMAX for 72 hours, protein levels were examined by Western blot analysis.

Protein digestion and phosphopeptide enrichment

A total of 500 mg of proteins were reduced, alkylated, and digested with trypsin (Promega) overnight at 37°C. Samples were acidified, adjusted to 80 mg/mL glycolic acid, and phosphopeptides purified by the addition of 5 mg of TiO2 beads (Titansphere, 10 μm) for 1 hour. Beads were extensively washed and phosphopeptides were eluted with consecutive 100 μL additions of 1% (v/v) ammonia (Sigma) with 0%, 30%, and 50% (v/v) acetonitrile. Samples were immediately dried and resuspended in 1% (v/v) trifluoroacetic acid, 5% (v/v) acetonitrile for LC/MS-MS.

Mass spectrometry (LC-MS/MS)

Samples were analyzed by LC-MS/MS using a Triple-TOF 5600 mass spectrometer (AB Sciex) and peptides identified using ProteinPilot v4.5 and Mascot v2.2 as described in Supplementary Methods. Label-free quantification (LFQ) using extracted ion chromatograms was conducted using Skyline (14) and statistical analysis carried out using the DanteR scripts. Parallel reaction monitoring was conducted on the TripleTOF 5600 and analyzed using Skyline as described in Supplementary Methods.

Phosphosite localization and kinase assignment

To localize modifications, search results were processed using Scaffold PTM (ProteomeSoftware), which uses A-score (15) and a probability function to assign confidence to amino acid modification location based on available peak depth present in MS/MS spectra. Upstream kinases were putatively assigned using the NetworkKIN algorithm (16), and the output was analyzed in excel and using the program phosphosite analyzer (17), which performs statistical enrichment analysis.

Viability assays

Cells were seeded in 96-well plates at 5,000 to 10,000 cells per well in triplicate for each drug treatment and time point. After 2 hours, cells were treated with dilutions 0.02, 0.2, 1, 2, 10 μmol/L for vemurafenib (PLX4032), and 0.02, 0.02, 0.1, 0.2, 1 μmol/L of selumetinib (AZD6244) and 0.8, 4, 8 μmol/L of CK2 inhibitor (CX-4945; Selleckchem) in thyroid cells. For melanomas, 0.5, 1, 2, 4, 8 μmol/L of all compounds were used either singularly or in combination.
After 72 hours, cell viability was assessed by Presto blue assay (Life Technologies).

**Western blot**

A total of 25 μg of protein was mixed with NuPAGE loading buffer (Life Technologies). Membranes were blocked with TBS/Tween-20 supplemented with 5% (v/w) skimmed milk, incubated with primary antibody O/N at 4°C, and with secondary antibody conjugated to fluorescent tag (LiCor) for 1 hour at room temperature and imaged using an Odyssey system (LiCor).

**Results**

**Mutations in the MAPK pathway predict sensitivity to MAPK inhibitors**

To measure the sensitivity of thyroid cell lines to MAPK inhibitors, we measured the relative effect on cell proliferation using the oncogenic BRAF inhibitor vemurafenib (PLX4032; ref. 18) and the MEK1/2 inhibitor selumetinib (AZD6244; ref. 19; Fig. 1A). The cell lines SW-1736 (BRAFV600E; ref. 3), TPC-1 (RET/PTC1 translocation; ref. 20), and the BRAF wild-type thyroid cell, Nthy-ori 3.1 (derived from normal tissue and SV40 immortalized; ref. 21), were used as a comparative system for MAPK activation in thyroid cancer, and treated for 48 to 72 hours with 0.2 μmol/L selumetinib and 2 μmol/L vemurafenib (Fig. 1B and C). The BRAFV600E-mutant cell line SW-1736 exhibited sensitivity (>40% growth inhibition at 1–2 μmol/L) to both BRAFV600E and MEK1/2 inhibition, whereas both TPC-1 and Nthy-ori 3.1 were insensitive to vemurafenib as expected (~10% growth inhibition at 1–2 μmol/L; Fig. 1B). Both cell lines with activating MAPK pathway mutations (SW-1736 and TPC-1) showed similar sensitivity to selumetinib (30%–40% growth inhibition at 1 μmol/L), demonstrating the convergence of pathway activation through the downstream component MEK1/2 (Fig. 1B). ERK1/2 output was detected by Western blot analysis of phospho-retinoblastoma binding protein (RB), total cyclin-D1, p27KIP, phospho-ERK and total-ERK in the presence or absence of 2 μmol/L vemurafenib (Fig. 1C). At 48 hours, clear reduction in phospho-ERK1/2 was evident in the BRAFV600E cells after treatment with vemurafenib. A concurrent reduction in phosphorylation of RB, cyclin-D1 and a moderate increase in p27KIP protein are consistent with reduced ERK1/2 output. Both TPC1 and Nthy-ori 3.1 exhibited no change in phospho-ERK, phospho-RB, and cyclin-D1, confirming that ERK1/2 output is not affected by vemurafenib in these cells. TPC1 cells exhibited loss of p27KIP expression upon exposure to vemurafenib; however, no significant change in proliferation was observed.

**Phosphoproteomic analysis of MAPK pathway inhibition**

In SW-1736 cells, signaling through the MAPK pathway is dominated by the oncogenic BRAFV600E mutation, so that screening for changes to protein phosphorylation after BRAFV600E inhibition will identify proteins that are regulated via this kinase. We used both vemurafenib and selumetinib to find common and distinguishable changes to phosphorylation regulated by either BRAF or MEK1/2, respectively. Following short-term drug exposure (15 and 30 minutes), proteins were extracted, digested, and phosphopeptides were enriched using TiO2 before being analyzed by LC-MS (Fig. 1D). In total, 2,329 unique peptide sequences, of which approximately 90% were phosphorylated (STY), were identified at an FDR of approximately 1%. From these, 2,457 p[S], p[T], p[Y] sites could be localized with high confidence (>95%) using MS/MS spectra (Supplementary Table S1). These class-one sites mapped to approximately 1,200 phosphoproteins. For the class-one sites that represent the highest confidence phospho-site assignments in the dataset, the NetworKIN algorithm was used to map the most likely kinase responsible for the phosphorylation of each site (Supplementary Table S2). NetworKIN uses motif analysis and context information to generate a score for each kinase–substrate relationship (16).

**Phosphoproteins regulated by BRAF and MEK inhibition**

We next quantified the difference in relative abundance of phosphopeptides in SW-1736 cells treated with vemurafenib or selumetinib (Supplementary Table S3). Generally, data were well matched across treatments and biologic replicates; however, each peak was manually validated to ensure accurate peak integration, resulting in approximately 1,800 phosphopeptides reliably quantified after drug inhibition (Supplementary Table S3). To assign high confidence changes in altered phosphopeptides, a robust ANOVA (P < 0.05) with a fold change of 1.5 was used as a cutoff; FDR was controlled by P value adjustment using the Benjamini and Hochberg method (Q < 0.2; Supplementary Table S3). At this level of stringency, 23 phosphopeptides (33 phosphorylation sites) showed significant changes in abundance in all treatment groups and most were coregulated by BRAFV600E and MEK inhibition (Table 1). Supplementary Table S4 describes the key function (derived from UniProt and PubMed) of each protein and the most likely (NetworKIN; ref. 16) or verified kinase(s) for these sites and also compares with expression observed previously for these phosphorylation sites in cell-cycle phases G1–M (22). In general, the direction in which sites were regulated by 30 minutes of MAPK inhibition showed limited overlap with G1 (5/14) or M (2/9) phase arrest observed in a previous study in HeLa cells (22). Several phosphoproteins with sites regulated in our study were annotated with roles in the regulation of chromatin structure/orchestration, control of transcription, and nuclear envelope organization. Gene ontology analysis using Ingenuity pathway analysis indicated that processes DNA methylation and transcriptional repression (P = 1.29 × 10−5; >50% pathway coverage) and ERK/MAPK signaling (P = 8.11 × 10−8) were highly represented (Supplementary Table S5). The relative change in abundance of these core MAPK components correlated with both treatments (30 minutes; R² = 0.62) indicating a high...
degree of similarity in response to MAPK inhibition at blockade through BRAF or MEK (Fig. 1E). Among these peptides, S695 in SMARCA4 and S297 in SGTB exhibited an early 15-minute response to MAPK inhibition with MEK and were nearer to control levels after 30-minute inhibition. Two other peptides, S331 of BIN1 and S389/393 in SRRM1, were regulated specifically by BRAF inhibition.

Explorative kinase enrichment

To investigate if any kinase(s) could account for a significant proportion of the regulation observed in the phosphopeptide dataset, we explored the NetworKIN results for phosphorylation sites changing in abundance versus the proportion in the entire dataset. Figure 2 summarizes the results of this analysis; we observed that approximately 50% (RRP1B S245, TMF1 S344, MAX S2 and S11 and OSTF1 S213) of the upregulated sites with NetworKIN kinase predictions could be attributed to the CK2 family (Fig. 2A–C). This pattern was confirmed using the phosphosite analyzer program, which performs a statistical test where the frequency of regulated clusters of phosphosites is compared with the background frequency of all phosphosites (17; Supplementary Fig. S1). CK2 is an acidophilic kinase that requires an acidic or phosphorylated residue at position +3 in their substrate (SXXE/D). In our data, one site OSTF1 S213 is the penultimate amino acid at the
Table 1. Phosphoproteins regulated by BRAF and MEK inhibition in SW1736 thyroid carcinoma cells

<table>
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<tr>
<th>Gene name</th>
<th>UniProt</th>
<th>Protein name</th>
<th>Peptide sequence</th>
<th>P-site</th>
<th>PLX&lt;sup&gt;a&lt;/sup&gt; (15 min)</th>
<th>PLX&lt;sup&gt;a&lt;/sup&gt; (30 min)</th>
<th>AZD&lt;sup&gt;a&lt;/sup&gt; (15 min)</th>
<th>AZD&lt;sup&gt;a&lt;/sup&gt; (30 min)</th>
<th>Site&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>BIN1</td>
<td>O00499</td>
<td>Myc box-dependent–interacting protein 1</td>
<td>VNHEPEPAGGATPGATLPK&lt;sup&gt;[pS]&lt;/sup&gt;PSQLR</td>
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<td>−0.9</td>
<td>−0.3</td>
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<td>PPP1R12A</td>
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<td>Protein phosphatase 1 regulatory subunit 12A</td>
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<td>TPR</td>
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<td>Nucleoprotein TPR</td>
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<td>S2155</td>
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<td>MAPK1</td>
<td>P28482</td>
<td>MAPK 1</td>
<td>VADPDHDTGF[pT]E[pY]VATR</td>
<td>T185, Y187</td>
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<td>EMD</td>
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<td>Emerin</td>
<td>DSAYQSTHYRPV&lt;sup&gt;[pS]&lt;/sup&gt;ASR</td>
<td>S171</td>
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<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>0.97</td>
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<td>SMARCA4</td>
<td>P51532</td>
<td>Transcription activator BRG1</td>
<td>KIPDP&lt;sup&gt;[pS]&lt;/sup&gt;DVSEVDDR</td>
<td>S695</td>
<td>−0.4</td>
<td>−0.3</td>
<td>−3.6</td>
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<td>MAX</td>
<td>P61244</td>
<td>Protein MAX</td>
<td>[pS]&lt;sup&gt;D&lt;/sup&gt;NNDIEVE&lt;sup&gt;[pS]&lt;/sup&gt;DADKR</td>
<td>S2, S11</td>
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<td>TMF1</td>
<td>P82094</td>
<td>TATA element modulatory factor</td>
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<td>S344</td>
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<tr>
<td>AHNK</td>
<td>Q09666</td>
<td>Neuroblast differentiation-associated protein</td>
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<td>S5110</td>
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<td>RRP1B</td>
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<td>Ribosomal RNA processing protein 1 homolog B</td>
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<td>S135</td>
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<td>EZH2</td>
<td>Q15910</td>
<td>Histone-lysine N-methyltransferase EZH2</td>
<td>VKESSIA&lt;sup&gt;[pT]&lt;/sup&gt;APAEVD&lt;sup&gt;[pT]&lt;/sup&gt;PR</td>
<td>T448</td>
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<td>Retinoblastoma-binding protein 6</td>
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<td>SRRM1</td>
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<td>Serine/arginine repetitive matrix protein 1</td>
<td>KIPAP&lt;sup&gt;[pS]&lt;/sup&gt;PVQ&lt;sup&gt;[S]&lt;/sup&gt;Q&lt;sup&gt;[S]&lt;/sup&gt;STNWSPAVPVK</td>
<td>S781, S787</td>
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<td>Osteoclast-stimulating factor 1</td>
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<td>Small glutamine-rich tetratricopeptide repeat-containing protein beta</td>
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NOTE: Q < 0.2 shown in bold.

<sup>a</sup>Log<sub>2</sub> fold change.

<sup>b</sup>P-site localization probability.
C-terminus, and thus no +3 position is possible; however, the phosphoacceptor site is surrounded by acidic residues in positions −4 to +1 and NetworKIN predicted it as a CK2 family substrate. Searching the literature revealed the sites regulated in protein MAX and RRP1B as verified CK2 substrates, whereas others have no bona fide kinase relationships assigned (23, 24). Other upregulated phosphosites were associated with CDK2/3, RCK, CLK family kinases. Among the downregulated sites, the majority were predicted substrates of the p38 family and CDK2/3 family. CDK2/3 and MAPK are highly related protein kinases and share similar substrate specificity (PXSP and S'PXK/R).

MAPK blockade with vemurafenib is mutation specific

Next, we investigated the regulation of several phosphosites by MAPK blockade in the background of the two most common thyroid cancer mutations (BRAF and RET). To perform this analysis, we prepared fresh cell lysates and used a relatively novel mass spectrometric technique called parallel-reaction monitoring (PRM) that quantitates with high-specificity, predetermined peptides present in the samples (25). We selected a subset of the phosphopeptides whose levels were altered upon MAPK inhibition and applied this method to quantitate the specific phosphopeptide and a detectable counterpart unmodified peptide from the same protein (Fig. 3A–D). This approach was less sensitive than the LFQ experiment but still enabled cross validation of 8 of the 23 phosphopeptides (Supplementary Table S6). Four of these, MAPK1, translocated promoter region (TPR), niban-like protein 1 (FAM129B), and osteoclast-stimulating factor 1 (OSTF1), were plotted alongside a phosphopeptide from chaperone DnaJ (DNAJ) measured as a loading control. These data were consistent with the original screen and confirmed that these phosphorylation sites are regulated by BRAFV600E inhibition with vemurafenib in the BRAFV600E cell line SW-1736. All but one (S213 in OSTF1) of the phosphopeptides responded to MEK inhibition in BRAFV600E, RET/TPC, and the BRAF wild-type normal thyroid cell line, indicating that these substrates were normally regulated by the MAPK pathway requiring activated MEK1/2. The RET/TPC mutant exhibited small increases in phosphorylation of MAPK1, AHNAK, TPR and a decrease in OSTF1 phosphorylation after treatment with vemurafenib. This is consistent with the paradoxical activation of the MAPK pathway observed in a BRAF wild-type background, with an activated Ras signal (26), and is corroborated by the loss of p27KIP determined by Western blot (Fig. 1C).

Inhibition of CK2 is synergistically lethal with vemurafenib or selumetinib in BRAF-mutated thyroid carcinoma and melanoma cell lines

Kinase enrichment analysis indicated that blocking BRAFV600E signaling with vemurafenib or selumetinib was associated with an increase phosphorylation of substrates of acidophilic kinase(s). Of the acidophilic kinases,
CK2 was predicted by NetworKIN to regulate several of these sites, and two, S2/11 in MAX and S245 in RRP1B, have been reported previously (23, 24). As CK2 can activate AKT signaling, a known mechanism for drug resistance to MAPK inhibitors, we hypothesized that CK2 activity might interact with the inhibition of BRAFV600E as a positive regulator of cell proliferation. To investigate this, the thyroid cell lines were treated with vemurafenib (2 μmol/L) or selumetinib (1 μmol/L) in combination with increasing concentrations of the specific CK2 inhibitor CX-4945, (0.8–8 μmol/L; ref. 27) and cell viability determined after 3 days (Supplementary Fig. S2). In the BRAFV600E-mutant SW-1736 cells, CK2 inhibition alone reduced cell growth by approximately 13%, vemurafenib reduced growth by approximately 36% consistent with our earlier observations. Coadministration of CX-4945 with vemurafenib showed only a minor effect on cell proliferation. In TPC1 cells treated with selumetinib, the addition of the CK2 inhibitor acted antagonistically and positively regulated cell proliferation. To determine if CK2 inhibition in the RET-mutant cell line TPC1 would be more effective in combination with a MAPK inhibitor tailored to this mutation, we assessed possible synergistic effects of vandetanib and CK2. TPC1 cells were sensitive to vandetanib (GI50 ~1 μmol/L); however, TPC1 was relatively insensitive to CK2 inhibition with CX-4945, and no synergistic effect of coinhibition was observed (Supplementary Fig. S3), confirming the requirement of the BRAF lesion for CK2 synergism to be effective. To test the effect of combined treatment in melanoma, four patient-derived cell lines were evaluated. Genotyping of C0045 and C0088 showed they carry the BRAFV600E/K mutation, whereas C0037 and C0084 are BRAF wild-type (14). For both oncogenic BRAF melanoma cell lines, the effects of combining CK2 inhibition with vemurafenib were additive (Supplementary Fig. S4). For these lines, we also calculated the combination index.
(CI) for serially diluted 1:1 mixtures of vemurafenib and CX-4945 (0.5–8 μmol/L) using the Chou and Talalay method (28). Synergism was observed for all combinations in both oncogenic BRAF melanoma cell lines, with strong synergism (CI = 0.21 at 4 μmol/L total dose) observed for V600E-mutant C0045, and synergism observed for V600K-mutant C0088 (Fig. 4B). Both BRAF wild-type melanoma lines were unaffected by vemurafenib alone (<10%), and no advantage was observed with coadministration of CX-4945, confirming the requirement of the BRAF lesion for drug synergism as observed above with thyroid cells. Interestingly, all melanoma cell lines were more sensitive to CK2 inhibition alone (~20%–35% at 4 μmol/L) when compared with thyroid carcinoma cell lines (2%–13% at 4 μmol/L). To confirm specificity of the observed BRAF/CK2 drug synergism, we knocked down the expression of CK2α using siRNA in two of the melanoma cell lines C0088 (BRAF wild-type) and C0037 (BRAFV600K-mutant) cell lines. After siRNA interference with 1 pmol of siCON, siCK2α, 4 μmol/L vemurafenib (PLX-4032), and combinations with siRNA treatments, however, when CK2 siRNA was combined with 4 μmol/L vemurafenib, an additive response was observed in the BRAFV600K-mutant C0088, whereas no significant change in viability was detected in BRAF wild-type C0037 cells, consistent with the observations we reported for CK2 inhibition with CX-4945.

**CK2 affects AKT signaling in oncogenic BRAF thyroid cells**

Several possible mechanisms may underlay the synergism observed between vemurafenib and CK2 inhibition in BRAF-mutant cells. To explore one avenue, we performed Western blot analysis of signaling through the PI3K–AKT pathway in the BRAFV600E thyroid cells. SW-1736 cells were treated with vemurafenib (2 μmol/L), CX-4945 (4 μmol/L), AKT inhibitor perfosine (4 μmol/L), and with binary combinations for 30 minutes (Fig. 5B). Phosphospecific antibodies for two well-known AKT phosphorylation sites (T308 and S473) were downregulated by CK2 treatment alone, and when CK2 inhibition was combined with vemurafenib (Fig. 5B). Phosphorylation of the CK2-regulated AKT priming site, pSer129, was reduced upon exposure to CX-4945, providing a clear indication of the importance of CK2 in regulating AKT signaling in these cells.

**Figure 4.** Response to combinatorial MAPK/CK2 inhibition. A, bar plot of growth inhibition in SW-1736, TPC-1, and Nthy-ori cells after 3-day exposure to 4 μmol/L CX-4945, 2 μmol/L vemurafenib (PLX-4032), 1 μmol/L selumetinib (AZD6244), and 2 μmol/L vemurafenib in combination with 4 μmol/L CX-4945, 1 μmol/L selumetinib in combination with 4 μmol/L CX-4945. Additivity >5% is marked with a dotted line and shaded, and was calculated as the theoretical combined fractional response effect \( \frac{(F_a + F_b)}{100-F_a} \), where \( F \) is the percentage of cells killed at given concentration, \( F_a \) is vemurafenib or selumetinib, and \( F_b \) is CX-4945. B, bar plot for the CI based on the fractional responses measured for four patient-derived melanoma cell lines after treatment with a 1:1 mixture of CX-4945 and vemurafenib 2-fold serially diluted (1.0–16 μmol/L). C, Western blot for CK2α after 72 hours of transfection with CK2α (siCK2α) or control (siCON) siRNA in melanoma cells C0037 (BRAF wild-type) and C0088 (BRAFV600K); β-tubulin is shown to represent total protein loading. D, bar plot of viability of melanoma cells C0037 (BRAF wild-type) and C0088 (BRAFV600K) after siRNA interference with 1 pmol of siCON, siCK2α, 4 μmol/L vemurafenib (PLX-4032), 4 μmol/L CX-4945, and combinations with siRNA treatments.
link between CK2 and the AKT prosurvival pathway. BRAF inhibition alone was unable to illicit changes to AKT phosphorylation, indicating the response to be dependent on CK2 activity. Interestingly, PTEN phosphorylation was not affected, nor was CRAF, a target of AKT1. Little effect was observed for PDK1, the kinase responsible for AKT1 phosphorylation at T308 after RTK activation. The inhibitory site Ser9 in the N-terminal of the AKT target GSK3β was downregulated, indicating CK2 activity is required for AKT signaling to GSK3β in SW-1736 cells.

Discussion

In this work, we measured the response of the phosphoproteome of BRAFV600E-activated thyroid cancer cells to pharmacologic blockade with the clinically tested inhibitors vemurafenib and selumetinib. Our work identified approximately 2,300 phosphopeptides and quantitated approximately 1,800, demonstrating comprehensive depth of analysis. Gene enrichment analysis of protein function indicated that a high abundance of components involved in the regulation of gene transcription and signaling through MAPK are constitutively phosphorylated. Using a PTM site localization algorithm, linear motif analysis, contextual and literature searches, phosphopeptides could be confidently assigned as putative and bona fide substrates to several upstream regulatory kinases. The LFQ mass spectrometry approach was able to quantify the relative effect of shutting down MAPK signaling by blocking BRAFV600E. We observed highly significant reduction in the MEK1/2 substrate of the MAPK ERK1/2, and several other MAPK signaling molecules showed high correlation between treatments, indicating highly overlapping responses to inhibiting at BRAF and MEK in thyroid carcinoma cells (Fig. 1E).
It was important to see if the phosphorylation sites identified to be inhibited by the compounds used in this study were also reported to be inhibited by other MEK inhibitors (29, 30). Site S135/136 in neuroblast differentiation-associated protein (AHNAK) was significantly downregulated after both BRAF and MEK inhibition and has previously been shown to be regulated in response to the MEK inhibitor I (CAS 297744-42-4) in the MEK-sensitive acute myelogenous leukemia (AML) cell line, MV4-11. In their study, phosphorylation of S135/136 was nonresponsive to MEK inhibition in the resistant AML cell line, HEL. In a second study, melanoma cells were treated with MEKi GDC-0973 and PBKi GDC-0941, and immunoprecipitation of phosphopeptides was used to profile DNA damage response signaling (29). The protein SMARC4 showed initial decrease (not significant) in phosphorylation after MEKi and increased after long-term treatment with PBKi singularly and in combination with MEKi (29). Consistent with data presented here, S695 in SMARC4 is affected at 15 minutes with the MEKi AZD-6244 and returns to control levels by 30 minutes. These data confirm that several phosphosites are consistently regulated in response to MEK inhibition across different cell types in agreement with our observations.

We investigated the kinases that are involved in driving the BRAFV600E phenotype and the response to kinase inhibition. The results were striking; the majority of phosphorylation sites downregulated were flanked by the highly conserved p38/CDK2/3 linear motif, indicating prominent activity of ERK1/2 and/or CDK2/3. However, surprisingly, many sites (−40%) were upregulated and were highly enriched (−4-fold over background) for substrates of the acidophilic kinases. Several of the phosphorylation sites proposed to be regulated by these kinases are from proteins with characterized functions in tumor biology; most function in chromatin regulation and, for a few, the function of the phosphorylation site has been reported (Supplementary Table S4). Figure 5A is a summary of these data and attempts to place the protein phosphorylation events detected in networks with respect to their function and compartmentalization (36, 37). The N-terminal phosphopeptide from protein MAX, a nuclear component of the c-MYC transcription factor complex identified in our screen, was upregulated after vemurafenib blocking in BRAFV600E thyroid cancer cells (38). The peptide was N-terminally acetylated and phosphorylated at positions S2 and S11. S11 is flanked by a CK2 consensus motif and phosphorylated by CK2 to prevent cleavage by caspases to inhibit caspase-5 Fas-mediated apoptosis (23, 39). A second protein that was predicted to be a CK2 substrate was OSTF1; phosphorylation of S213 increased after BRAF or MEK inhibition, and this was confirmed by PRM mass spectrometry. S213 is at the C-terminal of the OSTF1 protein, a human ortholog of SH3P2 in mice, and both are indirectly involved in osteoclast formation (40). Recently, phosphorylation of SH3P2 at S202 was shown to be ERK dependent and catalyzed by ribosome S6 kinase (RSK; ref. 41). RSK phosphorylation promoted cell motility by inhibiting the activity of SH3P2 (41). Our data indicate a role for regulation of the closely situated S213 by a CK2 family kinase. It would be interesting to investigate the role S213 may play in cell motility and investigate how the plasticity of phosphosite localization in SH3P2 relates to function and cell phenotype.

The increase in levels of phosphoprotein substrates predicted to be regulated by acidophilic kinases following MAPK pathway inhibition was intriguing. As the activity of the archetypal acidophilic kinase CK2 is usually associated with the maintenance of cell proliferation in cancer (42), this led us to evaluate the therapeutic potential of controlling this signaling axis in BRAF cancers. We treated cells with a relatively new and specific inhibitor of CK2

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Glioblastoma xenografts (54). A recent report showing that CX-4945 prevents AKT phosphorylation of the G1–S phase cyclins D, E, and transcription factors c-JUN (AP-1) and c-MYC, targeting them for degradation through potentiation of NF-κB pathway blockade. Consistent with this, we observed reduced phosphorylation of both the PDK1 and mTORC2 phosphorylation sites in AKT following CK2 inhibition. No change in the phosphorylation of the G1–S phase cyclins D, E, and transcription factors c-JUN (AP-1) and c-MYC, targeting them for proteolysis and enhancing cell-cycle arrest (53). The relevance of CK2 regulation of AKT activity is highlighted in a recent report showing that CX-4945 prevents AKT activation and promotes survival in mice with intracranial glioblastoma xenografts (54).

The development of many new anticancer drugs is based on the knowledge that cancers arise from specific genetic aberrations in genes controlling regulatory pathways (27). Logically targeting these pathways with high specificity should provide a personalized antitumor effect while reducing side effects of more generalized cytotoxic chemotherapy. Here, we reveal several novel sites of regulation that occur in the phosphoproteome of BRAFV600E-mutated thyroid cancer cells treated with therapeutic molecules designed to target specific components of MAPK signaling. CK2 inhibition has already been shown to be effective in combination with the chemotoxic agents gemcitabine, cisplatin, and carboplatin (27, 55), and our data suggest that cotargeting BRAFV600E and CK2 is a valid path for further investigation. This could be especially useful in some BRAF-mutant colon cancers that seem unresponsive to direct BRAF inhibition due to feedback mechanisms that reactivate RTK signaling (56). This feedback results in AKT activation, and our data suggest that the novel combination of BRAF and CK2 inhibition would suppress proliferation in tumors that are addicted to AKT-driven survival. Indeed, there is substantial interest in direct targeting of PI3K and AKT in combination with MAPK pathway inhibitors in BRAF-mutant tumors (57, 58). As CK2 regulates a myriad of pro-growth substrates, it would be of interest to determine whether controlling CK2 activity is advantageous compared with current approaches trialing PI3K/AKT targeting in combination with MAPK inhibition.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: R. Parker, R. Clifton-Bligh, M.P. Molloy
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Parker, R. Clifton-Bligh, M.P. Molloy
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Phosphoproteomics of MAPK Inhibition in BRAF-Mutated Cells and a Role for the Lethal Synergism of Dual BRAF and CK2 Inhibition

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