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BRAF^{V600E} induces *ABCB1*/P-glycoprotein expression and drug resistance in B-cells via AP-1 activation

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Abstract

A subset of patients with chronic lymphocytic leukemia (CLL) and nearly all patients with classic hairy cell leukemia (HCL) harbor somatic *BRAF* activating mutations. However, the pathological role of activated BRAF in B-cell leukemia development and progression remains unclear. In addition, although HCL patients respond well to the BRAF^{V600E} inhibitor vemurafenib, relapses are being observed, suggesting the development of drug resistance in patients with this mutation. To investigate the biological role of BRAF^{V600E} in B-cell leukemia, we generated a CLL-like B-cell line, OSUCLL, with doxycycline-inducible BRAF^{V600E} expression. Microarray and real-time PCR analysis showed that *ABCB1* mRNA is upregulated in these cells, and P-glycoprotein (P-gp) expression as well as function were confirmed by immunoblot and rhodamine exclusion assays. Additionally, pharmacological inhibition of BRAF^{V600E} and MEK alleviated the BRAF^{V600E}-induced *ABCB1*/P-gp expression. *ABCB1* reporter assays and gel shift assays demonstrated that AP-1 activity is crucial in this mechanism. This study therefore uncovers a pathological role for BRAF^{V600E} in B-cell leukemia, and provides further evidence that combination strategies with inhibitors of BRAF^{V600E} and MEK can be used to delay disease progression and occurrence of resistance.

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YTT designed, performed and interpreted experiments and wrote the paper; GL performed flow cytometry and mutation analysis; AL conducted the statistical analyses; EJS assisted with experiments; EH provided expertise and the OSUCLL cell line; SBS and CSC provided expertise and reagents; MRG designed the study and interpreted the data; JCB designed the study, interpreted the data, provided patient samples, and wrote the paper; DML oversaw the work, designed the study, interpreted the data, and wrote the paper. All authors reviewed the manuscript prior to submission.

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Keywords

BRAF; vemurafenib; B-cell; *ABCB1*; P-glycoprotein; leukemia; lymphoma

Introduction

Multiple solid tumors, particularly melanoma, have been found to carry somatic *BRAF* mutations that result in constitutive BRAF protein activation and cell transformation. Since the initial description of *BRAF* mutations in 2002[1], over 40 distinct point mutations affecting the BRAF kinase domain have been identified[2]. Among these, a mutation altering valine (V) to glutamic acid (E) at amino acid 600 in the activation segment of the kinase domain shows the highest incidence. While earlier studies found little or no occurrence of *BRAF* mutations in hematologic diseases, rarer disease types were not studied. More recently, Tiacci *et al.* found that nearly all classic hairy cell leukemia (HCL) cases bear the $BRAF^{V600E}$ mutation[3], and Dietrich and others now report that HCL can be successfully treated with the $BRAF^{V600E}$-selective inhibitor vemurafenib[4]. A subset of chronic lymphocytic leukemia (CLL) patients also show mutated BRAF[5–7], and *BRAF* mutations were identified as one of the acquired initiating mutations in early hematopoietic cells of CLL, leading to deregulation of B-cell receptor (BCR) signaling[8]. Furthermore, a *BRAF* pseudogene transcript is aberrantly expressed in human diffuse large B-cell lymphoma, positively correlates with BRAF expression, and results in MAPK activation. Expression of this pseudogene in a murine model results in aggressive B-cell lymphoma[9]. Together, these findings clearly implicate $BRAF^{V600E}$ in the development of a subset of B-cell malignancies.

Although not all *BRAF* mutations identified to date are V600E, most are activating mutations and result in MAPK stimulation. BRAF is upstream of MEK and ERK, which are involved in regulating cell proliferation, survival, differentiation and senescence following external signals[10]. Because $BRAF^{V600E}$ is active in the absence of external stimuli, it constitutively activates the MAPK pathway to promote cell transformation through enhanced transcription (*e.g.* via c-Fos, Elk-1) and translation (*e.g.* via RSK, eIF4E) of factors that subsequently drive survival and proliferation (*e.g.* cyclin D1, c-myc). $BRAF^{V600E}$ inhibitors including vemurafenib and dabrafenib show clinical responses in many cases of $BRAF^{V600E}$ mutated cancers. However, resistance to these agents commonly develops[11,12]. Emerging data also indicate that $BRAF^{V600E}$ HCL patients relapse following vemurafenib treatment that was initially effective[13]. Interestingly, it was recently reported that a patient with $BRAF^{V600E}$-driven melanoma who responded to vemurafenib developed CLL-like disease, possibly due to paradoxical BRAF inhibitor-associated ERK activation in B-cells via the BCR/SYK/RAS/RAF axis[14]. ERK is also a key downstream effector of the BCR pathway, and inhibition of this pathway by the BTK inhibitor ibrutinib leads to loss of ERK phosphorylation both *in vitro*[15] and in samples from ibrutinib-treated patients[16]. Thus in B-cells, ERK is a point of convergence of the MAPK pathway and the critical BCR pathway, further supporting the relevance of MAPK signaling in B-cell malignancies.

Despite the prevalence of $BRAF^{V600E}$ in HCL and the importance of mutant BRAF in leukemia development and potentially its treatment, downstream targets of this pathway in

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B-cells remain unclear. Here, we sought to identify transcriptional events resulting from constitutive BRAF activation in transformed B-cells. We demonstrate that $BRAF^{V600E}$ induces the expression of the multi-drug resistance (MDR) gene *ABCB1* and its product, P-glycoprotein (P-gp). Further, we determined that MAPK pathway-mediated induction of AP-1 could be a potential mechanism for this effect. This finding may have clinical implications for the long-term use of MDR substrate agents in patients with BRAF-mutated cancers.

Materials and Methods

Cells, cell culture, and reagents

The OSUCLL cell line, previously described, has characteristics similar to the donor's CLL cells[17]. OSUCLL cells were retrovirally infected using the Tet-On 3G inducible expression system (OSUCLL-Tet), then with pRetroX-tight-pur vectors (Clontech, Mountain View, CA) expressing wild-type BRAF (OSUCLL-BRAF) or mutant BRAF (OSUCLL-$BRAF^{V600E}$). The $BRAF^{V600E}$ cDNA construct was purchased from Addgene (Cambridge, MA). For constitutive expression, the pBABE-puro retroviral vector was used (Cell Biolabs, San Diego, CA). Cells were cultured at 37°C, 5% CO_2 in RPMI1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Sigma, St. Louis, MO). Vemurafenib (PLX-4032) was purchased from Selleck (Houston, TX), and CI-1040 (PD184352) was synthesized as described[18]. Doxycycline (dox) was purchased from Clontech, and verapamil, vincristine and rhodamine 123 from Sigma.

Viability and proliferation

MTS assays were performed per manufacturer instructions (CellTiter 96, Promega, Madison WI). Cells were incubated 48 hr in 96-well plates with or without dox (1 μg/ml) and other agents, and MTS reagent was added for an additional 2 hr before analysis. To assess growth rate, cells were cultured in 96-well plates for 20 hr with or without dox. BrdU was added and measured 4 hr later per manufacturer instructions (Millipore, Billerica, MA).

Microarray

RNA from OSUCLL-Tet and OSUCLL-$BRAF^{V600E}$ cells incubated 48 hr with or without dox was analyzed using U133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) in collaboration with the OSU Comprehensive Cancer Center Genomics Shared Resource.

Flow cytometry

Cells were incubated 48 hr with or without dox and labeled using anti-CD69 or isotype antibodies (BD Biosciences, San Jose, CA). Cells were analyzed on a FC500 flow cytometer and results processed using Kaluza software (Beckman Coulter, Brea, CA). To measure P-gp activity, cells were cultured 48 hr with dox then incubated 1 hr with 2.6 μM rhodamine 123. After two washes in media, cells were incubated 90 min with or without verapamil (10 μg/ml). Rhodamine-positive cells were detected by flow cytometry.

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Real-time reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA) and analyzed on an ABI ViiA 7 system (Applied Biosystems, Foster City, CA), using GAPDH as an endogenous control. TaqMan Universal Master Mix, primers, and labeled probes were used per manufacturer instructions (Applied Biosystems). Mean threshold cycle (Ct) values were calculated by ABI ViiA 7 software to determine fold differences.

Immunoblot

SDS-PAGE and immunoblotting were performed using standard procedures. Antibodies against phospho-MEK, MEK, phospho-ERK, ERK, phospho-c-Fos, c-Fos, FosB/B2, phosphor-Fra-1, Fra-1, phospho-c-Jun, c-Jun, JunB and JunD were obtained from Cell Signaling Technology (Danvers, MA), and BRAF, P-gp, and GAPDH from Santa Cruz (Santa Cruz, CA). Human specific $BRAF^{V600E}$ antibody was obtained from Spring Bioscience (Pleasanton, CA). Each immunoblot analysis was repeated a minimum of three times.

ABCB1 promoter activity

NEK-293T cells were transiently transfected with the *ABCB1* promoter luciferase reporter construct pTL-MDR1 (Affymetrix) and pBABE-puro plasmid using FuGENE6 reagent (Promega), then incubated 16 hr with vehicle (DMSO), vemurafenib (2 μM), CI-1040 (1 μM), or both. Luciferase activity (relative luciferase units, RLU) was normalized to protein amount as determined by BCA assay (ThermoFisher, Waltham, MA).

Electrophoretic mobility shift assay (EMSA)

AP-1 double-stranded probes [5'-CGCTTGATGAGTCAGCCGGAA-3' for wild-type and 5'-CGCTTGATAGTAGTGCCGGAA-3' for mutant[19,20]] were end-labeled with ^{32}P dCTP using Klenow DNA polymerase (Life Technologies, Grand Island, NY). Nuclear lysates (10 μg) were incubated 1 hr with antibodies to c-Jun, c-Fos, JunB, JunD, and Fra-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or MEK, phospho-c-Jun, and phospho-c-Fos (Cell Signaling). Labeled probes were added, incubations were continued at room temperature for another 30 min, and complexes were separated on native polyacrylamide gels. Gels were dried and signals detected using a phosphor screen (GE Healthcare, Pittsburgh, PA).

Statistical analysis

Differences in BrdU incorporation, *ABCB1* mRNA expression, percentage of rhodamine-positive cells, percentage of mitochondrial activity, and relative luciferase activity versus controls were assessed using mixed effects models. Log transformations were applied as appropriate before modeling to stabilize variances. All analyses were performed using SAS/STAT software, version 9.3 (SAS Institute, Inc., Cary, NC).

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Results

Generation of B-cells with inducible BRAF^{V600E} expression

BRAF mutations are found in a subset of CLL, and are early acquired events that may contribute to disease development or progression[8] and potentially drug resistance. To investigate effects of this mutant protein in malignant B-cells, we stably transfected OSUCLL cells with a dox-inducible vector system to generate OSUCLL-Tet. This cell line was infected with retroviral vectors containing wild-type or mutant BRAF to produce OSUCLL-BRAF and OSUCLL-BRAF^{V600E}. OSUCLL-BRAF^{V600E} cells strongly upregulated mutant BRAF following 24 hr dox treatment, as detected by immunoblot using a BRAF^{V600E}-specific antibody (Figure 1A). A moderate but consistent increase in wild-type BRAF was observed in dox-treated OSUCLL-BRAF versus OSUCLL-Tet cells. Under these conditions, dox-mediated BRAF^{V600E} induction was accompanied by increased MEK and ERK phosphorylation, indicating functional transfected protein. Minimal effects on MEK and ERK phosphorylation were detected in dox-treated OSUCLL-BRAF cells. However, a slight increase in ERK phosphorylation was detected in OSUCLL-BRAF^{V600E} cells in the absence of dox, and a low level of BRAF^{V600E} could be detected in these cells with prolonged exposures (Figure 2B), indicating transcription from this promoter is not completely absent in the absence of dox. In comparison with OSUCLL-Tet, both OSUCLL-BRAF and OSUCLL-BRAF^{V600E} cells showed a significant increase in growth rate as determined by BrdU proliferation assays (Figure 1B). OSUCLL-BRAF^{V600E} cells appeared to grow slightly faster than OSUCLL-BRAF cells, but this difference was not significant. Interestingly, the addition of dox did not further increase proliferation, suggesting that whatever effects transfected BRAF or BRAF^{V600E} have on growth rate occur at very low expression levels. These results demonstrate that transfected BRAF^{V600E} produces the expected downstream biochemical changes in OSUCLL cells, but its impact on proliferation relative to normal BRAF is limited.

Based on the increased signaling in BRAF^{V600E} transfected cells, we examined transcriptional changes resulting from the presence versus absence of BRAF^{V600E} expression using microarray. The top 30 genes increased or decreased in dox-treated OSUCLL-BRAF^{V600E} cells relative to dox-treated OSUCLL-Tet cells are shown in Supplementary Table S1. Among these, the lymphocyte activation marker *CD69* and the multidrug resistance gene *ABCB1* were observed to be increased in OSUCLL-BRAF^{V600E} cells versus the control. In validation of these results, CD69 expression was found to be markedly increased in dox-treated OSUCLL-BRAF^{V600E} compared to OSUCLL-Tet and OSUCLL-BRAF cells (Figure 1C).

BRAF^{V600E} induces ABCB1 mRNA and P-gp protein expression in OSUCLL cells

To confirm BRAF^{V600E}-mediated *ABCB1*/P-gp induction, OSUCLL-Tet, -BRAF, and -BRAF^{V600E} cells were incubated with or without dox and examined by real-time RT-PCR and immunoblot. As early as 24 hr, but consistently by 48 hr, *ABCB1* mRNA and P-gp protein were clearly up-regulated in dox-treated OSUCLL-BRAF^{V600E} cells (Figures 2A, 2B, 3A). Drug-resistant 697R cells that have increased P-gp expression[21] were included as a positive control. As previously noted, a low level of BRAF^{V600E} protein could be detected

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in OSUCLL-BRAF^{V600E} cells in the absence of dox treatment (Figure 2B), and *ABCB1* expression in these cells was slightly higher relative to OSUCLL-Tet cells (Figures 2A, 3A). However, increased P-gp expression was not clearly detected in OSUCLL-BRAF^{V600E} cells in the absence of dox, nor was *ABCB1* mRNA increased in dox-treated OSUCLL-BRAF cells (Figure 2A). As an additional validation, OSUCLL cells were transfected with cDNAs driven by a constitutive promoter. Cells transfected with BRAF^{V600E} again showed increases in MAPK signaling and in P-gp expression relative to cells transfected with normal BRAF or the empty vector (Supplementary Figure S1).

As P-gp expression may not necessarily correlate with its function[22], efflux of the fluorescent P-gp substrate rhodamine 123 was examined by flow cytometry in dox-induced OSUCLL cells. Within 90 minutes of removal from rhodamine-containing media, OSUCLL-BRAF^{V600E} cells were essentially negative for rhodamine compared to OSUCLL-Tet and -BRAF cells (Figure 2C, black bars; $p = 0.0003$). This effect was significantly reversed by the P-gp inhibitor verapamil (Figure 2C, grey bars; $p = 0.0025$). As a further determination of P-gp function, cells were examined for sensitivity to the P-gp substrate vincristine. Cells were incubated 24 hr without or with dox, then treated with vincristine in the presence or absence of verapamil. After an additional 48 hr, cell proliferation was evaluated by MTS assay. As shown in Figure 2D, dox-mediated induction of BRAF^{V600E} resulted in a significant increase in resistance to vincristine ($p<0.001$). Furthermore, the addition of verapamil significantly reduced this vicristine resistance ($p<0.001$). Together, these data indicate that BRAF^{V600E} induces P-gp expression and function in OSUCLL cells.

BRAF^{V600E} and MEK inhibition blocks P-gp induction in OSUCLL cells

Previous studies using various tumor cell lines have shown that *ABCB1* expression can be regulated through the MAPK pathway[23,24], involvement of the transcription factor AP-1[25] or reactive oxygen species[26], and/or NF-κB signaling and CRE transcriptional activity[27]. As the induction of BRAF^{V600E} causes MEK-ERK activation in our model, we first examined this pathway using the BRAF^{V600E} inhibitor vemurafenib and the MEK inhibitor CI-1040. Following a 24-hr incubation with dox, OSUCLL cells were treated with vehicle (DMSO), vemurafenib (2 μM), and/or CI-1040 (1 μM) for 16 hr. As shown in Figures 2A–B and 3A–B, the induction of BRAF^{V600E} by dox caused a notable increase in P-gp protein as well as *ABCB1* mRNA. This effect was inhibited by vemurafenib or CI-1040, and stronger inhibition was noted with the combination.

BRAF^{V600E} enhances ABCB1 promoter activity

To investigate the mechanism of BRAF^{V600E}-mediated *ABCB1* induction, a construct containing 1 kb of the *ABCB1* promoter driving a luciferase reporter (pTL-ABCB1) was transiently co-transfected with either an empty vector (pBABE-puro) or one containing BRAF^{V600E} (pBABE-puro-BRAF^{V600E}) into HEK293T cells. Cells were then treated without or with vemurafenib and/or CI-1040, and lysates were measured for luciferase activity (Figure 4A). *ABCB1* promoter activity was notably increased with BRAF^{V600E} expression, and BRAF^{V600E} and MEK inhibition reduced this effect. Based on data demonstrating the role of AP-1 in *ABCB1* regulation[25], a panel of AP-1 proteins were assessed by immunoblot in OSUCLL cells following 24-hr dox treatment. As shown in

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Figure 4B, c-Fos, FosB/B2, Fra1, c-Jun, JunB, and JunD all appeared to be upregulated and/or phosphorylated upon $BRAF^{V600E}$ induction, and these effects could be blocked by vemurafenib and/or CI-1040.

NF-κB and CRE have also been implicated in *ABCB1* regulation[27]. Here, phosphorylation of NFκB p65 was observed with $BRAF^{V600E}$ induction. However, this effect was inconsistent despite the consistent increase in *ABCB1* mRNA. Also, phosphorylation of CREB was unaltered upon $BRAF^{V600E}$ induction (Supplementary Figure S2). These results further support that $BRAF^{V600E}$-induced *ABCB1* expression in OSUCLL cells occurs via the MEK/ERK/AP-1 pathway. To identify AP-1 factors involved in *ABCB1* regulation in OSUCLL cells, EMSAs were performed using AP-1 elements from the *ABCB1* promoter as previously identified[19,20]. Following 24-hr dox incubation of OSUCLL cells, nuclear extracts were prepared, incubated with labeled probe in the absence or presence of antibodies to AP-1, and separated on a non-denaturing acrylamide gel (Figure 4C). A mobility shift was observed upon induction of $BRAF^{V600E}$ (lanes 1 vs. 7) that appeared to be specific for AP-1, as it was reduced following addition of excess cold probe or substitution of a mutated probe (lanes 2 and 3, respectively). While most of the antibodies to AP-1 family members produced no effect, an anti-JunD antibody resulted in a clear supershift in the AP-1 complex. Overall, these results indicate that $BRAF^{V600E}$ mediates *ABCB1*/P-gp expression in OSUCLL cells, and that JunD is likely to be an important component of this regulatory mechanism.

Discussion

A subset of B-cell malignancies carry activating *BRAF* mutations, but the role of activated BRAF in B-cell disease is unclear. We investigated the pathological role of $BRAF^{V600E}$ in B-cell leukemia using a model system in which we could control $BRAF^{V600E}$ expression to better assess specific signaling events and outcomes. While the list of genes affected by increased BRAF signaling includes several interesting candidates, here we focused on *ABCB1* because many melanoma patients with $BRAF^{V600E}$ are either resistant to therapy or relapse following treatment, hinting at a correlation between $BRAF^{V600E}$ and drug resistance. The protein product of *ABCB1*, P-gp, is a member of a class of glycoproteins that export xenobiotic agents across the cytoplasmic membrane. Thus, its presence in certain cell types acts to reduce intracellular accumulation of anti-cancer drugs, resulting in relative resistance to those agents. P-gp is normally expressed in the liver, pancreas, kidney, and gut, but is also aberrantly upregulated in certain solid tumors and hematologic malignancies by a variety of mechanisms[24,28]. Separately, drug resistance in $BRAF^{V600E}$ mutated cancers has also been reported to result from concurrent activation of the PI3K pathway through PTEN loss, amplification of cyclin D1, and feedback activation of EGFR[29–35]. However, the vast majority of these resistance studies have focused on increased gene expression as a consequence of chronic exposure to chemotherapeutic agents, and few studies describe the involvement of P-gp in *de novo* $BRAF^{V600E}$-mediated drug resistance. Here, we report that mutationally activated BRAF drives *ABCB1* transcription via AP-1 activity in a model of B-cell malignancy, leading to enhanced P-gp expression and function.

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Previous reports indicate that the expression levels of *ABCB1* mRNA and P-gp protein do not necessarily correlate with P-gp function[22]. Furthermore, the size and glycosylation of P-gp make this protein challenging to detect by immunoblot. Therefore, following real-time RT-PCR validation of increased *ABCB1* mRNA expression following $BRAF^{V600E}$ induction, we assessed P-gp function via rhodamine exclusion as well as drug sensitivity in the presence and absence of the P-gp inhibitor verapamil. These experiments confirmed increased P-gp function in $BRAF^{V600E}$-expressing cells. To investigate the relationship between BRAF activity and *ABCB1* regulation, we treated cells with the $BRAF^{V600E}$ inhibitor vemurafenib. Interestingly, this agent abolished ERK phosphorylation in OSUCLL cells only transiently (data not shown). This transient effect, which is also seen in melanoma and other $BRAF^{V600}$E-mutant cell lines[36,37], could be the result of P-gp-mediated drug elimination, as vemurafenib has been reported to be a substrate of efflux pumps such as P-gp. In support of this, brain distribution of vemurafenib is diminished via P-gp and BCRP/ABCG2 in patients with metastatic melanoma, and can be enhanced by inhibition of these proteins[38,39]. Furthermore, Wu *et al*. showed that vemurafenib blocked the efflux activity not only of P-gp, but also BCRP, and re-sensitized a $BRAF^{V600E}$ and BCRP-expressing melanoma cell line to the BCRP substrates topotecan and mitoxantrone[40]. These observations further support that elevated MDR protein expression might constitute an important mechanism of resistance to vemurafenib. Although we did not detect increases in other MDR proteins such as BCRP in $BRAF^{V600E}$-expressing cells, our data support that the $BRAF^{V600E}$ mutation might promote resistance to drugs that target it. In addition to V600E, other mutations in *BRAF* have been identified (*e.g.* G466, L597, K601) that result in enhanced BRAF kinase activity. While these are rarer and therefore less characterized, we hypothesize that these mutations would also increase *ABCB1* expression to cause resistance to chemotherapies including vemurafenib. However, more experiments are needed to address this.

$BRAF^{V600E}$ kinase inhibitor-induced paradoxical MAPK activation is known to stimulate receptor tyrosine kinases such as Ras or c-Raf to re-induce MAPK signaling. Interestingly, it was recently reported that a patient with $BRAF^{V600E}$-driven melanoma who responded to vemurafenib developed CLL-like disease, possibly due to paradoxical BRAF inhibitor-associated ERK activation in B-cells via the BCR/SYK/RAS/RAF axis[14]. To avoid this and enhance drug efficacy, combinations of MEK inhibitors with vemurafenib are now being explored, and studies are also emerging with new inhibitors of ERK. In this study, we found that MAPK pathway-induced AP-1 protein expression results in increased *ABCB1*/P-gp expression. We observed increased expression and/or phosphorylation of several proteins in the Fos and Jun family including c-Fos, FosB/B2, Fra-1, c-Jun, JunB, and JunD. Although only JunD was positively identified to interact with the *ABCB1* promoter element in EMSAs, other AP-1 components could also be involved in *ABCB1* expression, and combinations of MAPK pathway inhibitors will likely be needed to effectively prevent this effect in patients.

While we demonstrate that increased P-gp expression and function can result from constitutive BRAF activation in B-cells, the interpretations of these results are limited by the use of cell lines. It will be essential to demonstrate that these effects are noted in tumor cells

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derived from patients with the *BRAF*^{V600E} mutation, and such studies are underway. The strategy utilized here also does not address the role of BRAF^{V600E} in disease development, as transfections were performed in a cell line derived from malignant B-cells. Chung *et al*. reported that the *BRAF*^{V600E} mutation is expressed in hematopoietic stem cells in HCL patients and that these cells produce an HCL-like phenotype in immune-deficient mice[41]. However, pan-hematopoietic expression of BRAF^{V600E} via the Mx1 promoter appears to produce a non-lymphoid histiocytic malignant phenotype, rather than HCL-like disease[41–43], and *BRAF*^{V600E} mutations are found in approximately half the cases of histiocytic malignancies Langerhans cell histiocytosis[44] and Erdheim-Chester disease[45]. CD19-restricted BRAF^{V600E} expression in the absence of other mutations does not appear to be sufficient to induce disease[41]. Thus, exactly how and at what stage BRAF^{V600E} expression promotes the development of B-cell tumors remains unclear. A murine model with B-lineage restricted co-expression of BRAF^{V600E} and a relevant second hit will likely provide a more accurate model to investigate the pathological role of BRAF^{V600E} in B-cell malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A new cell line system with inducible expression of mutant BRAF was used to determine targets of this oncoprotein in B-cells.
- These findings indicate that activating *BRAF* mutations, now reported in several B-cell malignancies, can result in the increased expression of *ABCB1*/P-gp. Thus, incorporation of relevant BRAF pathway inhibitors into therapies, especially those that include P-gp substrate drugs, may benefit patients with such mutations.

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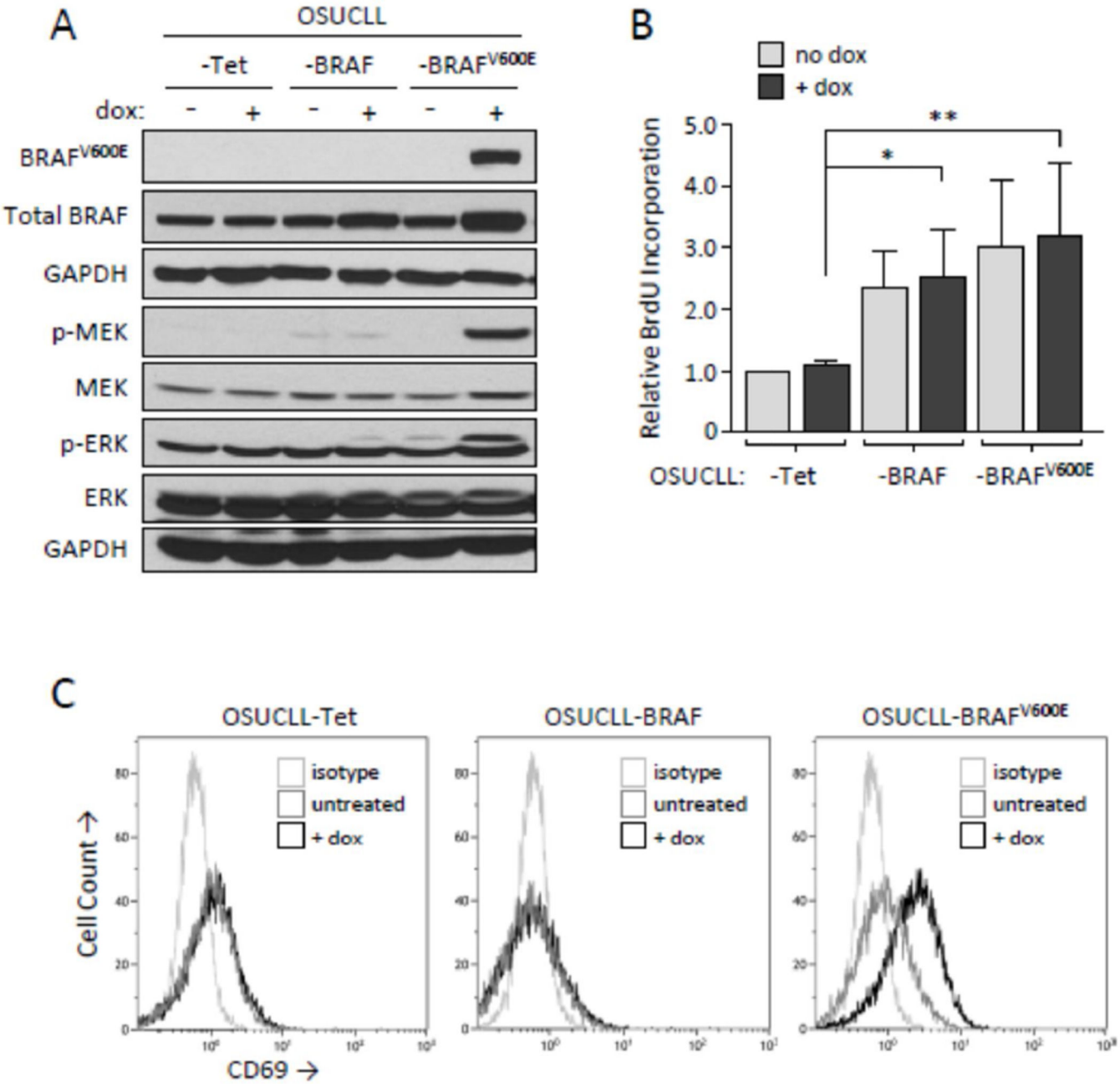

Figure 1. Effects of transfected BRAF^{V600E} on the MAPK pathway and cell growth

A. Immunoblot analysis of wild-type and mutant BRAF expression and MAPK signaling in transfected OSUCLL cells following 24 hr incubation without or with dox (1 μg/ml). **B.** Proliferation in OSUCLL-Tet, OSUCLL-BRAF, and OSUCLL-BRAF^{V600E} cells without or with dox treatment (1 μg/ml) for 24 hr. BrdU was added for the last 4 hr of the incubations. Data are shown relative to the OSUCLL-Tet control cell line without dox treatment (N=4, *p<0.05; **p<0.005). **C.** OSUCLL-Tet, OSUCLL-BRAF, and OSUCLL-BRAF^{V600E} cells were incubated 48 hr with or without dox, then stained with anti-CD69 or isotype antibodies and assessed by flow cytometry. Data are representative of three independent experiments.

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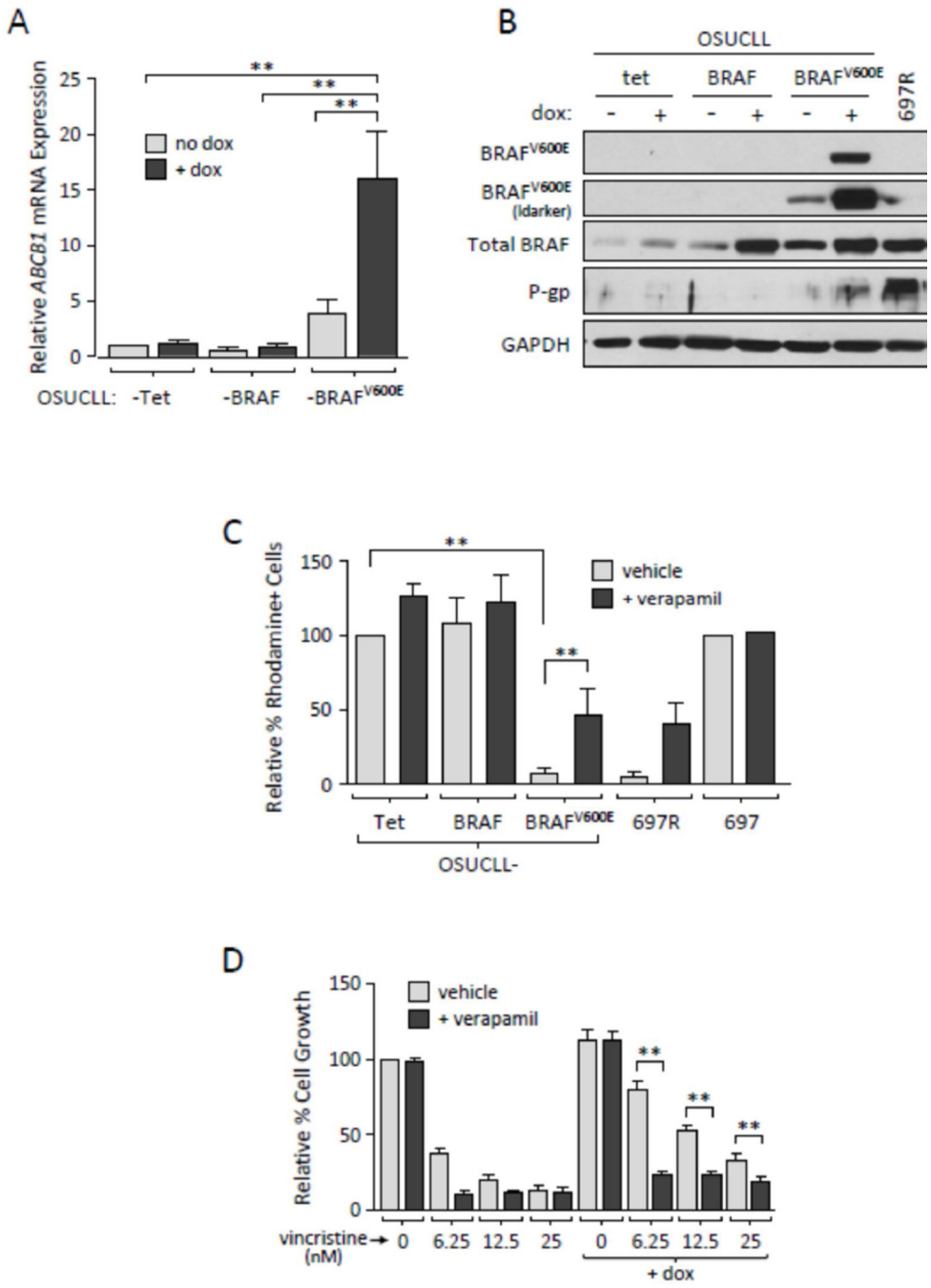

Figure 2. BRAF^{V600E} induces the expression of *ABCB1* and functional P-gp in OSUCLL cells

A. *ABCB1* mRNA levels were assessed in OSUCLL cells using real-time RT-PCR (N=3, **p<0.001). **B.** Immunoblot of normal BRAF, mutant BRAF, and P-gp in OSUCLL cells incubated 48 hr without or with dox. **C.** OSUCLL cells cultured 48 hr with dox were incubated 1 hr with the fluorescent P-gp substrate rhodamine 123, then transferred to rhodamine-free media with or without 10 μM verapamil. Retained rhodamine was assessed by flow cytometry after 90 min. Parental 697 cells and drug-resisant 697-R cells were included as controls. Results were averaged from three identical experiments (**p<0.005)

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and are shown as percent rhodamine-positive cells relative to vehicle-treated OSUCLL-Tet (far left). **D.** OSUCLL-BRAF^{V600E} cells were incubated 48 hr with or without dox, vincristine, and/or verapamil, and mitochondrial activity was evaluated by MTS assay. Results shown are averaged from three identical experiments. At each concentration of vincristine, comparisons were made between the dox and no dox conditions, as well as between the verapamil and control conditions in the presence of dox (**$p<0.001$).

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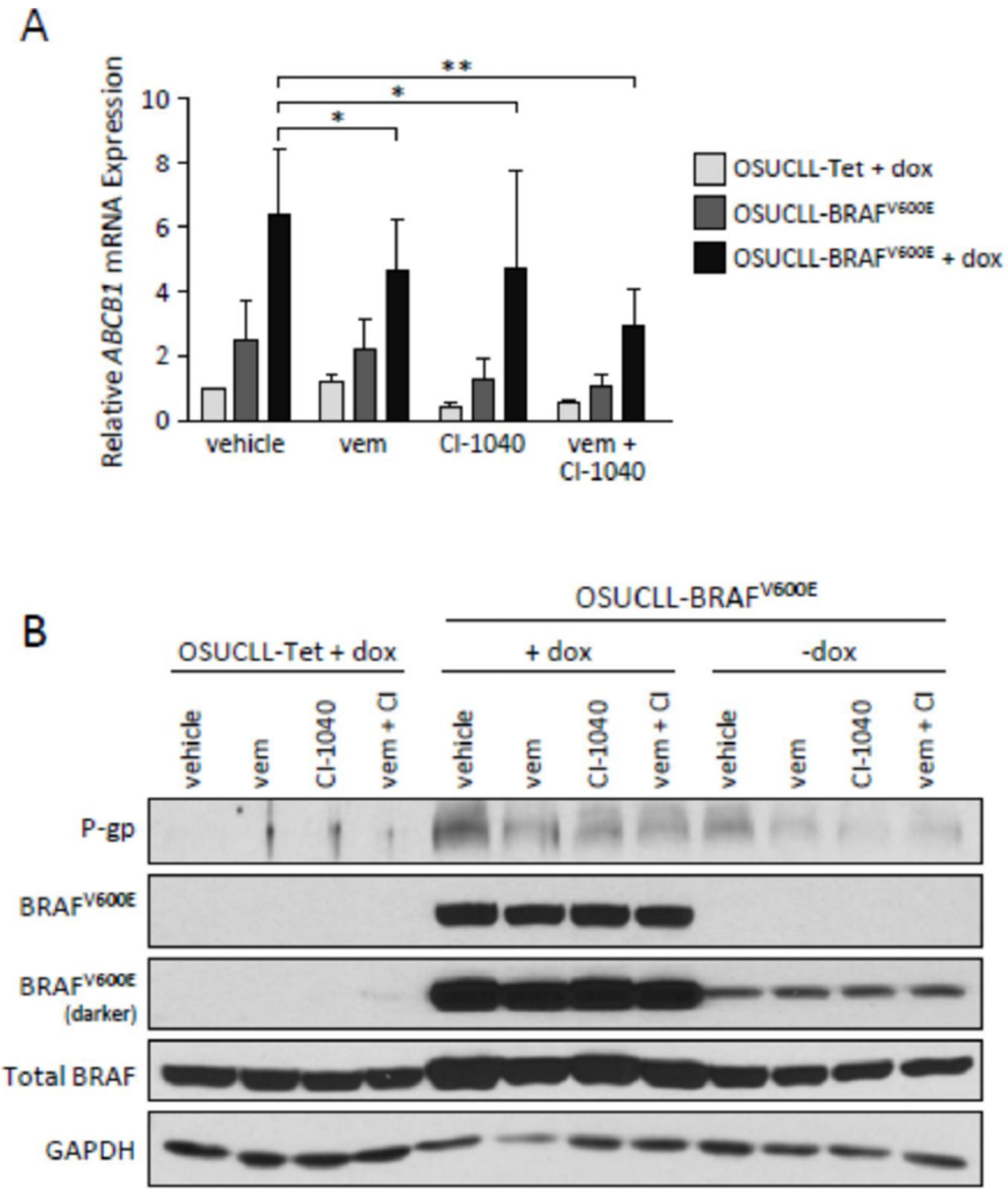

Figure 3. BRAF^{V600E} and MEK inhibition block *ABCB1*/P-gp expression in OSUCLL cells

A. Real-time RT-PCR analysis of *ABCB1* expression in OSUCLL cells in the presence or absence of BRAF^{V600E} or MEK inhibitors. Cells were incubated without or with dox 24 hr, then inhibitors (2 μM vemurafenib and/or 1 μM CI-1040) were added for an additional 16 hr. Inhibitor comparisons in the presence of dox were performed versus BRAF^{V600E} + dox (N=5, *p<0.05; **p<0.001). **B.** Immunoblot analysis of P-gp, normal BRAF, and BRAF^{V600E} expression in OSUCLL cells treated as in A. Results shown are representative of three individual experiments.

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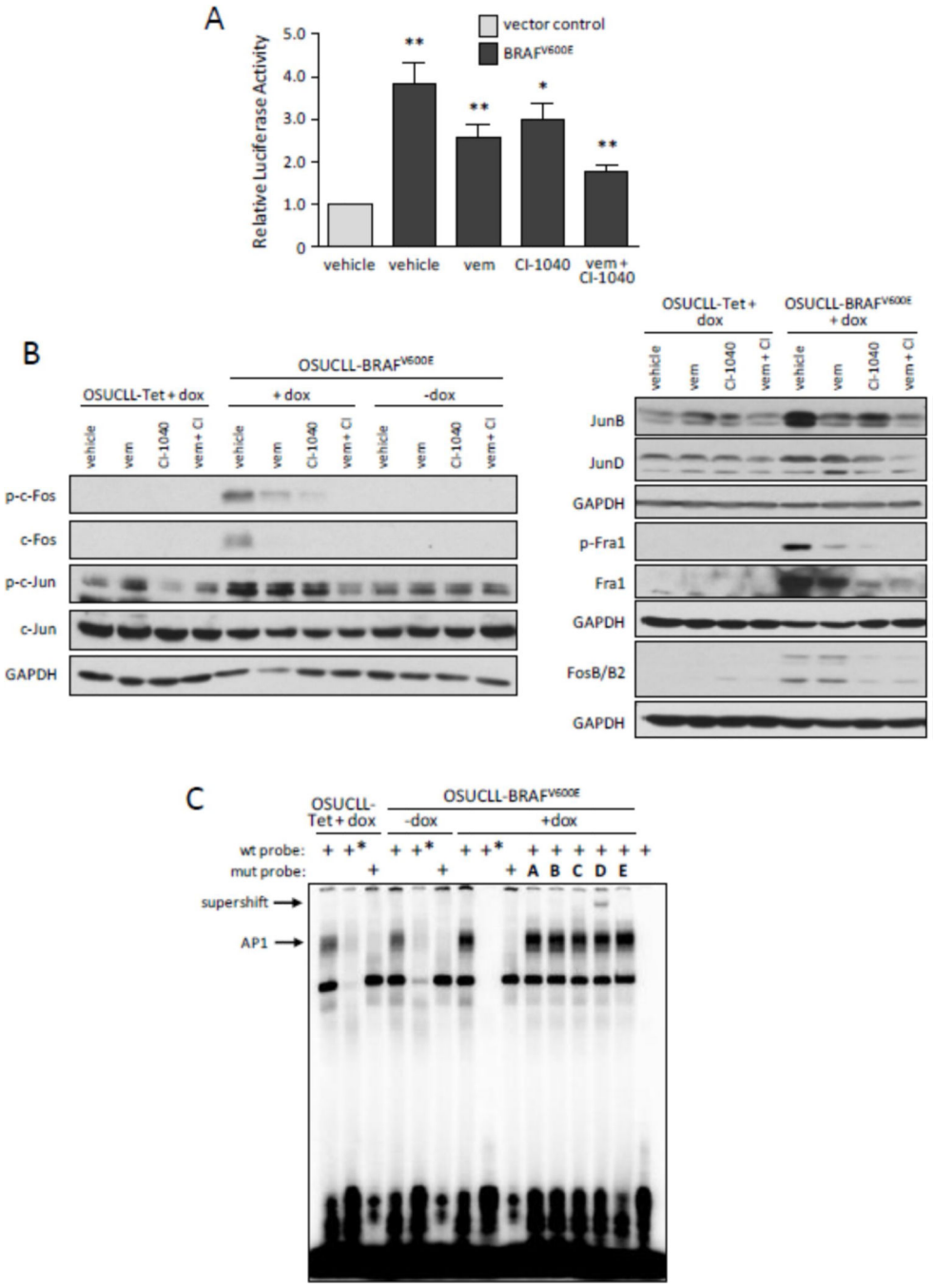

Figure 4. BRAF^{V600E} enhances *ABCB1* promoter activity via MAPK and AP-1

A. HEK293T cells were transiently co-transfected with 1 μg *ABCB1* reporter construct (pTL-MDR1) and 1 μg empty vector (pBabepuro) or mutant BRAF plasmid (pBabepuro-BRAF^{V600E}). After 8 hr, inhibitors were added as in Figure 3A. After an additional 16 hr, luciferase activity was assessed in total cell lysates. Results are shown normalized to the amount of lysate; each inhibitor was compared to control, and in addition, the control was compared to empty vector (N=3, *p<0.05; **p<0.001). **B.** The immunoblot from Figure 3B was additionally analyzed for c-Fos and c-Jun proteins. **C.** Nuclear extracts were prepared

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from OSUCLL cells incubated 24 hr with or without dox, then mixed with ^{32}P-labeled wild-type (wt) or mutant (mut) AP-1 probes, 100× cold probe (*), and antibodies as indicated: A. c-Fos; B. c-Jun; C. JunB; D. JunD; E. MEK (irrelevant control). The final lane is free probe without nuclear extract. Mixtures were separated by native acrylamide electrophoresis and bands detected by autoradiography. Results shown are representative of three individual experiments.