

# Protein Kinase CK2 $\alpha$ Maintains Extracellular Signal-regulated Kinase (ERK) Activity in a CK2 $\alpha$ Kinase-independent Manner to Promote Resistance to Inhibitors of RAF and MEK but Not ERK in BRAF Mutant Melanoma\*

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The protein kinase casein kinase 2 (CK2) is a pleiotropic and constitutively active kinase that plays crucial roles in cellular proliferation and survival. Overexpression of CK2, particularly the  $\alpha$  catalytic subunit (CK2 $\alpha$ , *CSNK2A1*), has been implicated in a wide variety of cancers and is associated with poorer survival and resistance to both conventional and targeted anticancer therapies. Here, we found that CK2 $\alpha$  protein is elevated in melanoma cell lines compared with normal human melanocytes. We then tested the involvement of CK2 $\alpha$  in drug resistance to Food and Drug Administration-approved single agent targeted therapies for melanoma. In BRAF mutant melanoma cells, ectopic CK2 $\alpha$  decreased sensitivity to vemurafenib (BRAF inhibitor), dabrafenib (BRAF inhibitor), and trametinib (MEK inhibitor) by a mechanism distinct from that of mutant NRAS. Conversely, knockdown of CK2 $\alpha$  sensitized cells to inhibitor treatment. CK2 $\alpha$ -mediated RAF-MEK kinase inhibitor resistance was tightly linked to its maintenance of ERK phosphorylation. We found that CK2 $\alpha$  post-translationally regulates the ERK-specific phosphatase dual specificity phosphatase 6 (DUSP6) in a kinase dependent-manner, decreasing its abundance. However, we unexpectedly showed, by using a kinase-inactive mutant of CK2 $\alpha$ , that RAF-MEK inhibitor resistance did not rely on CK2 $\alpha$  kinase catalytic function, and both wild-type and kinase-inactive CK2 $\alpha$  maintained ERK phosphorylation upon inhibition of BRAF or MEK. That both wild-type and kinase-inactive CK2 $\alpha$  bound equally well to the RAF-MEK-ERK scaffold kinase suppressor of Ras 1 (KSR1) suggested that CK2 $\alpha$  increases KSR facilitation of ERK phosphorylation. Accordingly, CK2 $\alpha$  did not cause resistance to direct inhibition of ERK by the ERK1/2-selective inhibitor SCH722984. Our findings support a kinase-independent

scaffolding function of CK2 $\alpha$  that promotes resistance to RAF- and MEK-targeted therapies.

The protein kinase casein kinase 2 or II (CK2)<sup>2</sup> is a highly conserved, ubiquitously expressed, pleiotropic, and constitutively active serine/threonine kinase that has crucial roles in cell survival, proliferation, and differentiation (2–4). The CK2 holoenzyme consists of two regulatory ( $\beta$ ) and two catalytic ( $\alpha$  or  $\alpha'$ ) subunits, the latter of which can also function independently of the tetramer (5). Although CK2 itself does not appear to be a proto-oncogene, its up-regulation has been shown to promote growth and prevent apoptosis, both of which promote cancer (4). Indeed, overexpression of CK2 at the transcript and/or protein level has been observed in many cancers (6), including multiple myeloma (7), chronic lymphocytic leukemia (8), breast cancer (9), colorectal cancer (10), liver cancer (11), etc. and is correlated with poorer patient survival (6, 10, 12). Similarly, CK2 exhibited 2.5-fold higher catalytic activity in metastatic melanoma than in dermal nevi (13).

In addition to its roles in tumor growth and progression, CK2 also promotes drug resistance to both conventional and targeted therapeutics. For example, pharmacological inhibition of CK2 kinase activity reverted multidrug resistance of a human T lymphoblastoid cell line (14) at least in part by down-regulating P-glycoprotein activity. In addition, siRNA-mediated knockdown of CK2 catalytic subunits enhanced chemosensitivity to gemcitabine in human pancreatic cancer cells (15). In cells depleted of CK2 $\alpha$ , gemcitabine induced MKK4/JNK signaling, resulting in cell death (15). Furthermore, CK2 displayed elevated protein expression and activity in chronic myeloid leukemia cells that are resistant to the small molecule kinase inhibitor imatinib (16). Either reduction of CK2 $\alpha$  expression or pharmacological inhibition of CK2 $\alpha$  kinase activity restored imatinib sensitivity, possibly through suppressing Akt activity (16). However, the role of CK2 $\alpha$  in drug resistance, particularly to targeted therapeutics, has remained underexplored.

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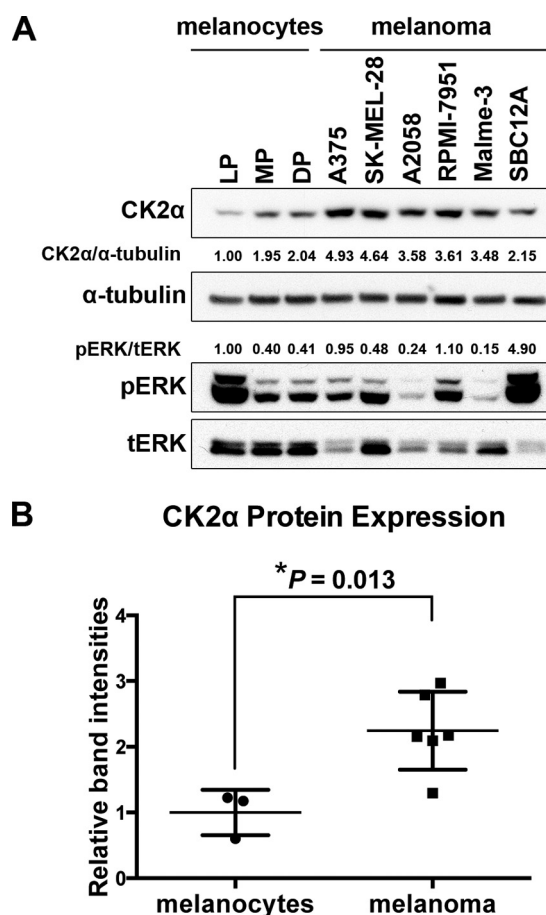
<sup>2</sup> The abbreviations used are: CK2, casein kinase 2; BRAFi, BRAF inhibitor; MEKi, MEK inhibitor; KSR, kinase suppressor of Ras; pERK, phosphorylated ERK; GI<sub>50</sub>, 50% growth inhibition; DUSP6, dual specificity phosphatase 6; ERKi, ERK inhibitor; RSK, ribosomal s6 kinase; pRSK, phospho-RSK.

Inhibitors of BRAF and MEK, members of the RAF-MEK-ERK kinase cascade, have achieved remarkable overall response rates in advanced melanomas harboring BRAF Val-600 mutations, but a significant proportion of patients is intrinsically resistant to such therapies, and those who respond almost inevitably develop resistance over a matter of months (17). Considerable efforts have been invested in identifying resistance mechanisms of *BRAF* mutant melanomas to BRAF inhibition, and some have demonstrated clinical relevance (18–21). In a recent whole-kinome siRNA screen for kinases that could induce resistance to ERK kinase inhibitors in pancreatic ductal adenocarcinoma cells, we identified CK2 $\alpha$  as a synthetic lethal partner of ERK inhibition (22). We postulated that kinase inhibitor resistance mechanisms can be shared by diseases that show hyperactivity of the same pathway. Given that the RAF-MEK-ERK pathway is strongly activated in both pancreatic cancer and melanoma, we sought to determine whether CK2 also plays a role in resistance to inhibition of this pathway in melanoma.

In the present study, we found that CK2 $\alpha$  overexpression was sufficient to drive resistance to both BRAF and MEK inhibitors in BRAF mutant melanoma cells. Conversely, depletion of CK2 $\alpha$  increased sensitivity to the BRAF inhibitor vemurafenib. Consistent with these results, CK2 $\alpha$  sustained ERK phosphorylation under conditions of pathway inhibition. Although we found that CK2 negatively regulated expression of the ERK-specific phosphatase dual specificity phosphatase 6 (DUSP6) in a kinase-dependent manner, the maintenance of ERK phosphorylation was not due to these decreased levels of DUSP6. Instead, we found that CK2 $\alpha$ -mediated maintenance of ERK phosphorylation and drug resistance were kinase-independent. The ability of both wild-type and kinase-inactive CK2 $\alpha$  to bind to the key RAF-MEK-ERK pathway scaffold protein kinase suppressor of Ras 1 (KSR1), which is required for optimal ERK phosphorylation and activation, supports a kinase-independent scaffolding role for CK2 $\alpha$  in facilitating optimal ERK signaling under conditions of pathway inhibition. That CK2 $\alpha$  overexpression did not cause resistance to a direct ERK inhibitor is further evidence that ERK inhibition may overcome resistance mechanisms that shorten the effectiveness of blocking upstream kinases in the RAF-MEK-ERK pathway.

## Results

**CK2 $\alpha$  Expression Is Up-regulated in a Subset of Melanomas**—To examine the expression of CK2 $\alpha$  in melanoma, we first surveyed the Cancer Genome Atlas skin cutaneous melanoma data set for CK2 $\alpha$  mRNA expression through cBioPortal (40). We found that the CK2 $\alpha$  transcript is up-regulated in a subset of those tumors (15% of 278 samples) and that 90% of that subset also harbor mutations in *BRAF*, *NRAS*, and/or *NF1* that lead to hyperactivation of ERK. Next, we measured CK2 $\alpha$  protein expression in a panel of neonatal human epidermal melanocytes, lightly pigmented, moderately pigmented, and darkly pigmented donors and melanoma cell lines (five BRAF mutants (A375, SK-MEL-28, A2058, RPMI-7951, and Malme-3) and one NRAS mutant (SBC12A)) (Fig. 1A). Melanoma cell lines had higher levels of CK2 $\alpha$  protein expression compared with melanocytes (Fig. 1B;  $p = 0.013$ ). In contrast, basal phosphorylated

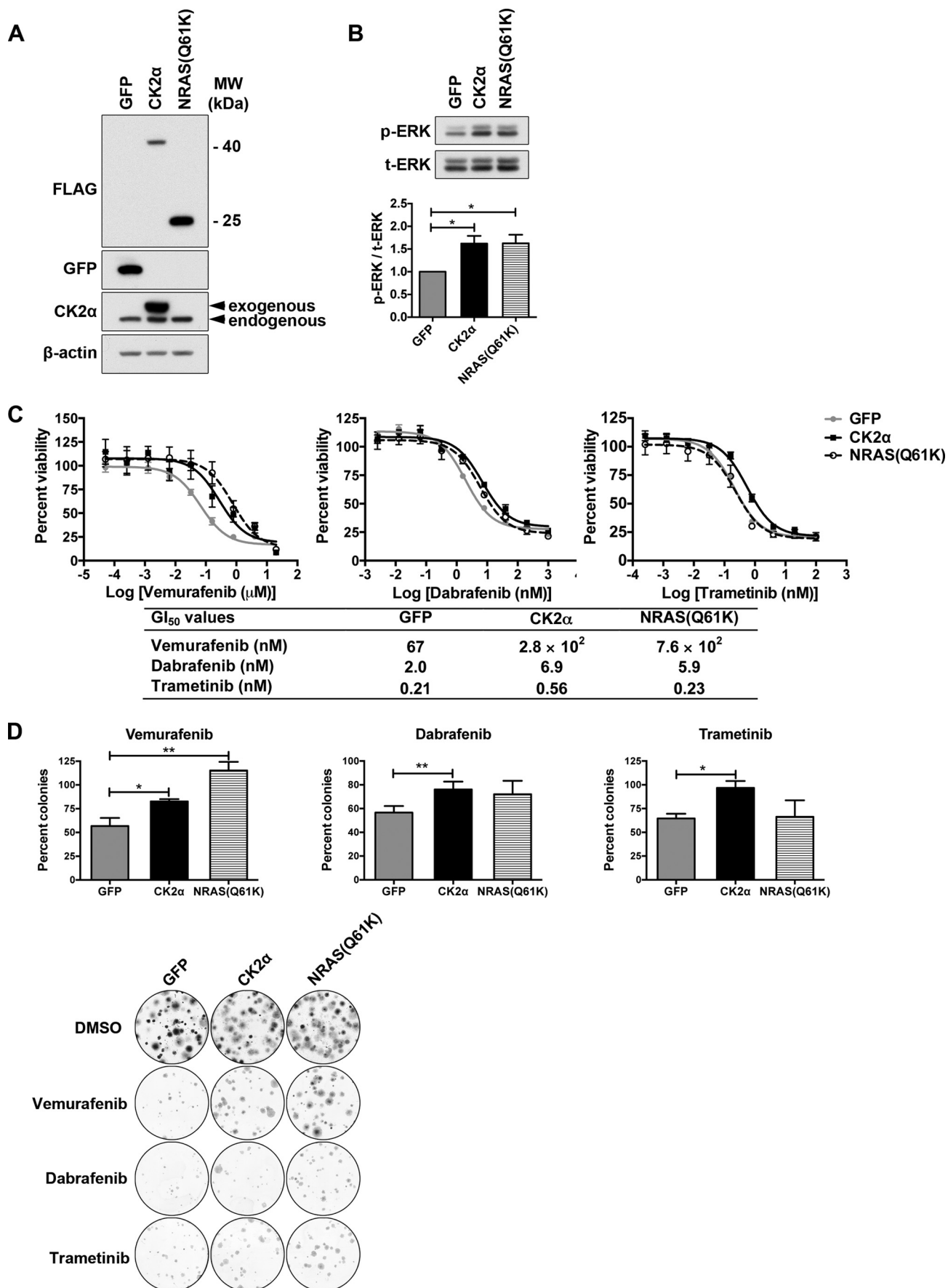


**FIGURE 1. CK2 $\alpha$  protein expression is elevated in melanoma cell lines compared with melanocytes.** A, cell lysates from a panel consisting of three types of neonatal epidermal melanocytes from lightly pigmented (LP), moderately pigmented (MP), and darkly pigmented (DP) donors and five BRAF and one NRAS mutant melanoma cell lines were probed for CK2 $\alpha$  protein by Western blotting with anti-CK2 $\alpha$  antibody.  $\alpha$ -Tubulin was used as a loading control. Normalized expression of CK2 $\alpha$ / $\alpha$ -tubulin is shown. Phosphorylation of ERK was measured and normalized to total ERK (tERK). B, statistical analysis of normalized CK2 $\alpha$  expression (\*,  $p = 0.013$ ). Error bars represent S.E.

ERK (pERK) levels were quite variable among the lines and were not predicted either by malignancy state ( $p = 0.5384$ ) or by CK2 $\alpha$  levels ( $R^2 = 0.06130$ ) (Fig. 1A), consistent with our previous findings (see Fig. 1 in Shields *et al.* (23)).

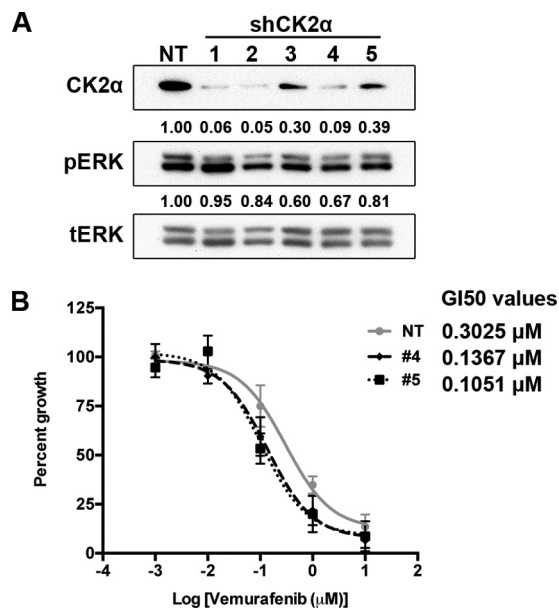
**CK2 $\alpha$  Promotes Resistance to Inhibitors of BRAF and MEK in BRAF Mutant Melanoma Cells**—We recently used a whole-kinome siRNA screen to search for mechanisms of resistance to ERK inhibition in pancreatic ductal adenocarcinoma cell, and found that CK2 $\alpha$  was one of the hits identified (22). To test whether CK2 $\alpha$  promotes resistance to approved single agent therapies targeting the RAF-MEK-ERK pathway in melanoma cells with hyperactivation of this pathway, we first stably expressed FLAG-tagged wild-type CK2 $\alpha$  in A375 melanoma cells (Fig. 2A). These cells possess a homozygous BRAF(V600E) mutation and are sensitive to both BRAFi and MEKi. We then assessed sensitivity to growth inhibition by multiple inhibitors of the pathway, including mutant BRAF-selective inhibitors vemurafenib and dabrafenib and MEK1/2-selective inhibitor trametinib. Oncogenically activated (mutant) NRAS has been identified in patients with BRAF mutant melanomas as one mechanism of resistance to BRAF but not MEK inhibition

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(24). Therefore, we stably expressed FLAG-tagged mutant NRAS(Q61K) (Fig. 2A) as a positive control. Expression of CK2 $\alpha$  or NRAS(Q61K) led to an  $\sim$ 1.62-fold increase in basal ERK phosphorylation (Fig. 2B). But, as expected, mutant NRAS promoted resistance to both BRAF inhibitors vemurafenib and dabrafenib as evidenced by increased values for 50% growth inhibition ( $GI_{50}$ ) of 11.4- and 2.97-fold, respectively, compared with the GFP negative control (Fig. 2D). In contrast, mutant NRAS did not increase the  $GI_{50}$  (1.1-fold change) for the MEKi trametinib, consistent with findings that BRAF mutant melanomas with secondary NRAS mutations still remain sensitive to MEK inhibition (24). Notably, expression of CK2 $\alpha$  increased the  $GI_{50}$  for vemurafenib, dabrafenib, and trametinib by 4.13-, 3.52-, and 2.63-fold, respectively, indicating reduced sensitivity to all three inhibitors (Fig. 2C). A summary of all  $GI_{50}$  values are provided in a table in Fig. 2C, lower panel. Expression of CK2 $\alpha$  also produced resistance to vemurafenib in another BRAF mutant melanoma cell line, SK-MEL-28 (data not shown). To further evaluate the effect of CK2 $\alpha$  expression on responses to the above mentioned inhibitors, we performed clonogenic cell survival assays, which assess the ability of individual cells to form colonies, a property that differs between tumor cells and their normal counterparts and that is distinct from the proliferation of an overall cell population as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Consistent with the increased  $GI_{50}$  values, CK2 $\alpha$  enhanced the clonogenic survival of A375 cells as measured by percent colony numbers in the presence of each inhibitor compared with DMSO vehicle control (Fig. 2D). In contrast to CK2 $\alpha$ , NRAS(Q61K) significantly enhanced clonogenic survival only in response to vemurafenib. The modest enhancement in clonogenic survival in response to dabrafenib did not reach statistical significance. Together, these results indicate that CK2 $\alpha$  overexpression but not NRAS mutation is sufficient to induce resistance to both BRAF and MEK inhibition, which implies a CK2 $\alpha$ -mediated resistance mechanism distinct from that mediated by mutant NRAS.

**CK2 $\alpha$  Depletion Sensitizes Melanoma Cells to BRAF Inhibition**—Given that CK2 $\alpha$  overexpression was sufficient to drive resistance, we asked whether, conversely, depletion of CK2 $\alpha$  would enhance sensitivity to pathway inhibition. We used a set of five shRNAs (1–5) to knock down CK2 $\alpha$  in A375 cells. Consistent with the mild increase in basal ERK phosphorylation when CK2 $\alpha$  was overexpressed (Fig. 2B), we found that ERK phosphorylation was mildly impaired upon CK2 $\alpha$  knockdown (data not shown). Complete knockdown of CK2 $\alpha$  was incompatible with cell survival (data not shown). Therefore, to obtain cells for subsequent experimentation, we utilized shRNAs 4 and 5, which yielded  $\sim$ 60% knockdown and suffi-



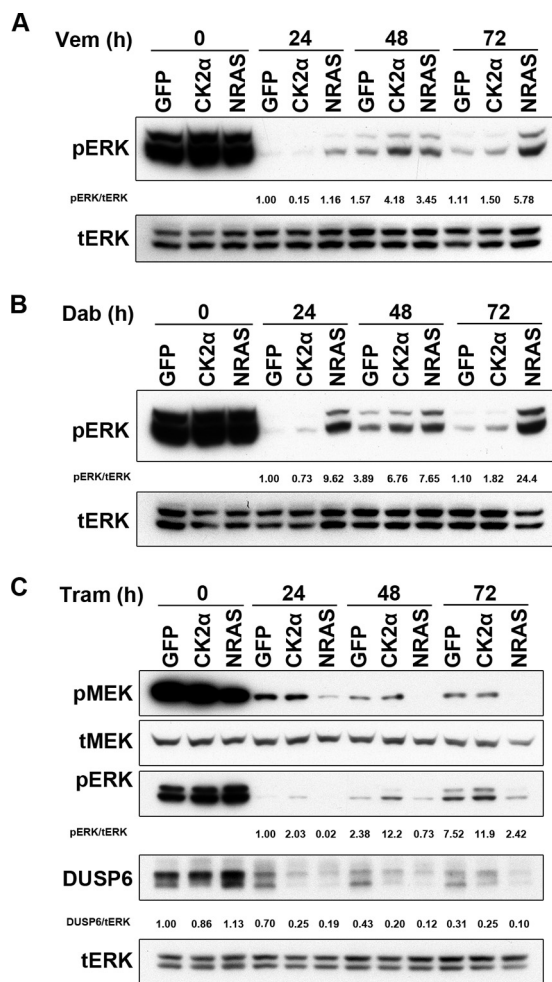
**FIGURE 3. Suppression of endogenous CK2 $\alpha$  increases sensitivity to the BRAF inhibitor vemurafenib.** A, endogenous CK2 $\alpha$  was suppressed in A375 cells by using five different shRNA sequences, and the degree of knockdown was assessed by Western blotting using anti-CK2 antibody.  $\beta$ -Actin served as a loading control. The percentage of knockdown achieved by each shRNA directed against CK2 $\alpha$ , normalized to the non-targeting (NT) shRNA, is indicated below each lane. tERK, total ERK. B, knockdown of  $\sim$ 60% of endogenous CK2 $\alpha$  (A) is sufficient to decrease the  $GI_{50}$  for vemurafenib.  $GI_{50}$  curves for A375 cells infected with either non-targeting shRNA or shRNA 4 (black dashed line) or shRNA 5 (black dotted line) are shown. Results are presented as means  $\pm$  S.E. ( $n = 3$ ). Error bars represent S.E.

cient viability (Fig. 3A). Even this incomplete depletion of CK2 $\alpha$  expression resulted in decreased  $GI_{50}$  for vemurafenib (55 and 65% decrease for shRNAs 4 and 5, respectively; Fig. 3B). This result indicates that CK2 $\alpha$  is necessary for resistance to BRAF inhibition.

**CK2 $\alpha$  Sustains ERK Phosphorylation under Conditions of RAF-MEK-ERK Pathway Inhibition**—Previous studies have identified multiple mechanisms of resistance to BRAF inhibition, the majority of which are characterized by ERK reactivation (25). Given that CK2 $\alpha$  and mutant NRAS both promote BRAFi resistance, we hypothesized that they are both capable of facilitating ERK reactivation following inhibition of BRAF and/or MEK. As anticipated, mutant NRAS induced strong ERK reactivation upon inhibition of BRAF with either vemurafenib or dabrafenib (Fig. 4, A and B). CK2 $\alpha$  also facilitated ERK rebound although not as strongly as NRAS(Q61K). Consistent with their trametinib resistance profiles, NRAS(Q61K) failed to reactivate ERK in the presence of trametinib, whereas CK2 $\alpha$  did sustain ERK phosphorylation (Fig. 4C). The maintenance of ERK phosphorylation by CK2 $\alpha$  suggested either main-

**FIGURE 2. Ectopic CK2 $\alpha$  promotes resistance to inhibitors of BRAF and MEK.** A, A375 cells were stably infected with lentiviral vectors to ectopically express GFP negative control or FLAG-tagged CK2 $\alpha$  or NRAS(Q61K), and cell lysates were subjected to Western blotting using anti-FLAG, anti-GFP, and anti-CK2 $\alpha$  antibodies.  $\beta$ -Actin served as a loading control. B, expression of CK2 $\alpha$  or NRAS(Q61K) increases the basal level of ERK phosphorylation in A375 cells by 1.62-fold as determined by Western blotting using anti-phospho-ERK(Thr-202/Tyr-204) antibody and normalized to total ERK (t-ERK) ( $n = 5$ ). C, CK2 $\alpha$  increases  $GI_{50}$  for BRAFi vemurafenib, BRAFi dabrafenib, and MEKi trametinib. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed after 72 h of treatment with nine different doses of inhibitors, and dose-response curves were generated by GraphPad Prism v5.0c. Results are presented as means  $\pm$  S.E. ( $n = 6$ ). A summary of all  $GI_{50}$  values is shown in the table below. D, CK2 $\alpha$  enhances clonogenic survival of inhibitor-treated A375 cells. Cells as in the previous panels were grown for 2 weeks on plastic as single colonies in the presence of vemurafenib (1  $\mu$ M), dabrafenib (100 nM), trametinib (1 nM), or DMSO vehicle control. Shown are the percentage of colonies formed in the presence of each inhibitor relative to the vehicle control. Results are presented as means  $\pm$  S.E. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$  ( $n = 3$ ). Crystal violet-stained images of colonies are shown in the lower panel. Error bars represent S.E.

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**FIGURE 4. Overexpressed CK2 $\alpha$  accelerates ERK rebound or sustains ERK phosphorylation in response to RAF-MEK-ERK pathway inhibition.** pERK was evaluated by Western blotting analysis of lysates from A375 cells ectopically expressing GFP, CK2 $\alpha$ , or NRAS(Q61K) treated for 24, 48, or 72h with vemurafenib (*Vem*) (BRAFi; 1  $\mu$ M) (A), dabrafenib (*Dab*) (BRAFi; 100 nM) (B), or trametinib (*Tram*) (MEKi; 1 nM) (C). Total ERK1/2 (tERK) served as a loading control. MEKi (trametinib)-treated cell lysates were additionally immunoblotted for phospho-MEK1/2 (pMEK) and for the ERK-specific phosphatase DUSP6. Densitometry values for pERK/total ERK are shown for each panel.

tained upstream activation or suppressed deactivation mechanisms. MEK is the only known direct activator of ERK (26), and phosphorylation of MEK at Ser-217 and Ser-221 is indicative of MEK activation. DUSP6/mitogen-activated kinase kinase phosphatase 3 is a key ERK-specific phosphatase that reverses MEK phosphorylation at the TEY motif of ERK (27). Therefore, we assessed the status of both MEK activation and DUSP6 expression by Western blotting. We found that MEK phosphorylation did not change in parallel with ERK phosphorylation, indicating that it is not the basis for maintained ERK activity (Fig. 4C). Intriguingly, DUSP6 expression was also strongly reduced in CK2 $\alpha$ -overexpressing cells even without inhibitor treatment (Fig. 4C). Based on this finding, we initially hypothesized that down-regulation of DUSP6 contributed to the sustained ERK phosphorylation. However, we first needed to confirm whether CK2 $\alpha$  truly regulates DUSP6 expression.

**CK2 $\alpha$  Regulates DUSP6 Protein Levels in a Kinase-dependent Manner**—CK2 $\alpha$  has been reported to directly phosphorylate DUSP6 at multiple sites *in vitro*, the consequences of which are

largely unknown (28). To control for CK2 $\alpha$  kinase activity, we generated a kinase-inactive mutant of CK2 $\alpha$  (29) and measured DUSP6 protein levels upon ectopic expression of either kinase-inactive (K68M) or wild-type (WT) CK2 $\alpha$ . As expected, CK2 $\alpha$ (WT) was constitutively active, and cells overexpressing this form of CK2 $\alpha$  exhibited elevated basal phosphorylation of EEF1D (Fig. 5A), a validated marker of CK2 activity (30). In contrast, cells expressing kinase-inactive CK2 $\alpha$ (K68M) exhibited mildly reduced levels of EEF1D phosphorylation (Fig. 5A), suggesting a weak dominant-negative effect. Interestingly, CK2 $\alpha$ (WT) drastically reduced DUSP6 abundance, whereas K68M did not have an effect (Fig. 5B), indicating that the decrease in DUSP6 protein is likely due to CK2 $\alpha$ -mediated phosphorylation. Conversely, shRNA-mediated knockdown of endogenous CK2 $\alpha$  enhanced DUSP6 protein levels (Fig. 5C). To determine whether the reduction in DUSP6 was the result of accelerated degradation or suppressed transcription, we first used MG132 to block proteasome-mediated protein degradation. Six hours after MG132 treatment, DUSP6 abundance was fully rescued (Fig. 5D). We also examined *DUSP6* mRNA levels by quantitative PCR and found that they did not change upon CK2 $\alpha$  expression (Fig. 5E). These results indicate that CK2 $\alpha$  kinase activity regulates DUSP6 abundance by facilitating its proteasomal degradation.

**CK2 $\alpha$ -mediated Maintenance of ERK Phosphorylation and Pathway Inhibitor Resistance Does Not Require Its Kinase Function**—Given that CK2 $\alpha$  kinase activity was essential to the decrease in DUSP6 expression (Fig. 5B), we hypothesized that kinase-inactive CK2 $\alpha$  would not be able to maintain ERK phosphorylation when the pathway was inhibited. Unexpectedly, in the presence of both BRAFis, ERK phosphorylation was sustained to a similar degree in the presence of either CK2 $\alpha$ (K68M) or CK2 $\alpha$ (WT) (Fig. 6A), suggesting a kinase-independent contribution of CK2 $\alpha$  to pERK. However, in the presence of MEKi, CK2 $\alpha$ (K68M) exhibited a lower level of pERK compared with CK2 $\alpha$ (WT). This potentially indicates a differential contribution of the kinase activity of CK2 $\alpha$  to maintaining pERK, depending on which node of the pathway is inhibited (Fig. 6A). We therefore anticipated that kinase-inactive CK2 $\alpha$  would also promote resistance in a similar fashion. Consistent with this, we found that CK2 $\alpha$ (K68M) promoted resistance to vemurafenib, dabrafenib, and trametinib to the same extent as CK2 $\alpha$  wild type as indicated by their respective GI<sub>50</sub> values (Fig. 6B), indicating that CK2 $\alpha$ -mediated BRAFi and MEKi resistance does not depend on its catalytic kinase activity. Instead, our findings indicate that the ability of CK2 $\alpha$  to maintain ERK phosphorylation when the pathway is inhibited and to promote resistance to BRAF and MEK inhibitors is more likely due to a protein binding or scaffolding function of CK2 $\alpha$ . Clearly, CK2 $\alpha$ (K68M), although catalytically inactive and incapable of degrading DUSP6, maintained ERK phosphorylation and inhibitor resistance. Therefore, DUSP6 regulation by CK2 $\alpha$  does not contribute to CK2 $\alpha$ -mediated resistance and maintenance of pERK or cell viability upon inhibitor treatment.

**CK2 $\alpha$ (WT) and CK2 $\alpha$ (K68M) Bind Equally Well to the RAF-MEK-ERK Scaffold Protein KSR1**—Our previous work (31) uncovered an essential role of CK2 $\alpha$  in maximally facilitating

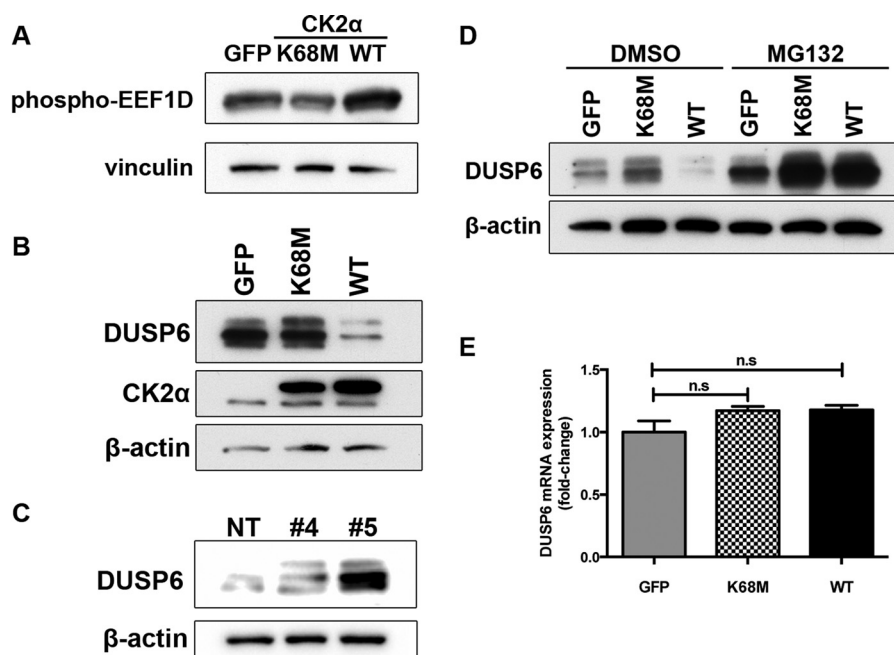


FIGURE 5. CK2 $\alpha$  decreases protein stability of the ERK phosphatase DUSP6 in a kinase-dependent manner. *A*, phosphorylation of the CK2 $\alpha$  substrate EEF1D upon expression of WT or kinase-inactive (K68M) CK2 $\alpha$  was detected by Western blotting with a phospho-EEF1D antibody. Levels of endogenous DUSP6 protein were determined by Western blotting of lysates from A375 cells ectopically expressing CK2 $\alpha$ (WT) or CK2 $\alpha$ (K68M) (*B*) or from A375 cells depleted of endogenous CK2 $\alpha$  by two different shRNAs (same lysates as shown in Fig. 3A) (*C*). *D*, to determine whether CK2 $\alpha$  regulates DUSP6 protein stability, the same cells as in *B* were immunoblotted for DUSP6 protein after treatment for 6 h with either the proteasome inhibitor MG132 (10  $\mu$ M) or DMSO vehicle control. *E*, to determine whether CK2 $\alpha$  also regulates DUSP6 at the transcriptional level, quantitative RT-PCR analysis of DUSP6 mRNA levels was done on cells expressing CK2 $\alpha$ (WT) or kinase-inactive CK2 $\alpha$ (K68M). Results are presented as means  $\pm$  S.E. ( $n = 3$ ). Error bars represent S.E. n.s., not significant.

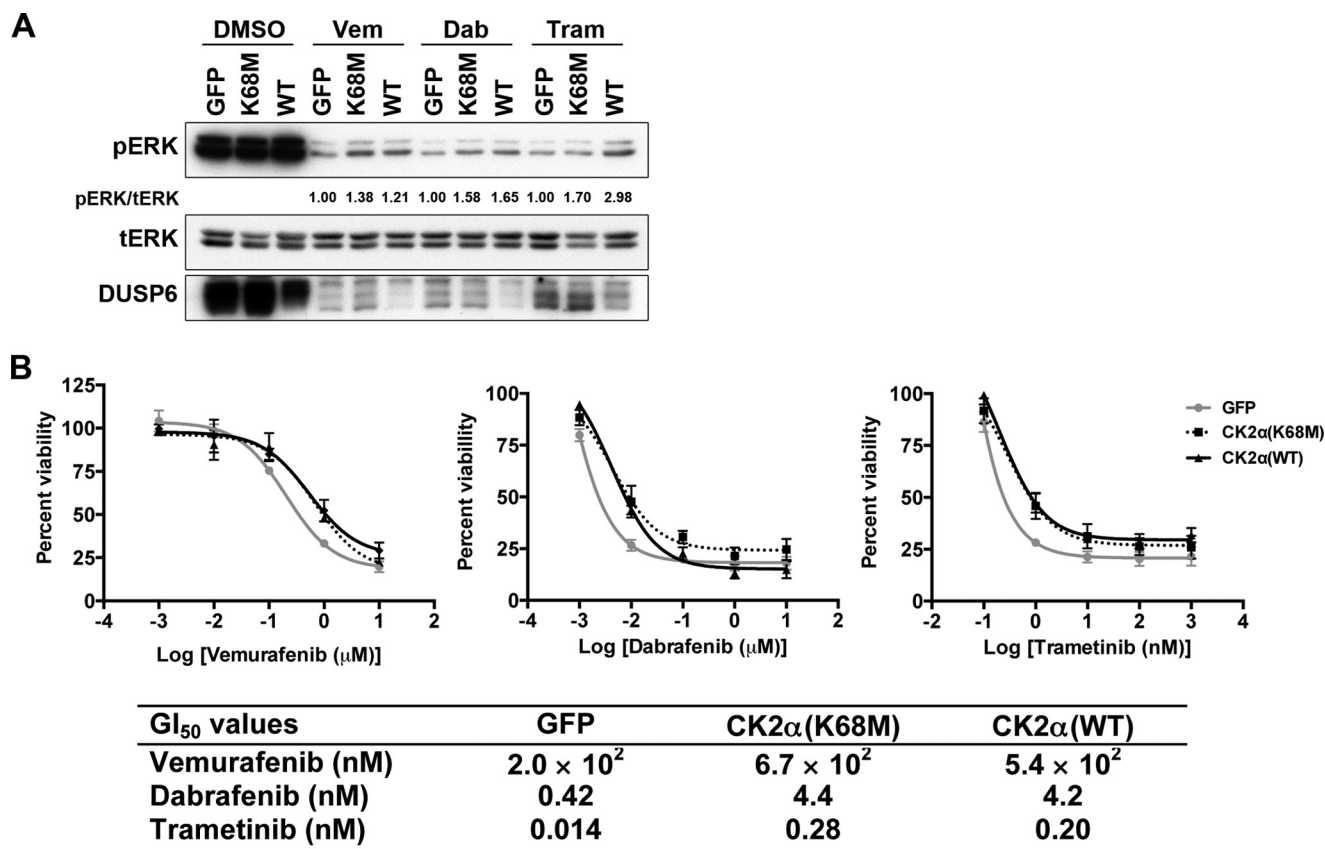
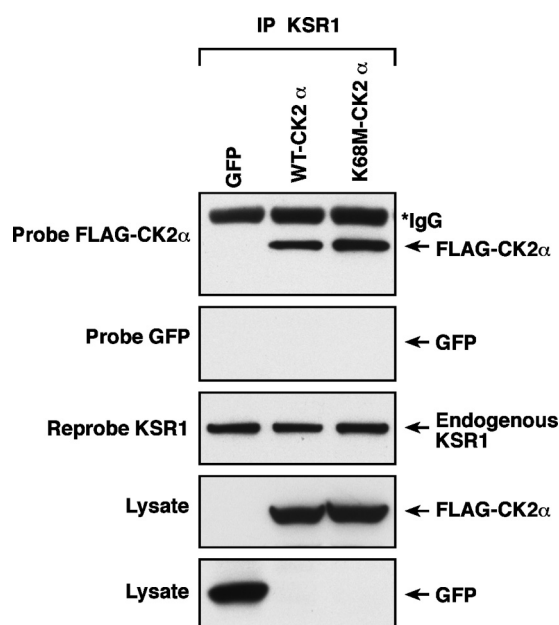


FIGURE 6. CK2 $\alpha$ -mediated maintenance of ERK phosphorylation upon pathway inhibition and resistance to BRAFi/MEKi are both kinase-independent. *A*, A375 cells ectopically expressing GFP, CK2 $\alpha$ (K68M), or CK2 $\alpha$ (WT) were treated with BRAFi and MEKi as in Fig. 4, then lysed, and immunoblotted for pERK and total DUSP6. Total ERK (tERK) served as loading control. *B*, cells were treated as in *A*, and GI<sub>50</sub> curves were generated after 72 h. A summary of GI<sub>50</sub> values is shown in a table below. Results are presented as means  $\pm$  S.E. ( $n = 4$ ). Error bars represent S.E. Vem, vemurafenib; Dab, dabrafenib; Tram, trametinib.

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**FIGURE 7. Both wild-type and kinase-inactive CK2 $\alpha$  interact with the RAF-MEK-ERK scaffold protein KSR1.** Endogenous KSR1 was immunoprecipitated (IP) from A375 cells expressing the GFP control or FLAG-tagged CK2 $\alpha$ (WT) or kinase-inactive CK2 $\alpha$ (K68M). Whole cell lysates and immunoprecipitates were then blotted for the presence of FLAG-CK2 $\alpha$  and reprobed for KSR1 to ensure that equal amounts of KSR1 were immunoprecipitated. \*IgG bands are from immunoprecipitation step. GFP served as a negative control to rule out nonspecific co-immunoprecipitation of the ectopic CK2 $\alpha$  proteins ( $n = 2$ ).

RAF-MEK-ERK pathway activation through its direct binding to KSR1 within the KSR1 scaffolding complex that also includes RAF, MEK, and ERK. CK2 $\alpha$  association with KSR1 enhances RAF phosphorylation of MEK. Given our finding here that kinase-inactive CK2 $\alpha$  maintains ERK phosphorylation and resistance to pathway inhibitors to the same extent as its wild-type counterpart, we speculated that it too retains binding to KSR1. Accordingly, when we immunoprecipitated endogenous KSR1 from A375 cells, we detected considerable levels of ectopically expressed CK2 $\alpha$ (WT) and CK2 $\alpha$ (K68M) but not the GFP control (Fig. 7). This result supports our hypothesis that CK2 $\alpha$  binding to KSR1 is kinase-independent, offering a potential mechanism by which both CK2 $\alpha$ (WT) and CK2 $\alpha$ (K68M) maintain ERK phosphorylation and resistance to BRAF and MEK inhibitors. Specifically, our findings are consistent with a model whereby CK2 $\alpha$  binding enhances the efficiency of KSR1 scaffolding to facilitate ERK activation. Testing of this model would require identification of the KSR1 binding site on CK2 $\alpha$ , which is currently unknown, to enable assessment of whether a KSR1 binding-deficient mutant of CK2 $\alpha$  is now impaired in conferring resistance to BRAFi and/or MEKi.

**ERK Inhibition Avoids CK2 $\alpha$ -mediated Resistance to RAF-MEK-ERK Pathway Blockade**—Our model predicts that CK2 $\alpha$  should not be able to cause resistance in melanoma cells to an inhibitor downstream of RAF and MEK that acts directly at the level of ERK. To test this model, we evaluated whether CK2 $\alpha$ (WT) or CK2 $\alpha$ (K68M) could cause resistance to the ERK inhibitor SCH772984 (1). Consistent with our model, overexpression of CK2 $\alpha$ (WT), CK2 $\alpha$ (K68M), or NRAS(Q61K) did not confer resistance to the ERKi as evidenced by the absence of

either increased GI<sub>50</sub> (Fig. 8A) or enhanced clonogenic survival (Fig. 8B). For evidence that the inhibitor correctly hit its ERK target, we examined the phosphorylation status of the ERK substrate p90 RSK (Fig. 8C). We (22) and others (1) have shown recently that decreased phospho-RSK (pRSK) is a more reliable marker of decreased flux through ERK than is ERK phosphorylation itself, not least because ERK phosphorylation rebounds quickly, whereas pRSK does not. Examination of pRSK in ERKi-treated cells at the same time point (72 h) as the GI<sub>50</sub> analysis revealed that neither overexpressed CK2 $\alpha$  nor mutant NRAS was able to restore ERK pathway activation in the presence of ERKi. Thus, although CK2 $\alpha$  induces resistance to BRAFi and MEKi in a kinase-independent manner, even kinase-intact CK2 $\alpha$  does not induce resistance to ERKi, which is an effective means of impairing the RAF-MEK-ERK pathway in these BRAF mutant melanoma cells. This finding is similar to other mechanisms that cause resistance to BRAFi or MEKi in BRAF mutant melanomas where ERKi sensitivity is retained (1).

## Discussion

Although targeted therapies in melanoma have substantially improved patient outcomes immediately following treatment in a subset of patients, even responsive patients are confronted with the inevitable development of resistance months later (18–21). Understanding the underlying mechanisms of innate or acquired resistance is key to developing new combination therapies to overcome tumor unresponsiveness or recurrence. In the present study, we demonstrate that abnormally elevated expression of CK2 $\alpha$  (*CSNK2A1*) is sufficient to cause resistance to each of three small molecule kinase inhibitors of the RAF-MEK-ERK pathway approved for treatment of melanoma: vemurafenib, dabrafenib, and trametinib. Furthermore, we show that this resistance correlates with the rebound/maintenance of ERK activity following pathway inhibition.

Protein kinase CK2 has been previously shown to affect RAF-MEK-ERK pathway signaling by various means whose complexity has yet to be fully elucidated. Aside from fine-tuning the signaling amplitude of the RAF-MEK-ERK pathway, CK2 has been reported to regulate ERK nuclear translocation and translocation of nuclear targets of ERK, which can also affect the signaling efficiency of the pathway (32). Mechanistically, CK2 kinase activity is needed to phosphorylate Ser-244 and Ser-246 in the nuclear translocation signal of ERK, allowing ERK binding to Importin7. Although nuclear ERK activation has not been directly linked to melanoma progression, lack of cytoplasmic ERK was associated with poor prognosis in primary cutaneous melanomas (33). Conversely, another study showed that the proliferation of A375 melanoma cells *in vitro* and *in vivo* was highly sensitive to an inhibitor of ERK dimerization (34) that is thought to act by decreasing ERK interactions specifically with its cytoplasmic but not nuclear substrates (35). The role of CK2 $\alpha$  kinase in this process has not been investigated.

The distinct resistance profiles of CK2 $\alpha$  and NRAS(Q61K) imply different mechanisms of promoting resistance. Specifically, it is known that secondary NRAS mutations that increase flux through the RAS-MEK-ERK pathway via CRAF activation can overcome inhibitor potency (24). Such a route of reactiva-

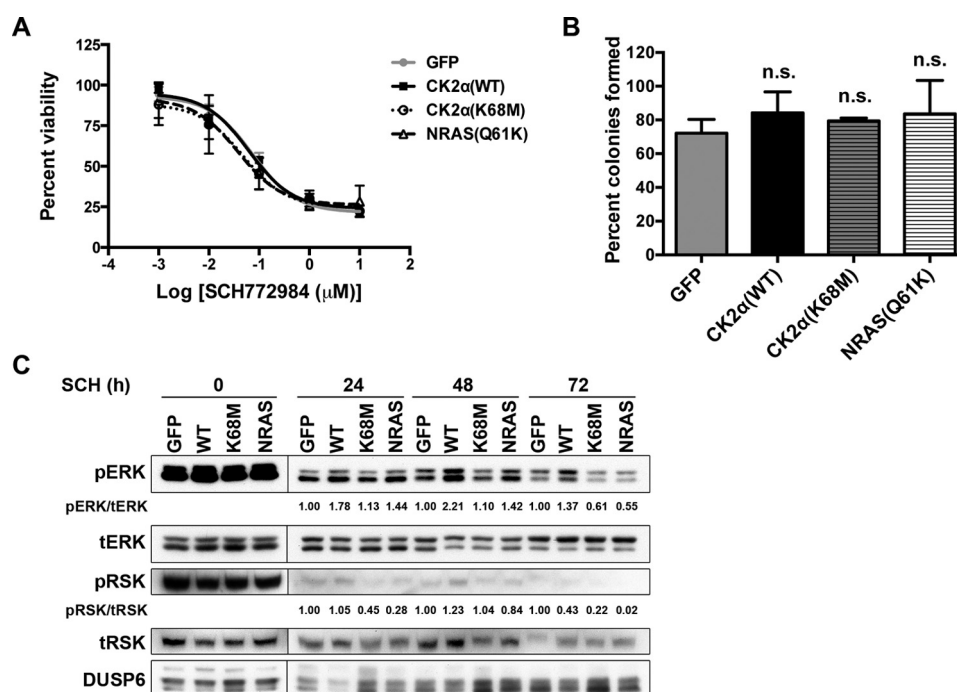


FIGURE 8. ERK inhibitor SCH772984 is insensitive to overexpression of either CK2 $\alpha$ (WT) or CK2 $\alpha$ (K68M). A, the GI<sub>50</sub> for ERKi SCH772984 is unchanged by overexpression of CK2 $\alpha$  or by mutant NRAS. GI<sub>50</sub> curves were generated after 72 h of ERKi treatment. Results are presented as means  $\pm$  S.E. ( $n = 3$ ). B, clonogenic survival in the presence of ERKi is not enhanced by overexpressed CK2 $\alpha$  or mutant NRAS. Shown are the percentages of colonies formed by A375 cells expressing GFP, CK2 $\alpha$ , or NRAS(Q61K) and treated with ERKi (100 nM) normalized to DMSO vehicle control. Results are presented as means  $\pm$  S.E. ( $n = 3$ ). n.s., non-significant compared with GFP. C, ERKi treatment shuts down ERK pathway signaling as indicated by loss of phosphorylated ERK substrate pRSK. A375 cells as in A and B were treated for 24, 48, or 72 h with 100 nM SCH772984. Error bars represent S.E. tERK, total ERK; tRSK, total RSK.

tion could easily be blocked by MEK inhibition. Consistent with this idea, NRAS(Q61K) did not confer resistance to MEK inhibitor trametinib. However, the fact that CK2 $\alpha$ -mediated resistance is MEK inhibitor-inert suggests two possible mechanisms. The first entails some unknown bypass that leads to sustained ERK phosphorylation. This is somewhat unlikely because MEK is still the only known direct activator of ERK. The second involves steric hindrance provided by CK2 $\alpha$  that prevents an MEK inhibitor from binding to its target effectively. Such a mechanism can be provided by a scaffolding function of CK2 $\alpha$  as discussed below.

Intriguingly, we found that wild-type CK2 $\alpha$  drastically reduced expression of the DUSP6, whereas CK2 $\alpha$  silencing elevated endogenous DUSP6 protein levels. DUSP6 is a key ERK-specific phosphatase that negatively regulates the RAF-MEK-ERK pathway (27). Indeed, DUSP6 has been previously reported to interact with and be phosphorylated by CK2 $\alpha$  (28). We further show here that CK2 $\alpha$ -facilitated proteasomal degradation accounts for the decreased abundance of DUSP6 protein. In light of our results, it would be interesting to know whether DUSP6 expression could serve as a biomarker of CK2 inhibition.

Much to our initial surprise, we determined, using a kinase-inactive mutant of CK2 $\alpha$ , that CK2 $\alpha$  kinase activity is not required for either CK2 $\alpha$ -mediated inhibitor resistance or sustained ERK phosphorylation in the context of these BRAF mutant melanoma cells. This was unexpected because CK2 is well known as a constitutively active kinase that has hundreds of endogenous substrates, and its kinase activity has largely been assumed to be responsible for its pleiotropic effects (2–4).

However, the whole-kinome screen by which we identified CK2 $\alpha$  as a potential resistance mechanism capable of inducing at least a 5-fold increase in resistance to ERK inhibitor was not performed by inhibition of the catalytic activity of the CK2 $\alpha$  kinase but rather by siRNA-mediated depletion of expression of the entire protein (22). Therefore, this screen would capture effects induced by loss of protein binding or scaffolding functions as well as by loss of catalytic activities of the depleted kinases.

Our data suggest that the above resistance phenotypes are the result of CK2 $\alpha$ -mediated protein-protein interactions rather than CK2 $\alpha$  kinase activity. Consistent with this notion, we reported previously that all subunits of CK2 bind to the RAF-MEK-ERK pathway scaffolding protein KSR1 (31) and that binding of the CK2 $\alpha$  subunit in particular to KSR1 is critical for maximal activation of the pathway (31). However, we had not tested whether the kinase activity of CK2 $\alpha$  was required. Because we have now found that kinase deficiency does not impair CK2 $\alpha$  binding to KSR1, we speculate that the catalytic activity-independent binding of CK2 $\alpha$  to KSR1 helps to promote formation of the KSR1 scaffold complex and to maintain the integrity and function of the RAF-MEK-ERK pathway, enabling the sustained ERK phosphorylation observed in the presence of overexpressed CK2 $\alpha$  even when the pathway is inhibited at the level of RAF or MEK. This hypothesis could be tested in the future once the region of CK2 $\alpha$  that mediates KSR1 binding has been identified as that would allow interrogation of the ability of KSR1 binding-deficient mutants of CK2 $\alpha$  to confer resistance to pathway inhibitors. Of note, overexpressed CK2 $\alpha$  exists pretreatment. It is also possible that



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the trametinib binding site on efficiently scaffolded MEK may be sterically hindered compared with that of free MEK, thereby reducing the effectiveness of MEK inhibitors. It would also be of great interest to determine whether the onset of BRAFi/MEKi resistance in melanoma patients is associated with increased levels of CK2 $\alpha$ .

The importance of CK2 $\alpha$  protein-protein interactions *versus* catalytic kinase activity may differ greatly depending on context. A recent study examined the effects of a CK2 $\alpha$ -selective kinase inhibitor, CX-4945, on the viability of BRAF mutant thyroid cancer cell lines and found synergism of CX-4945 with both the BRAFi vemurafenib and the MEKi selumetinib (36), suggesting that the kinase activity of CK2 $\alpha$  was important for the response to BRAFi/MEKi in this tumor type. Surprisingly, when they compared the combination of vemurafenib with CX-4945 or with siRNA directed against CK2 $\alpha$  in a patient-derived BRAF mutant melanoma cell line, they found an additive effect of each on cell death (36). The equivalent effects on vemurafenib responses of kinase-intact and kinase-inactive CK2 $\alpha$  that we observed argue that the kinase activity is not important in the vemurafenib response of BRAF mutant melanoma, but it is certainly possible that other genetic differences may also affect the relative roles of catalytic activity *versus* protein-protein interactions. Our study differs from work by Borgo *et al.* (16), who found a kinase-dependent role of CK2 in imatinib-resistant chronic myeloid leukemia. These differences may reflect the divergent genetic and epigenetic contexts of BCR-ABL mutant chronic myeloid leukemia *versus* BRAF mutant melanomas. For example, in our model system, BRAF(V600E) is the driver mutation, and CK2 $\alpha$  is an integral part of the KSR1 scaffolding complex that maximizes signaling efficiency through the RAF-MEK-ERK pathway. In contrast, in chronic myeloid leukemia, BCR-ABL is the driver mutation, and CK2 $\alpha$  activity has been shown to be directly regulated by BCR-ABL (37). Therefore, CK2 $\alpha$  can play distinct roles in different tumor contexts. It is also interesting that, although our original siRNA screen identified CK2 $\alpha$  as a mediator of resistance to ERKi in KRAS mutant pancreatic ductal adenocarcinoma cells (22), CK2 $\alpha$  did not mediate resistance to ERKi in BRAF mutant melanoma cells. We believe that this is also due to tumor heterogeneity. Indeed, when we performed our siRNA screen in two different pancreatic ductal adenocarcinoma cell lines, we found that there was little overlap of the hits; CK2 $\alpha$  was identified as a hit in CFPAC-1 but not in SW1990 cells. We were unable to determine the specific basis for such disparate results, but they certainly highlight the tremendous heterogeneity among tumor cell lines, even among those derived from the same cancer type (*e.g.* pancreatic ductal adenocarcinoma), let alone different cancer types (*e.g.* pancreatic ductal adenocarcinoma *versus* melanoma). Clearly, much remains to be elucidated about the role of CK2 $\alpha$  in responses to inhibitors of the RAF-MEK-ERK pathway, a role that is likely to be as complex as its hundreds of substrates and numerous biological activities portend.

In summary, our results identify a role for CK2 $\alpha$  in promoting resistance to BRAF and MEK but not ERK inhibitors in BRAF mutant melanoma. We also demonstrate, for the first time to our knowledge, a kinase-independent function of CK2 $\alpha$

**TABLE 1**  
Target sequences

TRC, The RNAi Consortium.

shRNA	TRC clone ID	Target sequence
1	TRCN0000000606	5'-GCTGCATTTAGGTGGAGACTT-3'
2	TRCN0000000607	5'-CGTAAACAACACAGACTTCAA-3'
3	TRCN0000000608	5'-CAAGAATATAATGTCCGAGTT-3'
4	TRCN0000000609	5'-AGAATTTGAGAGGAGGTCCTCA-3'
5	TRCN0000000610	5'-CCAAGAATATAATGTCCGAGT-3'

in modulating cellular signaling. These findings represent a novel mode of innate resistance to RAF-MEK-targeted therapy in BRAF mutant melanoma, which may not be easily addressed by inhibition of the dysregulated CK2 $\alpha$  kinase. Perhaps ongoing efforts to develop KSR inhibitors may achieve success in part via interference with CK2 $\alpha$ .

### Experimental Procedures

**Cell Culture and Reagents**—A375, A2058, Sbc12A, Malme-3, and 293T cell lines were grown in DMEM-H supplemented with 10% FBS (HyClone<sup>TM</sup>, Thermo Scientific) and 1% gentamycin/kanamycin (Tissue Culture Facility, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill). SK-MEL-28 and RPMI-7951 were grown in minimum Eagle's medium  $\alpha$  (Gibco) supplemented with 10% FBS and 1% gentamycin/kanamycin. Primary neonatal human epidermal melanocytes from lightly pigmented, moderately pigmented, and darkly pigmented donors were generously provided by Dr. Guang Hu (National Institute of Environmental Health Sciences, Research Triangle Park) and were grown in Medium 245 supplemented with human melanocyte growth supplement (a kind gift from Dr. Guang Hu). The BRAFi vemurafenib (PLX4032) was a generous gift from Gideon Bollag (Plexikon). The BRAFi dabrafenib (GSK2118436) and MEKi trametinib (GSK1120212) were purchased from Selleckchem. The ERKi SCH772984 was a generous gift from Ahmed Samatar (Merck). MG132 was purchased from Calbiochem (474790).

**Plasmid Constructs and Gateway Cloning**—The CK2 $\alpha$  expression construct, pDONR223-CSNK2A1 (Human ORFeome v5.1), was purchased from University of North Carolina's Tissue Culture Facility. pHAGE-FLAG (N-terminal tag) empty vector was a generous gift from Ben Major, University of North Carolina at Chapel Hill. Both CK2 $\alpha$  and NRAS(Q61K) were cloned into the pHAGE-FLAG vector by Gateway cloning using LR Enzyme Clonase Mix (Invitrogen) according to the manufacturer's protocol. A set of five shRNAs targeting CK2 $\alpha$  (CSNK2A1) in pLKO.1 vector was purchased from the Lentiviral Core Facility at University of North Carolina at Chapel Hill. Target sequences are indicated in Table 1.

**Lentivirus Production and Infection**—To produce lentivirus, 293T cells were transfected with pHAGE vector-based GFP, CK2 $\alpha$ , or NRAS(Q61K) in combination with psPAX2 and pMD2.G at a ratio of 4:3:1. After overnight transfection, the culture medium was changed to DMEM-H supplemented with 20% FBS. Thirty-six hours later, viral supernatants were harvested and filtered through a sterile 0.45- $\mu$ m filter to remove cell debris. Cleared supernatants were aliquoted and frozen at  $-80^{\circ}\text{C}$  until use. Cells were infected with 500  $\mu$ l of virus in 5  $\mu$ g/ml Polybrene (Millipore) overnight. Selection

of transduced cells in puromycin (1 or 2  $\mu\text{g}/\text{ml}$  for A375 or SK-MEL-28 cells, respectively) was complete at 48 h after infection.

**Western Blotting**—Cells were washed twice with ice-cold PBS and lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 1 $\times$  protease inhibitors (BaculoGold<sup>TM</sup> protease inhibitor mixture, BD Biosciences, 51-21426Z) and 1 $\times$  phosphatase inhibitors (Halt<sup>TM</sup> phosphatase inhibitor mixture, Thermo Scientific, 78420). Lysates were depleted of cell debris by centrifugation at maximum speed (4  $^{\circ}\text{C}$ , 10 min), and then proteins were quantified by Bradford assay (DC<sup>TM</sup> Protein Assay, Bio-Rad), normalized, reduced, denatured at 95  $^{\circ}\text{C}$  for 5 min, and resolved by SDS gel electrophoresis. Proteins were transferred to PDVF membranes (Millipore, IPFL00010) and probed with primary antibodies recognizing pERK1/2(Thr-202/Tyr-204) (Cell Signaling Technology, 4370), ERK1/2 (Cell Signaling Technology, 9102), phospho-MEK1/2(Ser-217/221) (Cell Signaling Technology, 9154), pRSK(Thr-259/Ser-263) (Cell Signaling Technology, 9344), DUSP6 (Abcam, ab54940),  $\beta$ -actin (Sigma, A5316), CK2 $\alpha$  (Santa Cruz Biotechnology, sc-373894), FLAG tag (Sigma, F3165; Novus, NBP1-06712SS), or GFP (Roche Applied Science, 11814460001). A rabbit antibody to the phosphorylated CK2 $\alpha$  substrate EE1D was a generous gift from David W. Litchfield (Western University). A rabbit-anti KSR1 antibody (home-made, Morrison laboratory) was used to detect KSR1 following immunoprecipitation. After incubation with the appropriate secondary anti-mouse (GE Healthcare, NA931V) or anti-rabbit (GE Healthcare, NA934V) antibody, proteins were detected by chemiluminescence (Thermo Scientific, 34075). Blots were developed by exposure to x-ray film or by the ChemiDoc<sup>TM</sup> MP Imaging System (Bio-Rad) for quantification. Film was scanned, and densitometry analysis was performed with ImageJ 1.45s.

**Pharmacologic  $GI_{50}$  Assay**—Growth inhibition assays were performed as described previously (24, 38) with minor modifications. Cultured cells were seeded into 96-well plates (2,000 cells/well). Sixteen hours after seeding (baseline), serial dilutions of inhibitors were prepared in DMSO and added to cells, yielding final drug concentrations ranging from 1 nM to 10  $\mu\text{M}$  for vemurafenib, dabrafenib, and SCH772984 and 0.1 nM to 1  $\mu\text{M}$  for trametinib with the final volume of DMSO not exceeding 1%. Cells were incubated for 72 h following addition of drug. To measure cell proliferation, 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, M5655) was added 1:10 into wells and incubated at 37  $^{\circ}\text{C}$  for 4 h. Formazan products were solubilized using acidified isopropanol (0.04 N HCl in isopropanol), and absorbance was measured at 562 nm with a background subtraction at 650 nm. Percent cell growth under each condition was calculated as follows: Cell growth (%) =  $100 \times (T - T_0) / (C - T_0)$  where  $T_0$  is absorbance at baseline,  $T$  is absorbance of drug-treated wells at 72 h, and  $C$  is absorbance of DMSO-treated wells at 72 h. A minimum of three replicates was performed for each cell line and drug combination. Data from growth inhibition assays were modeled using a non-linear regression curve fit with a sigmoidal dose response

(GraphPad Prism, v5.0c). The resulting curves were displayed and  $GI_{50}$  values were also generated using GraphPad Prism.

**Clonogenic Assay**—Clonogenic assays were performed as described previously (39) with slight modifications. Briefly, cells were plated in duplicate wells at 100 cells/well in 6-well plates and allowed to adhere for 3 h at 37  $^{\circ}\text{C}$  after which culture medium was carefully removed and replaced with medium containing either DMSO vehicle control or inhibitor. Two weeks following drug treatment, cells were washed once with PBS and then fixed and stained with crystal violet/paraformaldehyde for 30 min at room temperature. The stain was decanted, and plates were carefully rinsed with distilled water until background staining of the wells was minimized. Finally, plates were air-dried, and colonies were counted manually using a cell counter.

**Site-directed Mutagenesis**—Site-directed mutagenesis of CK2 $\alpha$  was performed using a QuikChange II XL site-directed mutagenesis kit (Agilent). Forward primer 5'-GCCATCAAC-ATCACAATAATGAAAAAGTTGTTGTTATGATTCTC-AAGCCAG-3' and reverse primer 5'-CTGGCTTGAGAAT-CATAACAACAACCTTTTTCATTATTTGTGATGTTGATGGC-3' were used to introduce a catalytic site mutation (K68M) into pDONR223-CSNK2A1 with bold nucleotides showing the site of mutagenesis. Reaction conditions strictly followed the manufacturer's protocol.

**RNA Isolation, Reverse Transcription, and Real Time PCR**—Total RNA was isolated from cells using the Thermo RNA kit (Thermo Scientific), and then 0.5  $\mu\text{g}$  of total RNA was reverse transcribed to generate cDNA using the iScript<sup>TM</sup> cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real time PCR was performed using the SsoFast<sup>TM</sup> EvaGreen<sup>®</sup> Supermix (Bio-Rad) on the Bio-Rad CFX-96 Real-Time PCR System.  $\beta$ -Actin was used for normalization. DUSP6 was amplified using forward primer 5'-CGACTGGAACG-AGAATACGG-3' and reverse primer 5'-TTGGAAC-TACTGAAGCCACCT-3'.

**Co-immunoprecipitation**—Co-immunoprecipitation assays were performed as described previously (31). In brief, two 10-cm dishes of A375 cells were washed twice with cold PBS and lysed in 1% Nonidet P-40 buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 alternative, 0.15 unit/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium vanadate, 20  $\mu\text{M}$  leupeptin) using 600  $\mu\text{l}$  of lysis buffer/10-cm dish. The lysates were clarified by centrifugation, and equivalent amounts of protein lysate were incubated with a mouse anti-human KSR1 antibody (Sigma-Aldrich, WH0008844M1) and protein G-Sepharose beads for 3 h at 4  $^{\circ}\text{C}$ . Immunoprecipitated complexes were collected by centrifugation, washed extensively with 1% Nonidet P-40 buffer, and then examined by immunoblotting analysis.

**Author Contributions**—B. Z., C. J. D., and A. D. C. conceived the study, designed the study, and wrote the paper. B. Z. designed, performed, and analyzed the experiments except Fig. 7, which was conceived by D. K. M. and designed and performed by D. A. R. All authors analyzed the results, contributed to editing, and approved the final version of the manuscript.

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