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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Abbreviations list: MAPK Mitogen activated protein kinase, PTC, Papillary thyroid cancer, ATC Anaplastic thyroid cancer, LFQ, Label free quantitation, FDR, False discovery rate, PRM Parallel reaction monitoring.

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Abstract

Activating mutations in the MAPK pathway are prevalent drivers of several cancers. The chief consequence of these mutations is a hyperactive ERK1/2 MAP kinase 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 co-inhibition of the pleiotropic acidophilic kinase 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 anti-proliferative effects of BRAF and MEK inhibition in BRAF cancers.
Introduction

Aberrant activity of protein kinases drive many of the hallmarks of cancer and also participate in the development of resistance to current treatments (1). In many cancers dysregulation of the mitogen activated kinase pathway (MAPK) 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, RET). The Braf gene is found mutated in ~1/4 of all anaplastic thyroid carcinomas (ATC), and 40-60% of papillary thyroid carcinomas (PTC) (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 pharmacological blocking 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, HMGB2, metalloproteases and other structural extracellular matrix (ECM) 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 post-translational 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 is 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.
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 ~2000 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. Whilst ERK1/2 and CDK1/2 substrates were expected in down-regulated sites after BRAF inhibition, we also observed increases in some phosphosubstrates 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 80-90% confluence. Cells were incubated with the respective inhibitor; 2 μM vemurafenib (PLX4032, Selleckchem) or 1-2 μM selumetinib (AZD-6244, Selleckchem) or DMSO (Sigma) for 30 min before cell lysis. Three independent cell cultures were used per cell line and condition. Cells were lysed as described in (12) with minor modifications (see Sup. Methods) and protein amounts determined. 1-25 pmol of siRNA targeting transcripts of the human CK2α (Cell Signaling) and non-target control siRNA were used to knock down CK2 expression in melanoma cells. siRNA was delivered using Lipofectamine RNAiMAX for 72 h, protein levels were examined by western blot analysis.

Protein digestion and phosphopeptide enrichment

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 addition of 5 mg of TiO2 beads (Titansphere, 10 µm) for 1 h. 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) TFA, 5 % (v/v) acetonitrile for LC-MS/MS.
Mass spectrometry (LC-MS/MS)

Samples were analysed by LC-MS/MS using a TripleTOF 5600 mass spectrometer (AB Sciex) and peptides identified using ProteinPilot v4.5 and Mascot v2.2 as described in Supplementary Methods. Label free quantitation (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 analysed 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 NetworKIN algorithm (16) and the output was analysed in excel and using the program phosphosite analyzer (17), which performs statistical enrichment analysis.

Viability assays

Cells were seeded in 96-well plates at 5000-10000 cells/well in triplicate for each drug treatment and time point. After 2 h cells were treated with dilutions 0.02, 0.2, 1, 2, 10 μM for vemurafenib (PLX4032), and 0.002, 0.02, 0.1, 0.2, 1 μM of selumetinib (AZD6244) and 0.8, 4, 8 μM of CK2 inhibitor (CX-4945) in thyroid cells. For melanomas 0.5, 1, 2, 4, 8 μM of all compounds were used either singularly or in combination. After 72 h cell viability was assessed by Presto blue assay (Life Technologies).

Western blot

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

Mutations in MAPK pathway predict sensitivity to MAP kinase 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) (18) and the MEK1/2 inhibitor selumetinib (AZD6244) (19) (Figure 1 A). The cell lines SW-1736 (BRAFV600E) (3), TPC-1 (RET/PTC1 translocation) (20), and the BRAF wild-type thyroid cell, Nthy-ori 3.1 (derived from normal tissue and SV40 immortalized) (21) were used as a comparative system for MAPK activation in thyroid cancer, and treated for 48-72 h with 0.2 μM selumetinib and 2 μM vemurafenib (Figure 1 B,C). The BRAFV600E mutant cell line SW-1736 exhibited sensitivity (>40% growth inhibition 1-2 µM) to both BRAF V600E and MEK1/2 inhibition, whilst both TPC-1 and Nthy-ori 3.1 were insensitive to vemurafenib as expected (~10% growth inhibition 1-2 µM) (Figure 1 B). Both cell lines with activating MAPK pathway mutations (SW-1736 and TPC-1) showed similar sensitivity to selumetinib (30-40% growth inhibition at 1 μM) demonstrating the convergence of pathway activation through the downstream component MEK1/2 (Figure 1 B). 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 μM vemurafenib (Figure 1 C). At 48 h 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 MAPK pathway is dominated by the oncogenic BRAFV600E mutation, so 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 min) proteins were extracted, digested and phosphopeptides were enriched using TiO₂ before being analyzed by LC-MS (Figure 1 D). In total, 2329 unique peptide sequences, of which ~90% were phosphorylated (STY), were identified at a FDR of ~1%. From these, 2457 p[S], p[T], p[Y] sites could be localized with high confidence (>95%) using MS/MS spectra. (Sup. Table 1). These class-one sites mapped to ~1200 phosphoproteins. For the class-one sites which 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 (Sup. Table 2). 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 (Sup. Table 3). Generally, data was well matched across treatments and biological replicates, however each peak was manually validated to ensure accurate peak integration, resulting in ~1800 phosphopeptides reliably quantified after drug inhibition (Sup Table 3). 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 cut-off; FDR was controlled by P-value adjustment using the Benjamini and Hochberg method (Q<0.2) (Sup Table 3). At this level of stringency, 23 phosphopeptides (33 phosphorylation sites) showed significant changes in abundance in all treatment groups and most were co-regulated by BRAFV600E and MEK inhibition (Table 1). Supplementary Table 4 describes the key function (derived from UniProt and PubMed) of each protein and the most likely (NetworKIN) (16) or verified kinase(s) for these sites and also compares to 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/organization, control of transcription, and nuclear envelope organization. Gene ontology analysis using Ingenuity pathway analysis indicated that processes ‘DNA methylation and transcriptional repression (P=1.29x10^-6), (>50% pathway coverage) and ERK/MAPK signaling (P=8.11x10^-6) were highly represented (Sup Table 5). The relative change in abundance of these core MAPK components correlated with both treatments (30 min, R^2=0.62) indicating a high degree of similarity in response to MAPK inhibition at blockade through BRAF or MEK (Figure 1 E). Amongst these peptides S695 in SMARCA4 and S297 SGTB exhibited an early 15 min response to MAPK inhibition with MEK and were nearer to control levels after 30 min inhibition. Two others 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 ~50% (RRP1B S245, TMF1 S344, Max S2 and S11 and OSTF1 S213) of the up-regulated sites with NetworKIN kinase predictions could be attributed to the casein kinase 2 (CK2) family (Figure 2 A-C). This pattern was confirmed using the phosphosite analyzer program which performs a statistical test where the frequency of regulated clusters of phosphosites are compared to the background frequency of all phosphosites (17) (Sup. Figure 1). CK2 is an acidophilic kinase that requires an acidic or phosphorylated residue at position +3 in their substrate (S^+XXE/D). In our data one site OSTF1 S213 is the penultimate amino acid at the 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 whilst others have no bona-fide kinase relationships assigned (23, 24). Other up regulated phosphosites were associated with CDK2/3, RCK, CLK family kinases. Amongst the down-regulated 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 (PX*S*P and S*PXK/R).

MAPK blockade with vemurafenib is mutation specific

Next we investigated the regulation of several phosphosites by MAP kinase blockade in the background of the two most common thyroid cancer mutations (BRAF, RET). To perform this analysis we prepared fresh cell lysates and employed 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 are altered upon MAPK inhibition and applied this method to quantitate the specific phosphopeptide and a detectable counterpart unmodified peptide from the same protein (Figure 3 A-D). This approach was less sensitive then the LFQ experiment but still enabled cross validation of 8 out of the 23 phosphopeptides. (Sup. Table 6). Four of these, mitogen activated protein kinase 1 (MAPK01), 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 MAPK01, ANHK, TPR and a decrease in OSTF1 phosphorylation after treatment with vemurafenib. This is consistent with the paradoxical activation of MAPK pathway observed in a BRAF WT background, with an activated Ras signal (26), and is corroborated by the loss of p27KIP determined by Western blot (Figure 1 C).

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 µM) or selumetinib (1 µM) in combination with increasing concentrations of the specific CK2 inhibitor CX-4945, (0.8-8 µM) (27) and cell viability determined after 3 days (Sup Figure 2). In the BRAFV600E mutant SW-1736 cells CK2 inhibition alone reduced cell growth by ~13%, vemurafenib reduced growth by ~36% consistent with our earlier observations. Co-administration of CX-4945 and vemurafenib was synergistic, reducing growth by ~60% compared to controls (Figure 4 A). The anti-proliferative activity was greater than (6-15 %) of Bliss additivity at all concentrations used (Sup. Figure 2 and Sup. Table 7). The level of synergism observed between selumetinib and CX-4945 was considerably lower (5% at 4 µM) (Figure 4 A). In cells with wild-type BRAF protein (TPC1 and Nthy-ori 3.1 cells), the combination 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 (~19%, Bliss additivity) (Figure 4 A). 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 ~1µM) however TPC1 was relatively insensitive to CK2 inhibition with CX-4945 and no synergistic effect of co-inhibition was observed (Sup Figure 3), 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, while 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 (Sup. Figure 4). For these lines we also calculated the combination index (CI) for serially diluted 1:1 mixtures of vemurafenib and CX-4945 (0.5-8 µM) 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 µM total dose) observed for V600E mutant C0045, and synergism observed for V600K mutant C0088 (Figure 4 B). Both BRAF wild-type melanoma lines were unaffected by vemurafenib alone (<10%) and no advantage was observed with co-administration 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 µM) when compared to thyroid carcinoma cell lines (2-13 % at 4 µM). To confirm specificity of the observed BRAF/CK2 drug synergism we knocked-down the expression of CK2 alpha using siRNA in two of the melanoma cell lines C0088 (BRAF) and C0037 (WT). We repeated the viability assay using 4 µM vemurafenib and compared it to effects of dual inhibition using CX-4945/vemurafenib (Figure 4 C-D). The cell viability response to CK2 siRNA alone was minimal in both C0037 and C0088 cells. However, when CK2 siRNA was combined with 4 µM vemurafenib an additive response was observed in the BRAFV600K mutant C0088 whilst no significant change in viability was detected in BRAF WT 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 µM), CX-4945 (4 µM), AKT inhibitor perfosine (4 µM) and with binary combinations for 30 min (Figure 5 B). Phosphospecific antibodies for two well-known AKT phospho-activation sites (T308 and S473) were down regulated by CK2 treatment alone, and when CK2 inhibition was combined with vemurafenib (Figure 5 B). Phosphorylation of the CK2 regulated AKT priming site, pSer129 was reduced upon exposure to CX-4945, providing a clear link between CK2 and the AKT pro-survival 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 down regulated 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 pharmacological blockade with the clinically tested inhibitors vemurafenib and selumetinib. Our work identified ~2300 phosphopeptides and quantitated ~1800, 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 (Figure 1 E).

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 down regulated 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 myeloid leukaemia (AML) cell line, MV4-11. In their study, phosphorylation of S135/136 was non-responsive to MEK inhibition in the resistant AML cell line, HEL. In a second study melanoma cells were treated with MEKi GDC-0973 and PI3Ki GDC-0941 and immunoprecipitation of phosphopeptides was used to profile DNA damage response (DDR) signalling (29). The protein SMARC4 showed initial decrease (not significant) in phosphorylation after MEKi and increased after long-term treatment with PI3Ki singularly and in combination with MEKi (29). Consistent with data presented here S695 in SMARC4 is affected at 15 min with the MEKi AZD-6244 and returns to control levels by 30 min. These data confirm that several phosphosites are consistently regulated in response to MEK inhibition across several 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 down-regulated 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 up-regulated 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 (Sup Table 4). Figure 5 A is a summary of these data and attempts to place the protein...
phosphorylation events detected in networks with respect to their upstream regulatory kinases and the downstream processes they affect.

Of those phosphoproteins affected by MAPK inhibition the cytosolic protein, PPP1R12A, is a key regulatory component of protein phosphatase 1 (PP1); the site S445 was assigned by NetworKIN as a substrate of the AMPK group of kinases and is a *bona fide* substrate of the AMPK-related kinase NUAK1 (31). Phosphorylation of S445 by NUAK1 inhibits targeting of PP1 to myosin suppressing phosphatase activity and maintaining phospho-myosin essential for the actin-cytoskeletal re-arrangements required for cell migration (32). Another protein involved in cell migration/invasion was niban-like protein (FAM129B). Kinase mediated regulation of the sites S692 and S696 was confirmed by PRM mass spectrometry, with no detectable change in the unmodified protein amount. These sites were also found phosphorylated in an inhibitor screen for BRAFV600E regulated phosphoproteins in a melanoma cell line. In that report, physiological characterization demonstrated that phosphorylation drives cell invasion by an unknown mechanism (33). In our dataset, gene enrichment analysis demonstrated the large part played by phosphoproteins in the regulation of gene transcription. EZH2, BIN1 and TPR all regulate chromatin structure and organization and were regulated by MAPK inhibition at sites corresponding to p38/CDK1/2 phosphorylation. PRM mass spectrometry confirmed the phosphorylation of S2155 in TPR, a nuclear pore complex protein important for nuclear transport and mitosis (34). S2155 is a confirmed substrate of ERK1/2, flanked by ERK1/2 docking domains; phosphorylation enhances ERK1/2 binding and is thought to sequester ERK1/2 to nuclear targets enabling gene transcription (35). These data indicate several processes BRAFV600E drives through promoting ERK1/2 or CDK1/2 activity in the nucleus. The phosphorylation of EZH2, TPR and BIN1 may be key in modifying chromatin interacting proteins to regulate gene expression necessary for proliferation (Figure 5 A).

The enrichment of putative phosphopeptide substrates of CK2 following BRAFV600E inhibition indicates that blocking MAPK has consequences for CK2 activity. CK2 is constitutively active with >300 known substrates (36). The specificity of CK2 is achieved by regulatory β-subunits 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 up-regulated after vemurafenib blocking in BRAFV600E thyroid cancer cells (38). The peptide was N-terminally acetylated and phosphorylated at position 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, both are indirectly involved in osteoclast formation (40). Recently, phosphorylation of SH3P2 at S202 was shown to be ERK dependent and catalysed by ribosome S6 kinase (RSK) (41). RSK phosphorylation promoted cell motility by inhibiting the activity of SH3P2 (41). Our data indicates 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 signalling axis in BRAF cancers. We treated cells with a relatively new and specific inhibitor of CK2 (CX-4945) (43) alone and in combination with vemurafenib or selumetinib. Growth inhibition was additive when combined with vemurafenib, increasing cell death by 15-25% in both thyroid and melanoma cell lines harbouring the BRAFV600E mutation. This suggests that the observed increase in abundance of CK2 phospho-substrates was potentially a survival adaptation to MAPK pathway blockade.

CK2 promotes anti-apoptotic, pro-survival mechanisms through potentiation of NF-κβ, Wnt/β-catenin, PI3K/AKT pathways and inhibition of caspase mediated apoptosis (37, 42, 44). CK2 regulation is key to the development of multi-drug resistance (MDR) phenotypes and plays a role in ‘non-oncogene addiction’ in several cancers including T-cell leukemia, prostate, colon and breast cancer (42). In melanoma patients with the BRAFV600 mutation the long-term effectiveness of vemurafenib is limited by the development of acquired drug resistance. In humans and cell models the development of resistance is often associated with the activation of the PI3K/AKT pathway usually suppressed by MAPK activation (45, 46). So far co-targeting with AKT, mTOR and MEK can reduce resistance in melanoma cell lines (46, 47). The phosphoproteomic data presented here highlights CK2 activity as a mechanism by which BRAFV600E cells may adapt to vemurafenib treatment. Our data is consistent with a role for CK2 in activating pro-survival mechanisms through pathways including AKT signaling (48), potentiating the ERK nuclear localization (49) and regulation of c-Myc transcription (23).

Western analysis of several components from the PI3K/AKT pathway indicated that CK2 regulates AKT phosphorylation in BRAF mutated thyroid cancer cells. We have shown that AKT pSer129, a site directly regulated by CK2 (50) is indeed greatly reduced by treatment with CX-4945 (Figure 5 B). S129 phosphorylation has been shown to hyperactivate AKT through a priming step that recruits HSP90 and inhibits phosphatase activity, increasing occupancy at the activation site T308 and most likely S473 (50). Consistent with this we observed reduced phosphorylation of both the PDK1 and mTORC2 phosphorylation sites in AKT following CK2 inhibition. No change in PDK1 phosphorylation or PTEN phosphorylation was observed indicating that PI3K was still active and CK2 did not act upstream of PDK1 as reported previously for PTEN (51, 52). CK2 inhibition demonstrated reduction of AKT kinase activity evidenced by reduced phosphorylation of GSK3β Ser9 (Figure 5 B). GSK3β is a multi-functional protein kinase that is constitutively active until phosphorylated by AKT at position Ser9 (53). Reduced GSK3β phosphorylation observed here would enhance 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 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 anti-tumor effect whilst 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 EGFR inhibition by erlotinib in lung carcinoma, and to prevent DNA repair after treatment with the chemotoxic agents gemcitabine, cisplatin, and carboplatin (27, 55), and our data suggests that co-targeting BRAFV600E and CK2 is a valid path for further investigation. This could be especially useful in some BRAF mutant colon cancers that appear unresponsive to direct BRAF inhibition due to feedback mechanisms that reactivate RTK signalling (56). This feedback results in AKT activation and our data suggests that the novel combination of BRAF and CK2 inhibition would suppress proliferation in tumours 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 tumours (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 trialling PI3K/AKT targeting in combination with MAPK inhibition.

**Acknowledgements.** The authors would like to acknowledge Chris Schmidt, Cathy Lanagan, Linda O’Connor, Ken Dutton-Regester and Nick K Hayward at QIMR Berghofer Medical Research Institute for establishing and genotyping the melanoma cell lines. Jimmy Parker for providing scripts facilitating the compiling of datasets.
References


Table 1. Phosphoproteins regulated by BRAF and MEK inhibition in SW1736 thyroid carcinoma cells.

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Figure legends.

**Figure 1. Experimental design and phosphoproteomics.** (A) Three thyroid cell lines were chosen for their known mutations in the ERK1/2 MAP kinase pathway. Nthy-ori provided a wild-type MAPK pathway response. TPC1 is activated by RET/TPC translocation and SW-1736 is activated by BRAFV600E, representing two major mutations found in the MAPK pathway in thyroid cancer. The points of inhibition by vemurafenib and selumetinib in MAPK are indicated (light grey). (B) The viability of each cell line was measured after exposure to vemurafenib and selumetinib for 3 days, error bars are Stdev. (C) Western blot of ERK1/2 signaling output after treatment for 2 days with 2 µM vemurafenib. (D) Phosphoproteomic workflow showing SW-1736 cells treated with MAPK inhibitors and MAPK activity assessed by western blot for p-ERK, samples were digested with trypsin and phosphopeptides enriched by TiO$_2$ and analysed by LC-MS. (E) Scatterplot of the log2 relative effect measured for phosphopeptides after 30 min exposure to vemurafenib and selumetinib. Phosphopeptides labelled (solid boxes) are from proteins involved in the canonical MAPK pathway.

**Figure 2. Kinase enrichment analysis.** (A) Clustered heat-map of the log2 relative effect of phosphosites regulated by MAPK inhibition (Q<0.2) (red>0, green <0). (B) Web logo display of stacked histograms generated from the 11 amino acid linear motifs surrounding up-regulated and down-regulated sites, with background (all) sites shown for comparison. (C) Pie charts for the top 5 (occurrence) kinase groups found to match (using NetworKIN) the substrates of sites up-regulated and down-regulated phosphoproteins.

**Figure 3. Inhibitor-based phosphopeptide regulation in BRAFV600E, RET/TPC1, and wild type genetic backgrounds quantified by mass spectrometry.** (A-D) Bar plot for log2 ratio of 4 phosphopeptides, counterpart non-phosphopeptides and phospho-DNAJ (loading control) relative to control after 30min treatment with vemurafenib and selumetinib in SW-1736 (BRAFV600E), TPC1 (RET/TPC) and Nthy-ori (WT) thyroid 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 µM CX-4945, 2 µM vemurafenib (PLX-4032), 1 µM selumetinib (AZD6244), 2 µM vemurafenib in combination with 4 µM CX-4945, 1 µM selumetinib in combination with 4 µM CX-4945. Additively >5% is marked with a dotted line and shaded, and was calculated as the theoretical combined fractional response effect $(Fa+Fc)*(100-Fa)/100$ where $F$= % cells killed at given concentration, $Fa$ = vemurafenib or selumetinib and $Fb$ = CX-4945. (B) Bar plot for combination index 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 ($1.0 - 16 \mu M$). (C) Western blot for CK2$\alpha$ after 72 h of transfection with CK2$\alpha$ (siCK2$\alpha$) or control (siCON) siRNA in melanoma cells C0037 (BRAF WT) and C0088 (BRAFV600K), $\beta$-tubulin is shown to represent total protein loading. (D) Bar plot of viability of melanoma cells C0037 (BRAF WT) and C0088 (BRAFV600K) after siRNA
interference with 1 pmol of siCON, siCK2α, 4 µM vemurafenib (PLX-4032), 4 µM CX-4945 and combinations with siRNA treatments.

**Figure 5. Summary of phosphoproteomic pathways.** (A) Network diagram consisting of several proteins with sites regulated (red=up, green=down) in this study; connections place regulatory significance in context of knowledge about specific substrate-kinase relationships. Several regulatory kinases are implicated and the potential mechanistic sites of regulation (grey dashed boxes) and biological processes are hypothesised. (B) Western blot analysis of PI3K/AKT pathway after CK2, BRAF and AKT inhibition for 30 minutes in SW-1736 thyroid cancer cells. (C) Network interpretation of Western blot of PI3K/AKT pathway.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
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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