The Role of eIF4E in Response and Acquired Resistance to Vemurafenib in Melanoma

Yao Zhan[1], Michael S. Dahabieh[1], Arjuna Rajakumar[1], Monica C. Dobocan[2], Marie-Noël M'Boutchou[2], Christophe Goncalves[2], Lucy Shiru L.[1], Filippa Pettersson[2], Ivan Topisirovic[1,2], Léon van Kempen[1,2], Sonia V. del Rincón[2] and Wilson H. Miller Jr.[1,2]

In eukaryotic cells, the rate-limiting component for cap-dependent mRNA translation is the translation initiation factor eIF4E. eIF4E is overexpressed in a variety of human malignancies, but whether it has a role in melanoma remains obscure. We hypothesized that eIF4E promotes melanoma cell proliferation and facilitates the development of acquired resistance to the BRAF inhibitor vemurafenib. We show that eIF4E is overexpressed in a panel of melanoma cell lines, compared with immortalized melanocytes. Knockdown of eIF4E significantly repressed the proliferation of a subset of melanoma cell lines. Moreover, in $BRAF^{V600E}$ melanoma cell lines, vemurafenib inhibits 4E-BP1 phosphorylation, thus promoting its binding to eIF4E. Cap-binding and polysome profiling analysis confirmed that vemurafenib stabilizes the eIF4E–4E-BP1 association and blocks mRNA translation, respectively. Conversely, in cells with acquired resistance to vemurafenib, there is an increased dependence on eIF4E for survival; 4E-BP1 is highly phosphorylated and thus eIF4E-4E-BP1 associations are impeded. Moreover, increasing eIF4E activity by silencing 4E-BP1/2 renders vemurafenib-responsive cells more resistant to BRAF inhibition. In conclusion, these data suggest that therapeutically targeting eIF4E may be a viable means of inhibiting melanoma cell proliferation and overcoming vemurafenib resistance.

Journal of Investigative Dermatology (2015) **135,** 1368–1376; doi:10.1038/jid.2015.11; published online 19 February 2015

INTRODUCTION

Cutaneous melanoma is the most predominant form of skin cancer, with approximately 50% of melanomas carrying activating BRAF mutations (Bastian, 2014). The $BRAF^{V600E}$ mutation leads to a 500-fold activation of BRAF and constitutive activation of the mitogen-activated protein kinase signaling pathway (Davies *et al.*, 2002; Maurer *et al.*, 2011). In 2011, the US Food and Drug Administration approved vemurafenib, a BRAF V600E inhibitor, for clinical use in advanced melanoma (Young *et al.*, 2012). Although patients initially respond well, with pronounced tumor regressions (Chapman *et al.*, 2011) drug resistance almost inevitably develops, with a median response duration of 6 months. Multiple mechanisms involved in vemurafenib resistance have been reported, including acquired EGFR upregulation (Sun *et al.*, 2014), mitogen-activated protein kinase signaling pathway reactivation (Romano *et al.*, 2013; Trunzer *et al.*, 2013), BRAF V600E and COT (MAP3K8) copy number gains (Johannessen *et al.*, 2010; Shi *et al.*, 2012), BRAF V600E splice variants (Poulikakos *et al.*, 2011), as well as phosphatidylinositol 3′-kinase–AKT–mTOR (mammalian target of rapamycin) activation (Greger *et al.*, 2012).

One potential vemurafenib resistance mechanism that has not been well defined in melanoma involves the eIF4F complex, which consists of eIF4A, an RNA-dependent ATPase and helicase, eIF4G1, a scaffolding protein that mediates 40S ribosomal bridging with eIF4F, and eIF4E, a m^7GpppN (N is any nucleotide) cap-dependent RNA-binding protein (Topisirovic *et al.*, 2011). eIF4E facilitates the translation of pro-oncogenic mRNAs, such as vascular endothelial growth factor, cyclin D3, and Mcl-1 (Topisirovic *et al.*, 2011). *In vitro,* overexpression of eIF4E is sufficient to induce transformation (Lazaris-Karatzas *et al.*, 1990; Avdulov *et al.*, 2004), whereas *in vivo* overexpression is associated with prostate cancer and lymphoma (Ruggero *et al.*, 2004; Graff *et al.*, 2009), among other cancers. Moreover, in human breast cancer cells, blocking eIF4E decreases mRNA translation and anchorage-independent growth (Soni *et al.*, 2008) and also inhibits cell proliferation (Pettersson *et al.*, 2011). Although eIF4E has been reported to be overexpressed in various hematological malignancies and solid tumors (Mamane *et al.*, 2004), its role in melanoma remains largely unknown.

The activity of eIF4E is regulated by its binding proteins and upstream signaling pathways. Several eIF4E-binding proteins have been documented in the literature, but the best characterized are eIF4E-binding proteins (4E-BP) 1 and 2 (Pause *et al.*, 1994; Richter and Sonenberg, 2005). When 4E-BP1 is

[1]*Experimental Medicine, Faculty of Medicine, McGill University, Montréal, QC, Canada and* [2]*Segal Cancer Centre, Lady Davis Institute, Jewish General Hospital, McGill University, Montréal, QC, Canada*

Correspondence: Wilson H. Miller Jr., Experimental Medicine, Faculty of Medicine, McGill University, and Segal Cancer Centre, Lady Davis Institute, Jewish General Hospital, Montreal, QC, Canada.
E-mail: wmiller@ldi.jgh.mcgill.ca

Received 8 August 2014; revised 4 January 2014; accepted 7 January 2015; accepted article preview online 23 January 2015; published online 19 February 2015

hypophosphorylated, it binds to eIF4E, inhibiting cap-dependent translation (Gingras *et al.*, 1998). Conversely, when 4E-BP1 is hyperphosphorylated via activated mTOR, eIF4E is liberated, allowing cap-dependent translation to proceed (Lawrence and Abraham, 1997). Thus, the phosphatidylinositol 3′-kinase /Akt/mTOR pathway activates eIF4E via hyperphosphorylation of 4E-BP1. When this pathway is constitutively activated, the eIF4E-mRNA complex forms and cap-dependent translation is active, which leads to continuous cell proliferation (Boussemart *et al.*, 2014).

In this report, we characterize the role of eIF4E and its binding partners in a panel of melanoma cell lines. Importantly, we provide evidence that melanoma cells can escape the effects of vemurafenib by a mechanism involving increased phosphorylation of 4E-BP1 and bioavailability of eIF4E. Our findings provide the groundwork for novel combinatorial therapeutic approaches targeting BRAF V600E and eIF4E in melanoma.

RESULTS

Melanomas with elevated phospho-4E-BP1 and phospho-AKT protein levels are more sensitive to eIF4E knockdown

To determine the role of eIF4E in melanoma, we first analyzed the expression of eIF4E and eIF4G1, components of the eIF4F complex, within a panel of melanoma cell lines, versus immortalized melanocytes, MelST (Gupta *et al.*, 2005). As shown in Figure 1a, various melanoma cell lines, expressing either wild-type or mutant BRAF, expressed high levels of eIF4E compared with immortalized MelST melanocytes. We also analyzed the eIF4F complex scaffolding protein, eIF4G1, and found that its expression varied across cell lines (Supplementary Figure S1a online), as did the expression of 4E-BP1 (Figure 1a). Interestingly, profiling of the phosphorylation status of extracellular signal–regulated kinase (ERK) and AKT in the panel of melanoma cells revealed a striking correlation between 4E-BP1 hyperphosphorylation and phospho-AKT (Figure 1b). Increased phospho-4E-BP1 suggests that the bioavailabilty of eIF4E is increased in a subset of melanoma cell lines, as the phosphorylated form of 4E-BP1 fails to bind and repress eIF4E.

We then investigated whether eIF4E is a regulator of cell proliferation in a subset of melanoma cells. Following eIF4E knockdown via small interfering RNA (siRNA), we found that four lines were highly sensitive to eIF4E silencing: A375M, MM117, MM102, and MM111 (Figure 1c, Supplementary Figure S1b and d online). Although BLM and SKMel28 exhibited intermediate responsiveness, the remaining cell lines (WM164, MM57, 451Lu, and A375) continued to proliferate in comparison with control siRNA (siCTL), despite eIF4E knockdown (Figure 1c, Supplementary Figure S1b online). Interestingly, the cell lines with the greatest cell proliferation inhibition upon eIF4E silencing expressed the highest levels of phospho-AKT and phospho-4E-BP1 (Figure 1b–d, Supplementary Figure S1c online), suggesting that cells with increased eIF4E activity were more dependent on this pathway for survival. All 10 cell lines examined had a similar efficiency of eIF4E depletion; thus, the differences in proliferation following eIF4E knockdown were not simply due to differential silencing of eIF4E in one cell line versus another (Figure 3a and Supplementary Figure S1d online).

Vemurafenib reduces the phosphorylation of the eIF4E inhibitory protein 4E-BP1 in $BRAF^{V600E}$ mutant lines

Having demonstrated that silencing eIF4E can block the proliferation of some but not all melanoma cell lines examined, we next wanted to determine whether vemurafenib had any effect on the eIF4F complex. We assessed the phosphorylation of 4E-BP1 after treatment with vemurafenib in three $BRAF^{V600E}$ mutant melanoma cell lines: A375, SKMel28, and A375M. In all three cell lines, we observed a time-dependent decrease in the phosphorylation of 4E-BP1 using a phospho-specific 4E-BP1 antibody and by detecting a reduction in the hyperphosphorylated, slower migrating forms of 4E-BP1 following vemurafenib treatment (Figure 2a). Furthermore, as shown in Figure 2a, there is a marked decrease in ERK activation (phospho-ERK) upon 4-hour vemurafinib treatment, demonstrating an early inhibitory role of vemurafenib on the mitogen-activated protein kinase pathway. We next assessed the levels of phospho-p70S6K and phospho-S6, which lie downstream of mTOR. Consistent with previously published data, we found that the phosphorylation of S6 was decreased by vemurafenib treatment (Figure 2b) (Atefi *et al.*, 2011; Corcoran *et al.*, 2013). No consistent changes in AKT and P70S6K activation (phospho-AKT and phospho-P70S6K) were detected in these cell lines treated with vemurafenib (Figure 2b).

To further explore the effect of vemurafenib on the eIF4E/4E-BP1 association, we performed cap-binding assays using 7-methyl-GTP-bound agarose beads (Figure 2c). In this assay, eIF4E present in cell lysates binds to cap-mimicking beads, also enabling proteins bound to eIF4E to be assessed upon elution (Sonenberg *et al.*, 1979). In vemurafenib-treated cells, we observed increased binding between eIF4E and 4E-BP1, which occurs when 4E-BP1 is dephosphorylated, leading to decreased cap-dependent translation. Of note, PP242, an mTORC1/2 inhibitor, was used as a positive control, leading to 4E-BP1 hypophosphorylation and increased eIF4E:4E-BP1 association (Figure 2a and c).

Dephosphorylation of 4E-BP1 and subsequent eIF4E:4E-BP1 complex formation typically corresponds to an inhibition of translation initiation. To further investigate this, we assessed the effect of vemurafenib on the polysome distribution in $BRAF^{V600E}$ mutant melanoma cells. Treatment of A375 cells with vemurafenib for 4 hours did not result in an inhibition of translation (data not shown). However, after 24 hours, there was a decrease in the abundance of polysomes (Figure 2d), consistent with a block in eIF4F complex formation. Western blotting analysis showed that the expression of the eIF4E translational targets vascular endothelial growth factor, cyclin D3, c-Myc, and Bcl-2 expression was drastically repressed by vemurafenib treatment (Figure 2d). Cyclin D3 is a well-characterized eIF4E-sensitive mRNA (Dowling *et al.*, 2010; Alain *et al.*, 2012). In keeping with our results that vemurafenib can block mRNA translation, we observed a shift in the polysome loading, from heavy to light polysomes, of cyclin D3 mRNAs in A375 cells treated with vemurafenib

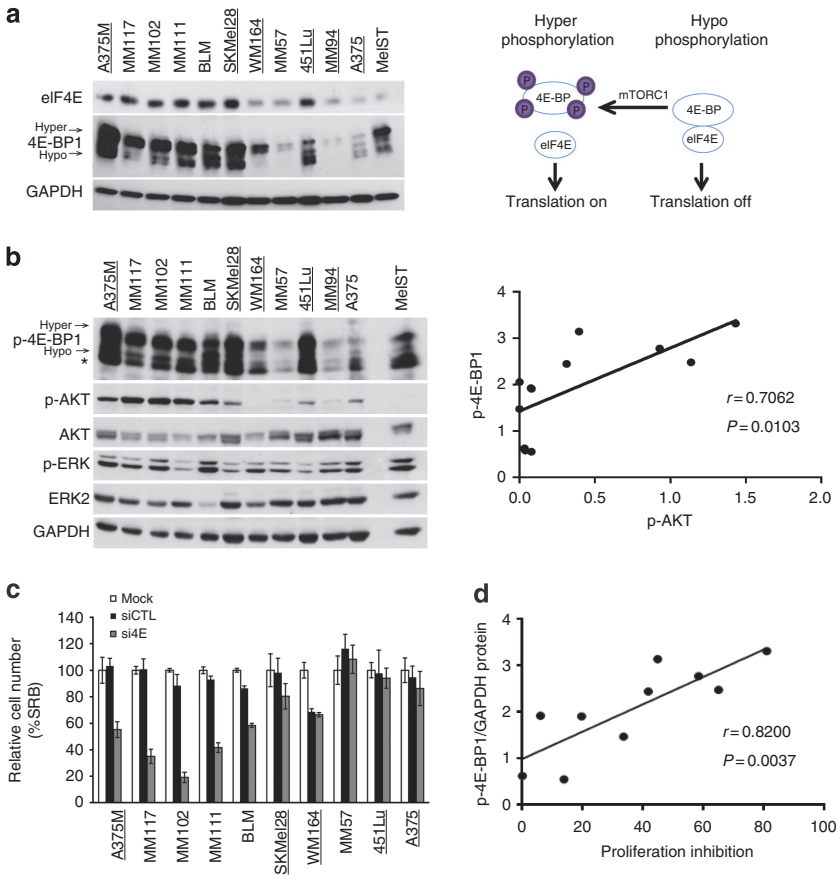

Figure 1. eIF4E knockdown inhibits proliferation in melanoma cell lines that highly express eIF4E, p-AKT, and hyperphosphorylated 4E-BP1. (**a**) Western blottings displaying eIF4E and 4E-BP1 protein expression in a panel of melanoma cell lines compared with immortalized melanocytes (MelST). Underlined are the cell lines that possess a BRAF V600E mutation. The schematic (right) shows the relationship between the phosphorylation status of 4E-BP and its ability to bind and inhibit eIF4E. (**b**) Western blotting of p-AKT, AKT, phospho-extracellular signal–regulated kinase (p-ERK), ERK2, and p-4E-BP1 in melanoma cell lines versus immortalized melanocytes (MelST). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a loading control in all immunoblots. Four bands can be seen on the p-4E-BP1 blot: the upper band represents the hyperphosphorylated form, whereas the lower band represents the hypophosphorylated form. The lowest band identified with an asterisk shows the phosphorylation of 4E-BP2. Correlation between the expression of p-AKT and p-4E-BP1 in the melanoma cell lines is shown (right). Pearson's correlation statistics have been used. (**c**) Cell proliferation assay plot following 4-day eIF4E siRNA treatment, assessed by sulforhodamine B (SRB) staining. Error bars are defined as mean ± SD, $n = 3$. (**d**) Correlation between the levels of proliferation inhibition upon eIF4E siRNA silencing versus p-4E-BP1 expression. Pearson's correlation statistics have been used. Note: p-4E-BP1 and p-AKT protein levels were normalized to corresponding GAPDH levels.

(Figure 2e, Supplementary Figure S2 online). Moreover, vemurafenib had no impact on the polysome loading of two eIF4E-insensitive mRNAs: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (Figure 2e and Supplementary Figure S2 online). These data suggest that vemurafenib-induced reduction in cell proliferation is associated with defects in eIF4E-mediated translation initiation.

eIF4E contributes to vemurafenib resistance in A375 BRAF^{V600E} melanomas

Having demonstrated that vemurafenib functions in part by repressing 4E-BP1 phosphorylation and increasing eIF4E:4E-BP complex formation, we next examined the status of the eIF4F complex in cells with acquired resistance to vemurafenib. Here, we obtained the parental BRAF^{V600E} mutant human melanoma cell line, A375, and corresponding vemurafenib-resistant cell lines, denoted A375(A)R1 and AR2 (Su *et al.*, 2012). To ensure that AR1 and AR2 cell lines were valid models of acquired resistance, and not merely chronically adapted to vemurafenib, we maintained the AR1 and AR2 cell lines in the absence of vemurafenib for 1 month and referred to these as AR1 and AR2 wash-off (WO) cell lines, AR1WO and AR2WO, respectively. We next challenged AR1WO and AR2WO cells with vemurafenib, and, as shown in Supplementary Figure S3a online, withdrawal of vemurafenib from the media for 1 month does not cause AR1 and AR2 cell lines to regain sensitivity to vemurafenib. Next, to determine whether eIF4E activity is associated with acquired resistance to vemurafenib, we performed eIF4E knockdown in both parental and resistant lines, with or without vemurafenib, and confirmed the efficiency of silencing by western blotting (Figure 3a). The proliferation of AR1 and AR2 is significantly inhibited by eIF4E silencing, compared with their parental counterpart (Figure 3a). Furthermore, evidence supporting a

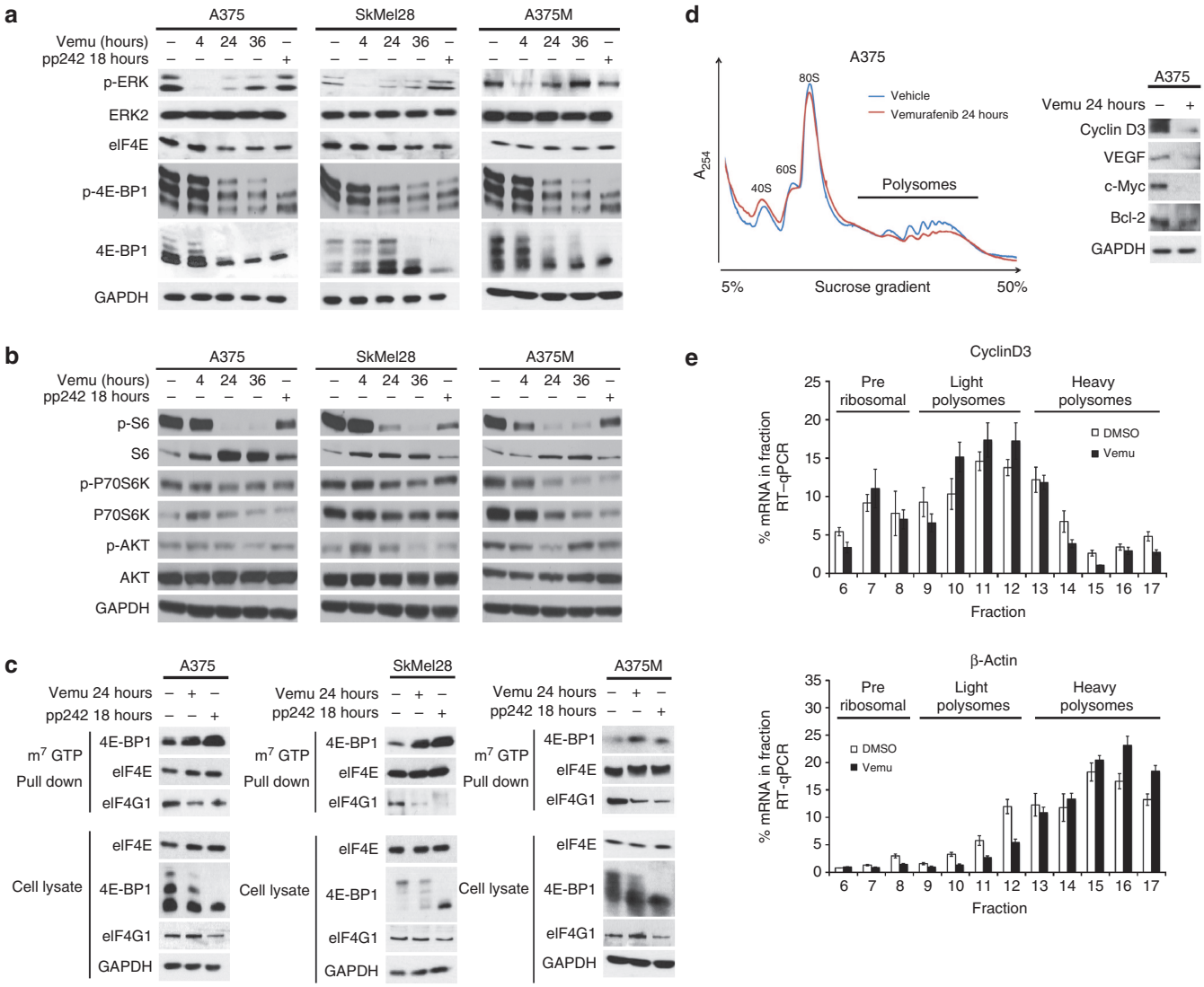

Figure 2. Vemurafenib drastically inhibits the phosphorylation of the eIF4E inhibitory protein 4E-BP1 in BRAF^{V600E} melanomas. (**a**) Western blotting of eIF4E, p-4E-BP1, 4E-BP1, and p-ERK and ERK2 in A375, SkMel28, and A375M upon 2.5 μM vemurafenib treatment at the times indicated. A concentration of 1 μM PP242 was used as a positive control to promote 4E-BP1 hypophosphorylation. (**b**) Western blotting of p-S6, S6, p-P70S6K, P70S6K, and p-AKT and AKT in A375, SkMel28, and A375M upon 2.5 μM vemurafenib treatment at the times indicated. (**c**) Cap-binding assay in A375, SkMel28, and A375M upon 2.5 μM vemurafenib treatment. (**d**) Polysome profile (% sucrose gradient vs. 254 nm rRNA absorbance) in A375 cells in the absence and presence of 2.5 μM vemurafenib for 24 hours (left panel). Western blotting analysis of cyclin D3, vascular endothelial growth factor (VEGF), c-Myc, and Bcl-2 following treatment with 2.5 μM vemurafenib for 24 hours (right panel). (**e**) Quantitative real-time reverse-transcriptase–PCR (qRT–PCR) was used to determine the distribution of cyclin D3 and β-actin mRNAs in polysome fractions (heavy vs. light polysomes) isolated from A375 cells treated with DMSO or 2.5 μM vemurafenib for 24 hours.

role of vemurafenib working via suppression of eIF4E activity is demonstrated by data (e.g., AR1WO vs. AR1 + V) showing that the addition of the BRAF inhibitor does not potentiate the effect of eIF4E silencing in the resistant cell lines (Figure 3a).

Silencing of eIF4E can inhibit the proliferation of vemurafenib-resistant cells. We investigated the integrity of the eIF4F complex in order to provide mechanistic insight toward this observation. Cap-binding analysis demonstrated that, compared with the parental A375, vemurafenib-resistant lines AR1, AR2, AR1WO, and AR2WO cell lines exhibited decreased eIF4E:4E-BP1 complex formation, leading to increased eIF4E:eIF4G1 association (Figure 3b). Furthermore, compared with the parental A375 cell line, vemurafenib-resistant lines overexpressed cyclin D3 and vascular endothelial growth factor, two well-documented eIF4E downstream targets (Dowling *et al.*, 2010; Topisirovic *et al.*, 2011). (Figure 3c). Our results suggest an increased role of eIF4E in the survival of cells with acquired resistance to vemurafenib.

4E-BP1/2 stable knockdown contributes to vemurafenib resistance in A375 cells

The data we have shown support the hypothesis that acquired resistance to vemurafenib is facilitated by hyperphosphorylation of 4E-BP1, leading to increased cap-dependent mRNA

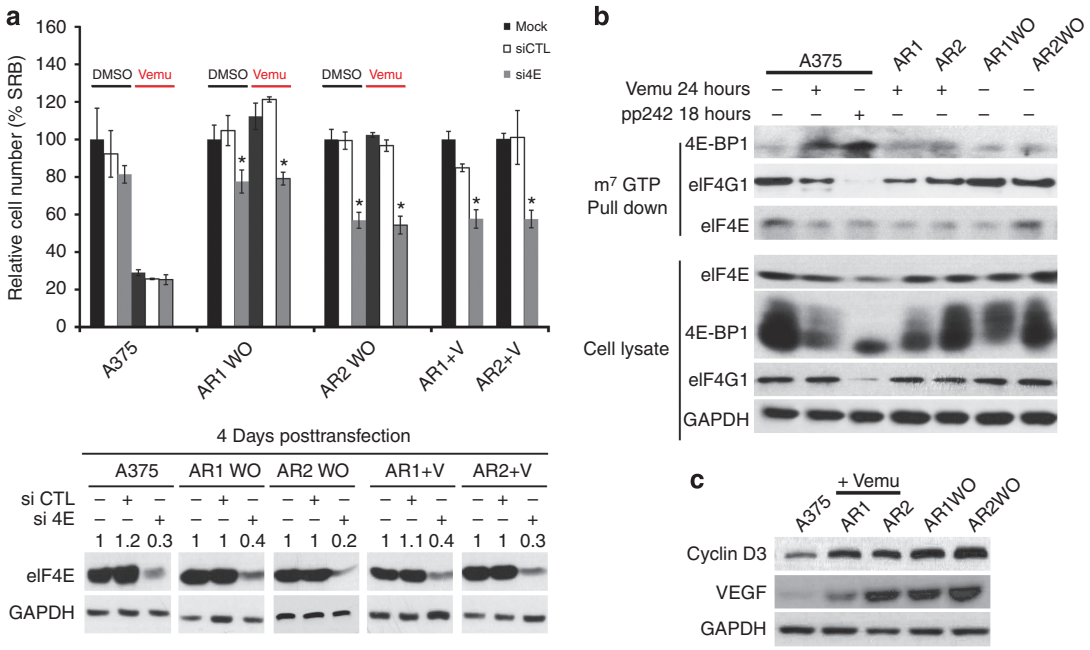

Figure 3. eIF4E has a key role in vemurafenib resistance in A375 BRAF^{V600E} human melanoma cell lines. (**a**) Cell proliferation assessment by sulforhodamine B (SRB) staining 4 days after si4E transfection with either vehicle (DMSO) or 2.5 μM vemurafenib cotreatment in A375, AR1WO (wash-off), AR2WO, AR1, and AR2. Note: AR1 and AR2 are continuously maintained in 2.5 μM vemurafenib. Error bars are defined as mean ± SD, $n=3$. Statistical significance was determined by the Student's *t*-test. (**b**) Cap-binding assay in parental A375 and resistant lines AR1 and AR2 (maintained in 2.5 μM vemurafenib), and corresponding vemurafenib WO lines AR1WO and AR2WO. PP242 is a positive control, for 4E-BP1 hypophosphorylation. (**c**) Western blotting of cyclin D3, vascular endothelial growth factor (VEGF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in A375 parental, resistant lines AR1 and AR2, and corresponding WO lines, AR1WO and AR2WO, respectively. *$P<0.05$.

translation, relative to parental A375. To further investigate the role of 4E-BP1 in resistance to vemurafenib, we transduced cells with retroviral particles expressing sh4E-BP1 and sh4E-BP2 RNAs. Knockdown of 4E-BP1/2 would have a similar effect on eIF4E activity as a 4E-BP1/2 hyperphosphorylation. Following puromycin selection and immunoblot confirmation, positive 4E-BP1/2 stable knockdown clones were established (Figure 4a). Consistent with increased eIF4E activity, the 4E-BP1/2 double knockdown cell line expressed elevated levels of eIF4E downstream targets c-Myc compared with the control scrambled knockdown (shCTL) cell line (Figure 4a). To determine the sensitivity to vemurafenib in the 4E-BP1/2 knockdown cell line, we assessed cell proliferation. While the proliferation rates between A375 shCTL and A375 shBP1/2 cell lines were similar, the 4E-BP1/2 double knockdown cells were more resistant to vemurafenib compared with their control knockdown counterparts (Figure 4b). Furthermore, in long-term clonogenic (proliferation) assays, we showed that the A375 shBP1/2 cell line is more resistant to vemurafenib compared with the A375 shCTL cell line (Figure 4c). To summarize, these data demonstrate that depletion of 4E-BP1/2 can cause a partial rescue of vemurafenib-induced inhibition of proliferation.

DISCUSSION

Gain-of-function BRAF mutations are common in melanoma, and although patients with tumors harboring mutant BRAF initially respond to targeted agents, resistance develops in most cases (Wiesner *et al.*, 2012; Zebary *et al.*, 2013; Boussemart *et al.*, 2014). Significant efforts have been made to understand the sensitivity and resistance to vemurafenib in this context, with several investigations focusing on the ERK and phosphatidylinositol 3′-kinase –Akt/mTOR pathways in melanoma progression (Nazarian *et al.*, 2010; Villanueva *et al.*, 2010). Interestingly, the profiles of downstream events, such as MNK phosphorylation (directly upstream of eIF4E) and, specifically, eIF4E expression, remain poorly documented in melanoma.

We found that a subset of cell lines expressed high levels of phospho-Akt, which correlated with elevated expression of hyperphosphorylated 4E-BP1 (Figure 1b). This led us to reason that the survival of this subset of melanoma cells (A375M, MM117, MM102, MM111, BLM, and SKMel28) would be driven by less sequestered, and more bioavailable, eIF4E (Pause *et al.*, 1994). The proliferation of these cell lines was significantly decreased when eIF4E was silenced, suggesting that this translation factor helps drive melanoma progression (Figure 1c, Supplementary Figure S1b online). Our data show that treatment of A375 cells with vemurafenib causes (1) a time-dependent reduction in the phosphorylation of 4E-BP1 (Figures 2a), (2) increased eIF4E:4E-BP1 association (Figure 2c), and (3) decreased abundance of light and heavy polysome fractions, as well as the reduced loading of eIF4E-sensitive mRNA cyclin D3 in heavy polysomes (Figure 2d and

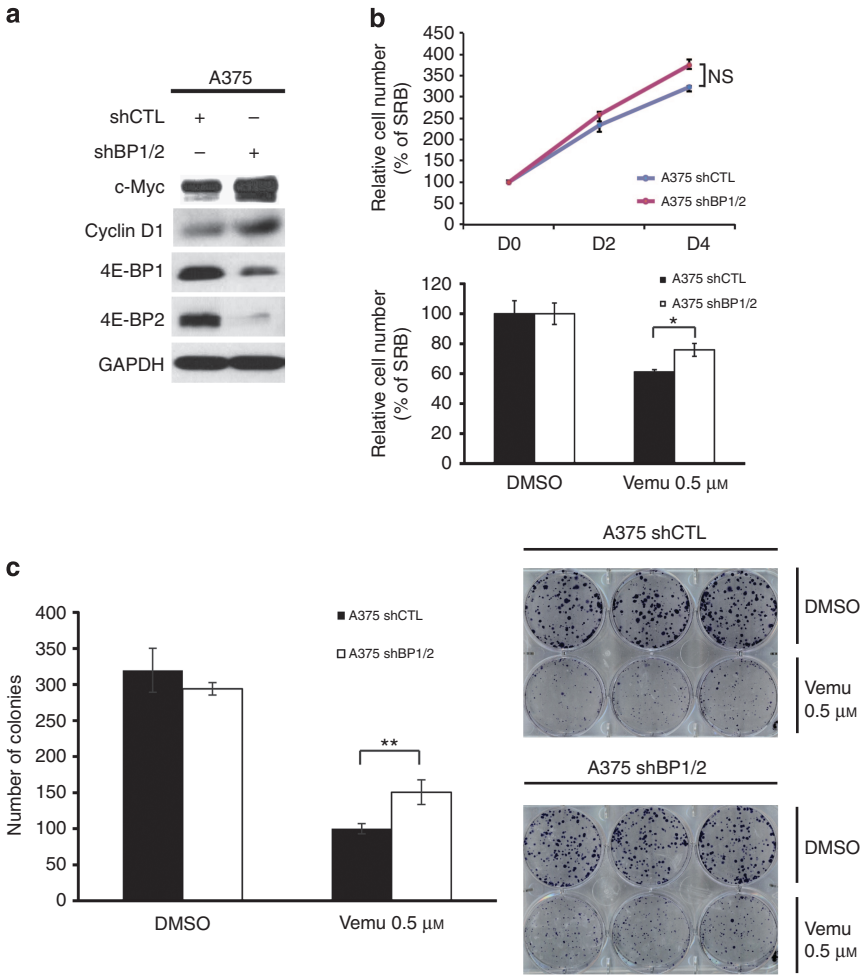

Figure 4. Stable knockdown of 4E-BP1/2 contributes to the development of vemurafenib resistance in A375 cells. (**a**) Western blotting of c-Myc, 4E-BP1, 4E-BP2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; loading control) in A375 shCTL and shBP1/2 cell lines. (**b**) Cell proliferation assay after 4 days with or without 0.5 μM vemurafenib treatment in A375 shCTL and shBP1/2 stable cell lines. Error bars are defined as mean ± SD, $n = 3$. Statistical significance was determined by one-way analysis of variance followed by the Newman–Keuls *post-hoc* test using Prism version 3.0 (GraphPad Software, San Diego, CA). (**c**) Clonogenic assay after 14 days with or without 0.5 μM vemurafenib treatment in A375 shCTL and shBP1/2 stable cell lines. The number of colonies was counted manually and graphed. Error bars are defined as mean ± SD, $n = 3$. Statistical significance was determined by one-way analysis of variance followed by the Newman–Keuls *post-hoc* test using Prism version 3.0 (GraphPad Software). $^*P < 0.05$, $^{**}P < 0.01$. Representative pictures are shown on the right. NS, not significant.

e, and Supplementary Figure S2 online); these results point to a role of vemurafenib in inhibiting eIF4E-mediated mRNA translation.

Conversely, in terms of resistance to chemotherapeutics, overexpression of eIF4E has been documented following anthracycline treatment in breast cancer (Heikkinen *et al.*, 2013), rapamycin treatment in murine lymphoma models (Wendel *et al.*, 2004, 2006), and, here, upon vemurafenib resistance in melanoma. Significant work by other groups has been put forward to address chemotherapy resistance in melanoma cells. Specifically, the observance of elevated phospho-4E-BP1 has been documented in cross-resistance to BRAF or mitogen-activated protein kinase/ERK kinase (MEK) inhibitors, which could be overcome by treating resistant cells with the mTOR inhibitor rapamycin (Atefi *et al.*, 2011). Furthermore, the use of second-generation mTOR inhibitors, such as everolimus and temsirolimus, alongside vemurafenib has come to fruition in clinical trials (see NCT01596140). Although targeting mTOR in such cases has the potential to regress and/or eradicate tumors, this may be insufficient, as elevated phospho-4E-BP1 levels are often associated with relatively high levels of eIF4E. Thus examining the eIF4E/4E-BP ratio upon administration of mTOR inhibitors (Alain *et al.*, 2012), or perhaps, more effectively, directly targeting eIF4E should provide a more pronounced effect clinically.

During preparation of this paper, a mechanism of resistance to anti-BRAF and anti-MEK treatment of melanomas was shown to involve heightened activation of the eIF4F complex (Boussemart *et al.*, 2014). As a means of targeting the eIF4F complex in response to standards of care, flavagline derivatives were developed, and were shown to depress translation of exogenous 5′-capped mRNA and reduce tumor volume in a Mel624 xenograft model (in concert with anti-BRAF). Although this investigation is promising in terms of targeting eIF4A, further examination of the effect of flavaglines on known eIF4E translational targets (e.g., c-Myc, cyclin D3)

and their effect on multiple melanoma cell lines and xenograft models would add to this approach.

In our study of genetically blocking eIF4E, rather than chemical inhibition of eIF4A with flavaglines (Basmadjian *et al.*, 2013), we observed the most pronounced inhibition of proliferation in cell lines with elevated phospho-4E-BP1 levels (Figure 1d). Exploring the effect of flavaglines within our panel of cell lines, in terms of proliferation inhibition and eIF4E target expression, would further support the clinical delivery of flavaglines in concert with anti-MEK and anti-BRAF therapies.

Overall, our data demonstrate that eIF4E promotes melanoma cell proliferation and may have a role in developing acquired resistance to the BRAF V600E inhibitor vemurafenib. Thus, targeting eIF4E in melanoma may be a novel therapeutic option geared toward cells expressing high basal levels of phospho-4E-BP1 and/or eIF4E upon acquired resistance to vemurafenib. Moreover, we hypothesize that eIF4E may promote vemurafenib resistance by promoting translation initiation of specific mRNAs, such as those intimately linked to cell survival. The literature supports this hypothesis, showing that proliferation and pro-survival mRNAs are less efficiently translated when cap-dependent (eIF4E-mediated) translation is blocked (Larsson *et al.*, 2007; Hsieh *et al.*, 2012). Future work in our lab will also focus on isolating translating ribosomes (polysomes) to define specific mRNAs, whereby translation is dependent on activated eIF4E in cells with acquired resistance to vemurafenib. Identification of specific mRNAs that are essential for eIF4E-mediated resistance to vemurafenib may suggest novel therapies, in addition to eIF4E inhibitors, to prevent or overcome resistance.

The work presented herein supports the importance of validating phospho-4E-BP1 and eIF4E as markers of resistance to BRAF inhibitors, using patient-derived prerelapse and postrelapse melanoma samples. We anticipate that a combinatorial drug treatment approach involving vemurafenib and novel eIF4E-targeting therapies will significantly reduce melanoma progression.

MATERIALS AND METHODS

Reagents

Vemurafenib was obtained from Plexxikon (Berkeley, CA), and PP242 was purchased at Sigma Aldrich (St. Louis, MO). All drugs were dissolved in DMSO to 10 mM, and aliquots were stored at −80 °C. Antibodies eIF4G1, 4E-BP1, phospho-4E-BP1 (p-4E-BP1; T37/46), phospho-eIF4E (p-eIF4E; Ser209), phospho-ERK (p-ERK; T202/Y204), 4E-BP2, c-Myc, phospho-AKT (p-AKT; Ser 473) AKT, cyclin D3, phospho-S6 (p-S6; S240/244), S6, phospho-P70S6K (p-P70S6K; Thr 389), P70S6K, and GAPDH were purchased from Cell Signalling Technology (Danvers, MA). The eIF4E antibody was purchased from BD Biosciences (Mississauga, CA). Immobilized r-Aminophenyl-m^7GTP agarose beads were purchased from Jena Science (Jena, Germany). Transfection reagents Lipofectamine 2000 or Lipofectamine RNAiMax were purchased from Invitrogen (Burlington, CA).

Cell culture

All cell lines used in this paper, except A375 and vemurafenib-resistant lines R1 and R2, were kindly provided by Dr Ghanem Ghanem (Institut Jules Bordet, Bruxelles, Belgium). The A375M cell line is a metastatic derivative of the A375 cell line originally reported by Dr JM Kozlowski (Kozlowski *et al.*, 1984). A375M, BLM, SkMel28, A375, and MelST were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin; MM117, MM111, MM102, WM164, MM57, 451Lu, and MM94 were cultured in HAM's F10 with 10% FBS and 1% Penicillin/Streptomycin. Vemurafenib-resistant cell lines AR1 and AR2 were maintained in 2.5 μM vemurafenib in DMEM with 10% FBS and 1% Penicillin/Streptomycin. For vemurafenib WO cell lines termed AR1WO and AR2WO, AR1 and AR2 cells, were maintained in DMEM with 10% FBS and 1% Penicillin/Streptomycin for 1 month in the absence of vemurafenib. All cell lines were maintained at 37 °C in a humidified incubator in 5% CO_2.

Proliferation assay

Cell proliferation was tested by sulforhodamine B assay. Cells were seeded on a 96-well plate the day before treatment. Cells were then treated with vemurafenib for 24, 48, or 96 hours. For each time point, 96-well plates were harvested and fixed with 10% trichloroacetic acid for 1 hour. Plates were then washed and sterilized with water three times and allowed to air-dry overnight. Once the fixation was completed, plates were stained with 0.4% (w/v) sulforhodamine B 100 μl per well in 1% acetic acid for at least 30 minutes. After staining, plates were washed with 1% acetic acid three times and air-dried overnight. Bound sulforhodamine B was solubilized by adding 100 μl per well of 10 mM unbuffered Tris base, pH 10.5, for 10 minutes. Absorbance at 564 nm was read using FLUOstar OPTIMA plate reader.

Plasmids, virus production, stable cell selection

Human sh4E-BP1 and sh4E-BP2 vectors were purchased from Sigma Aldrich (MISSION shRNAs). shRNAs were co-transfected with three Lentivirus packaging constructs, PLP1, VSVG, and PLP2, into 293FT cells using Lipofectamine 2000 (Invitrogen). Viral supernatants were harvested 48-hours posttransfection and spun at 1,000 r.p.m. for 5 minutes. For establishing shBP1 + BP2 stable knockdown cell lines, control or shBP1/2 viral supernatants were added to 10-cm dishes with adhered A375 cells for overnight infection. After two rounds of infection, cells were treated with puromycin (1 μg ml^{-1}) for 48 hours, and positive subclones were maintained.

Polysome profiling

Polysome profiling was performed as previously described by Gandin *et al.* (2014). For sucrose gradient fractionation and polysome isolation, A375 cells were seeded in 15-cm dishes with or without 2.5 μM vemurafenib for 24 hours. Cells were treated with cycloheximide (100 μg ml^{-1}) 5 minutes prior to harvesting, washed in cold PBS containing 100 μg ml^{-1} cycloheximide, and then spun for 5 minutes at 1,500 r.p.m. Cell pellets were lysed in hypotonic buffer (5 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM KCl, and 1 × protease inhibitor cocktail (EDTA-free), containing 1 mM dithiothreitol and RNAse inhibitor (100 units)). Samples were kept on ice for 12 minutes and then centrifuged at 13,000 r.p.m. for 8 minutes. The supernatants were harvested and added to 10–50% sucrose gradient. Gradients were centrifuged at 35,000 r.p.m. for 2 hours at 4 °C. Fractions were collected (24 fractions, 12 drops each) using a Foxy JR ISCO collector (Lincoln, NE), and data (absorbance, 254 nm) were collected.

Western blotting analysis

Cells were treated with vemurafenib (2.5 μM) or PP242 (1 μM) at the indicated times, and pellets were harvested to obtain protein extracts. Briefly, cell pellets were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). After sonication, cell lysates were centrifuged at 13,000 r.p.m. for 15 minutes. The supernatants were collected and protein concentrations were quantified. Equal amounts of protein were loaded on 10% SDS-PAGE. After transferring to a nitrocellulose membrane (Bio-Rad, Mississauga, CA), 5% milk/TBS was used to block for 1 hour and then probed for target antibodies overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature, the signals of targeted protein were developed with chemiluminescence substrate (Amersham, GE Healthcare, Buckinghamshire, UK).

RNA interference

Cells were seeded in 10-cm dishes at 80% confluency. eIF4E siRNA or control siRNA was added to the dishes after 20 minutes of incubation with transfection reagent Lipofectamine RNAiMAX following the manufacturer's instructions. After 16 hours, cells were washed with 1 × PBS, and fresh medium was added. At day 4 of transfection, cell pellets were harvested for western blotting. The sequences of the previously validated eIF4E siRNA pair were as follows: 5′-AGAGUG-GACUGCAUUUAAAUUUGdAdT-3′ and 5′-AUCAAAUUUAAAUG-CAGUCCACUCUGC-3′ (Pettersson *et al.*, 2011). AllStars Negative Control siRNA (Qiagen, Germantown, MD) was used as non-silencing control.

m^7GTP pull-down assay

Cells were treated with vemurafenib (2.5 μM) or PP242 (1 μM) at the indicated times, and whole cell lysate was harvested. For eIF4E pull-down assay, 20 μl m^7GTP agarose beads were added to each tube and washed with IP buffer (Tris-HCl pH 7.5 50 mM, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, TritonX-100 1%, and NP-40 0.5%) three times. Quantified protein extracts were then added on top of the m^7GTP agarose beads at equal amounts in each tube and were incubated with beads on the rotator overnight at 4 °C. Western blotting was performed to determine the association between eIF4E and 4E-BP1 or eIF4G1.

RNA isolation

To isolate mRNAs in each polysome fractions, Trizol (Invitrogen) was added into each fraction tube. After 5-minute incubation at room temperature, 200 μl of chloroform was added into each tube and mixed well for 15 seconds. Following centrifugation at 12,000*g* for 15 minutes at 4 °C, the clear phase was carefully obtained and placed into a clean tube. A volume of 500 μl of isopropanol was added to the clear phase, and this mixture was centrifuged for 30 minutes (12,000*g*, 4 °C). The isopropanol was then removed, and the remaining pellets were washed with 1 ml of 75% ethanol (in diethylpyrocarbonate water) and centrifuged for 5 minutes (12,000*g*, 4 °C). The liquid was then carefully removed, and the pellets were allowed to air-dry. A volume of 20 μl of diethy-lpyrocarbonate water was added to dissolve the RNA pellets, which were then quantitated (Thermo scientific Nanodrop 1000, Wilmington, DE).

Semiquantitative reverse transcription PCR (sqRT-PCR) and real-time qRT-PCR

Before performing the reverse transcription, 0.3 μg of mRNA was visualized by ethidium bromide agarose gel (2%) to check the quality of mRNAs (integrity of 28 S and 18 S bands). For cDNA production, a one-step RT-PCR kit (Bio-Rad) was used following the manufacturer's instructions. The sequences of human cyclin D3, GAPDH, and β-actin primers were as follows: cyclin D3 forward 5′-CTGGATCGC TACCTGTCTTG-3′, cyclin D3 reverse 5′-TCCCACTTGAGCTTCCC TAG-3′; GAPDH forward 5′-AATCCCATCACCATCTTCCA-3′, GAPDH reverse 5′-TGAGTCCTTCCACGATACCA -3′; and β-actin forward 5′-ACCACACCTTCTACAATGAGC-3′, β-actin reverse 5′-GATAGCACAGCCTGGATAGC-3′. To perform sqRT-PCR, the Taq DNA Polymerase Kit (Invitrogen) was used. For each of the transcripts (cyclin D3, GAPDH, β-actin), two cycle numbers (25 and 35 cycles) were performed to make sure the PCR results were in the linear range. Furthermore, cDNA was amplified for cyclin D3, GAPDH, and β-actin by real-time PCR analysis (ABI Prism7500; Applied Biosystems, Life Technologies, Burlington, CA) using SYBR green technology according to the manufacturer's instructions.

Clonogenic assay

A total of 300 cells per well were seeded in sixwell plates the day before treatment. After overnight incubation, the attached cells were treated with DMSO or vemurafenib at the indicated concentration, in triplicate. After 14 days, the incubating medium was removed, and the cells were stained with 0.5% (W/V) crystal violet in 70% ethanol. After 1 hour of incubation at room temperature, staining dye was washed, and the number of colonies was determined manually.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Dr Gideon Bollag from Plexxikon for providing us with the parental melanoma cell line A375 and acquired vemurafenib-resistant lines AR1 and AR2. We thank Dr Jessica N. Nichol for critically reading this research article. WHM is a Chercheur National du Fonds de la Recherche en Santé du Quebec (FRSQ). This project was funded in part by CIHR operating grant MOP-115002 and a grant from the Canadian Cancer Research Society (2012-17280). YZ was supported by the McGill Integrated Cancer Research Training Program (MICRTP) and Lady Davis Institute/TD bank studentship Award. MSD was supported by a MICRTP scholarship and a predoctoral award from the Fonds de la Recherche en Santé du Québec (30253).

Author contributions

YZ and SDR designed the research. YZ performed the research. MNB, LSL, and LVK provided critical reagents. FP, MCD, LVK, SDR, and WHM provided critical advice. YZ, MSD, and SDR wrote the article.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Alain T, Morita M, Fonseca BD *et al.* (2012) eIF4E/4E-BP ratio predicts the efficacy of mTOR targeted therapies. *Cancer Res* 72:6468–76

Atefi M, von Euw E, Attar N *et al.* (2011) Reversing melanoma cross-resistance to BRAF and MEK inhibitors by co-targeting the AKT/mTOR pathway. *PLoS One* 6:e28973

Avdulov S, Li S, Michalek V *et al.* (2004) Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells. *Cancer Cell* 5: 553–63

Basmadjian C, Thuaud F, Ribeiro N *et al.* (2013) Flavaglines: potent anticancer drugs that target prohibitins and the helicase eIF4A. *Future Med Chem* 5:2185–97

Bastian BC (2014) The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. *Annu Rev Pathol* 9:239–71

Boussemart L, Malka-Mahieu H, Girault I *et al.* (2014) eIF4F is a nexus of resistance to anti-BRAF and anti-MEK cancer therapies. *Nature* 513:105–9

Chapman PB, Hauschild A, Robert C *et al.* (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364:2507–16

Corcoran RB, Rothenberg SM, Hata AN *et al.* (2013) TORC1 suppression predicts responsiveness to RAF and MEK inhibition in BRAF-mutant melanoma. *Sci Transl Med* 5:196ra98

Davies H, Bignell GR, Cox C *et al.* (2002) Mutations of the BRAF gene in human cancer. *Nature* 417:949–54

Dowling RJ, Topisirovic I, Alain T *et al.* (2010) mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. *Science* 328:1172–6

Gandin V, Sikstrom K, Alain T *et al.* (2014) Polysome fractionation and analysis of mammalian translatomes on a genome-wide scale. *J Vis Exp* 17

Gingras AC, Kennedy SG, O'Leary MA *et al.* (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev* 12:502–13

Graff JR, Konicek BW, Lynch RL *et al.* (2009) eIF4E activation is commonly elevated in advanced human prostate cancers and significantly related to reduced patient survival. *Cancer Res* 69:3866–73

Greger JG, Eastman SD, Zhang V *et al.* (2012) Combinations of BRAF, MEK, and PI3K/mTOR inhibitors overcome acquired resistance to the BRAF inhibitor GSK2118436 dabrafenib, mediated by NRAS or MEK mutations. *Mol Cancer Ther* 11:909–20

Gupta PB, Kuperwasser C, Brunet JP *et al.* (2005) The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* 37:1047–54

Heikkinen T, Korpela T, Fagerholm R *et al.* (2013) Eukaryotic translation initiation factor 4E (eIF4E) expression is associated with breast cancer tumor phenotype and predicts survival after anthracycline chemotherapy treatment. *Breast Cancer Res Treat* 141:79–88

Hsieh AC, Liu Y, Edlind MP *et al.* (2012) The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* 485:55–61

Johannessen CM, Boehm JS, Kim SY *et al.* (2010) COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 468: 968–72

Kozlowski JM, Hart IR, Fidler IJ *et al.* (1984) A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. *J Natl Cancer Inst* 72:913–7

Larsson O, Li S, Issaenko OA *et al.* (2007) Eukaryotic translation initiation factor 4E induced progression of primary human mammary epithelial cells along the cancer pathway is associated with targeted translational deregulation of oncogenic drivers and inhibitors. *Cancer Res* 67:6814–24

Lawrence JC Jr., Abraham RT (1997) PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem Sci* 22:345–9

Lazaris-Karatzas A, Montine KS, Sonenberg N (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. *Nature* 345:544–7

Mamane Y, Petroulakis E, Rong L *et al.* (2004) eIF4E–from translation to transformation. *Oncogene* 23:3172–9

Maurer G, Tarkowski B, Baccarini M (2011) Raf kinases in cancer-roles and therapeutic opportunities. *Oncogene* 30:3477–88

Nazarian R, Shi H, Wang Q *et al.* (2010) Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 468:973–7

Pause A, Belsham GJ, Gingras AC *et al.* (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. *Nature* 371:762–7

Pettersson F, Yau C, Dobocan MC *et al.* (2011) Ribavirin treatment effects on breast cancers overexpressing eIF4E, a biomarker with prognostic specificity for luminal B-type breast cancer. *Clin Cancer Res* 17:2874–84

Poulikakos PI, Persaud Y, Janakiraman M *et al.* (2011) RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature* 480:387–90

Richter JD, Sonenberg N (2005) Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* 433:477–80

Romano E, Pradervand S, Paillusson A *et al.* (2013) Identification of multiple mechanisms of resistance to vemurafenib in a patient with BRAFV600E-mutated cutaneous melanoma successfully rechallenged after progression. *Clin Cancer Res* 19:5749–57

Ruggero D, Montanaro L, Ma L *et al.* (2004) The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. *Nat Med* 10:484–6

Shi H, Moriceau G, Kong X *et al.* (2012) Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun* 3:724

Sonenberg N, Rupprecht KM, Hecht SM *et al.* (1979) Eukaryotic mRNA cap binding protein: purification by affinity chromatography on sepharose-coupled m7GDP. *Proc Natl Acad Sci USA* 76:4345–9

Soni A, Akcakanat A, Singh G *et al.* (2008) eIF4E knockdown decreases breast cancer cell growth without activating Akt signaling. *Mol Cancer Ther* 7:1782–8

Su F, Bradley WD, Wang Q *et al.* (2012) Resistance to selective BRAF inhibition can be mediated by modest upstream pathway activation. *Cancer Res* 72:969–78

Sun C, Wang L, Huang S *et al.* (2014) Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature* 508:118–22

Topisirovic I, Svitkin YV, Sonenberg N *et al.* (2011) Cap and cap-binding proteins in the control of gene expression. *Wiley Interdiscip Rev RNA* 2:277–98

Trunzer K, Pavlick AC, Schuchter L *et al.* (2013) Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients with metastatic melanoma. *J Clin Oncol* 31:1767–74

Villanueva J, Vultur A, Lee JT *et al.* (2010) Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* 18:683–95

Wendel HG, De Stanchina E, Fridman JS *et al.* (2004) Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* 428:332–7

Wendel HG, Malina A, Zhao Z *et al.* (2006) Determinants of sensitivity and resistance to rapamycin-chemotherapy drug combinations in vivo. *Cancer Res* 66:7639–46

Wiesner T, Murali R, Fried I *et al.* (2012) A distinct subset of atypical Spitz tumors is characterized by BRAF mutation and loss of BAP1 expression. *Am J Surg Pathol* 36:818–30

Young K, Minchom A, Larkin J (2012) BRIM-1, -2 and -3 trials: improved survival with vemurafenib in metastatic melanoma patients with a BRAF(V600E) mutation. *Future Oncol* 8:499–507

Zebary A, Omholt K, Vassilaki I *et al.* (2013) KIT, NRAS, BRAF and PTEN mutations in a sample of Swedish patients with acral lentiginous melanoma. *J Dermatol Sci* 72:284–9