Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress

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Running Title: Modulation of the UPR by ERK in melanoma

Key Words: Endoplasmic Reticulum Stress, Melanoma, BRAF, ERK

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; ATF6, activating transcription factor 6; IRE1, inositol requiring enzyme 1; PERK, double-stranded RNA-activated protein kinase-like ER kinase; GRP78, glucose-regulated protein 78; eIF4E, eukaryotic initiation factor 4E.

Abstract

Cancer cells are commonly undergoing chronic endoplasmic reticulum (ER) stress, to which the cells have to adapt for survival and proliferation. We report here that in melanoma cells intrinsic activation of the ER stress response/unfolded protein response (UPR) is, at least in part, caused by increased outputs of protein synthesis driven by oncogenic activation of MEK/ERK, and promotes proliferation and protects against apoptosis induced by acute ER stress. Inhibition of oncogenic $BRAF^{V600E}$ or MEK attenuated activation of IRE1 and ATF6 signaling of the UPR in melanoma cells. This was associated with decreased phosphorylation of eIF4E and nascent protein synthesis, and was recapitulated by knockdown of eIF4E. In line with this, introduction of $BRAF^{V600E}$ into melanocytes led to increases in eIF4E phosphorylation and protein production, and triggered activation of the UPR. Similar to knockdown of GRP78, inhibition of XBP1 decelerated melanoma cell proliferation and enhanced apoptosis induced by the pharmacological ER stress inducers tunicamycin and thapasigargin. Collectively, these results reveal that potentiation of adaptation to chronic ER stress is another mechanism by which oncogenic activation of the MEK/ERK pathway promotes the pathogenesis of melanoma.

Introduction

The endoplasmic reticulum (ER) responds to ER stress that is characterized by accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen by activation of a range of signaling pathways to alter transcriptional and translational programs (Harding *et al.*, 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012). This couples the ER protein folding load with the ER protein folding capacity and is termed the ER stress response or the unfolded protein response (UPR) (Harding *et al.*, 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012). The UPR of mammalian cells is initiated by three ER transmembrane proteins, activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like ER kinase (PERK) (Harding *et al.*, 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012), which are anchored by the ER chaperon glucose-regulated protein 78 (GRP78) at their luminal domains, but upon ER stress, sequestration of GRP78 by unfolded proteins causes their activation through inducing phosphorylation and homodimerization of IRE1 and PERK and relocation of ATF6 to the Golgi where it is cleaved and activated as a transcriptional factor (Harding *et al.*, 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012).

The UPR is fundamentally a cyto-protective response, but excessive or prolonged UPR can trigger cell death predominantly by induction of apoptosis (Boyce and Yuan, 2006; Xu *et al.*, 2005). This is associated with attenuation of IRE1 and ATF6 activities, whereas PERK signaling that is essential for activation of pro-apoptotic proteins such as CHOP and Bim is maintained (Puthalakath *et al.*, 2007; Tabas and Ron, 2011; Zhang *et al.*, 2011). The UPR is often constitutively activated in cancer cells, indicative of adaptation to ER stress (Jiang *et al.*, 2009b; Jiang *et al.*, 2009c; Lee, 2007; Ma and Hendershot, 2004; Wang *et al.*, 2010). Indeed, cells in a developing solid cancer may undergo hypoxia, nutrient starvation, and acidosis, thus resulting in ER stress (Lee, 2007; Ma and Hendershot, 2004; Wang *et al.*, 2010). Moreover, increased synthesis of proteins that are often in mutated forms required to sustain malignancy of cancer cells may also directly uncouple the ER protein folding load

with the ER protein folding capacity (Lee, 2007; Ma and Hendershot, 2004).

The rate of protein synthesis is primarily controlled at the stage of mRNA translation initiation (Bilanges and Stokoe, 2007; Bitterman and Polunovsky, 2012; Grzmil and Hemmings, 2012; Silvera *et al.*, 2010). Initiation of translation of most mRNAs is mediated by the cap-dependent mechanism, which is governed by the eukaryotic initiation factor (eIF) 4F (eIF4F) complex that consists of the cap-binding protein eIF4E, the RNA helicase eIF4A, and the scaffolding protein eIF4G (Bilanges and Stokoe, 2007; Bitterman and Polunovsky, 2012; Grzmil and Hemmings, 2012; Silvera *et al.*, 2010). Among them, eIF4E is the rate-limiting factor (Jackson *et al.*, 2010; Silvera *et al.*, 2010; Sonenberg and Hinnebusch, 2009). While the activity of eIF4E is negatively regulated by eIF4E binding proteins (4E-BPs), it is enhanced with phosphorylation at serine 209 by MAP kinase signal-integrating kinase 1 (MNK1) and MNK2 upon binding to eIF4G (Hou *et al.*, 2012; Jackson *et al.*, 2010; Phillips and Blaydes, 2008; Silvera *et al.*, 2010; Sonenberg and Hinnebusch, 2009).

A characteristic of human melanoma is constitutive activation of the MEK/ERK pathway (Davies *et al.*, 2002; Platz *et al.*, 2008). This stems primarily from oncogenic mutations of BRAF with the most common mutation being a glutamic acid for valine substitution at position 600 ($BRAF^{V600E}$) (Davies *et al.*, 2002; Platz *et al.*, 2008). Mutations in N-RAS, H-RAS, c-Kit, ERBB4, or the G-protein α-subunit GNAQ are also responsible in subsets of melanomas (Flaherty *et al.*, 2010). Activation of MEK/ERK is critical for melanoma cell survival under acute, pharmacological ER stress (Hersey and Zhang, 2008; Jiang *et al.*, 2007), but its potential role in adaptation of melanoma cells to chronic, constitutive ER stress remains to be determined. It is known that MEK/ERK signaling promotes protein synthesis (Bilanges and Stokoe, 2007; Bitterman and Polunovsky, 2012; Grzmil and Hemmings, 2012; Silvera *et al.*, 2010), which may contribute to increased ER protein folding load in cells with MEK/ERK constitutively activated. However, it was recently reported that the RAF inhibitor vemurafenib induced ER stress, implicating that activation of MEK/ERK may alleviate ER stress in $BRAF^{V600E}$ melanoma cells (Beck *et al.*, 2013).

In this study, we have examined the potential interaction between the constitutively activated MEK/ERK pathway and the UPR in melanoma cells. We show here that MEK/ERK signaling is necessary and sufficient for intrinsic activation of the UPR as a consequence of ER stress triggered by enhanced protein synthesis, which in turn promotes proliferation and protects against apoptosis induced by acute ER stress in melanoma cells. These results indicate that potentiation of adaptation to chronic ER stress is another mechanism by which activation of the MEK/ERK pathway promotes the pathogenesis of melanoma.

Results and Discussion

The mutant BRAF inhibitor PLX4720 attenuates activation of the IRE1 and ATF6 branches of the UPR in BRAF^{V600E} melanoma cells

Past studies have shown that activation of MEK/ERK is required for induction of high levels of UPR and contributes to resistance of melanoma cells to acute, pharmacological ER stress (Hersey and Zhang, 2008; Jiang *et al.*, 2007). To study whether activation of MEK/ERK similarly contributes to chronic, constitutive activation of the UPR in melanoma cells, we tested the effect of the mutant BRAF inhibitor PLX4720 on the expression of the active (spliced) form of XBP1 (XBP1s) mRNA and GRP78, two commonly used indicators of activation of the IRE1 and ATF6 branches of the UPR (Lee, 2007; Ma and Hendershot, 2004), in melanoma cell lines. Strikingly, while XBP1s and GRP78 were downregulated in Mel-RMu and MM200 cells harboring BRAF^{V600E}, they were both increased in Mel-RM cells carrying wide-type BRAF, by PLX4720 (Figures 1a-d). Moreover, PLX4720 triggered decreases in phosphorylated (activated) IRE1α and cleaved (activated) ATF6 in Mel-RMu and MM200 cells (Figures 1e and f), but caused, albeit moderately, increases in activated IRE1α and ATF6 in Mel-RM cells (Figure S1a). These contrasting effects of PLX4720 were associated with its different impacts on activation of ERK, which was, as anticipated, inhibited in BRAF^{V600E}, but enhanced in wild-type BRAF, melanoma cells (Figure S1b) (Hatzivassiliou *et al.*, 2010). PLX4720 did not reduce the levels of phosphorylated eIF2α and ATF4, downstream targets of PERK signaling, in Mel-RMu and MM200 cells, nor did it increase their expression in Mel-RM cells, suggesting that it preferentially impinges on activation of IRE1 and ATF6 signaling (Figure S2). The inhibitory effect of PLX4720 on activation of IRE1 and ATF6 signaling was confirmed in another 4 BRAF^{V600E} cell lines as shown by downregulation of XBP1s and GRP78 (Figure S3).

In contrast to inhibition of IRE1α and ATF6 signaling by PLX4720, the mutant BRAF inhibitor vemurafenib, a close relative of PLX4720 (Tsai *et al.*, 2008), has recently been reported to induce ER stress in BRAF^{V600E} melanoma cells (Beck *et al.*, 2013). To clarify this paradox, we treated Mel-

RMu, MM200, and Mel-RM cells with vemurafenib. Indeed, vemurafenib upregulated XBP1s and GRP78 in Mel-RMu and MM200 cells, but strikingly, also caused upregulation of XBP1s and GRP78 in Mel-RM cells (Figures S4a-d). Nonetheless, vemurafenib inhibited ERK activation in Mel-RMu and MM200 cells, and enhanced activation of ERK in Mel-RM cells, recapitulating the different effects of PLX4720 on ERK activation in $BRAF^{V600E}$ and wild-type BRAF melanoma cells (Figure S4e and S4f). These results suggest that induction of ER stress by vemurafenib may be disassociated with its effects on activation of MEK/ERK signaling.

Inhibition of MEK or knockdown of ERK1/2 blocks IRE1 and ATF6 signaling in $BRAF^{V600E}$ and wild-type BRAF melanoma cells

To confirm that the contrasting effects of PLX4720 on activation of IRE1 and ATF6 signaling are due to its different effects on activation of MEK/ERK in $BRAF^{V600E}$ and wild-type BRAF melanoma cells, we treated Mel-RMu and MM200 ($BRAF^{V600E}$) and Mel-RM (wild-type BRAF) cells with the MEK inhibitor U0126. Inhibition of MEK downregulated XBP1s and GRP78 not only in $BRAF^{V600E}$ but also in wild-type BRAF melanoma cells (Figures 2a, 2b and S5). In line with this, concurrent knockdown of ERK1/2 with siRNA reduced the levels of XBP1s and GRP78 in both Mel-RMu and Mel-RM cells (Figures 2c-e).

We also examined the effect of the other mutant BRAF inhibitor dabrafenib on XBP1 and GRP78 in melanoma cells. Dabrafenib downregulated XBP1s and GRP78 and inhibited ERK activation in MM200, but upregulated XBP1s and GRP78 and enhanced ERK activation in Mel-RM cells, recapitulating the contrasting effects of PLX4720 on activation of IRE1 and ATF6 signaling in $BRAF^{V600E}$ and wild-type BRAF melanoma cells (Figures S6a-c and S7a-c). In contrast, the BRAF inhibitor CEP32496 that inhibits both $BRAF^{V600E}$ and wild-type BRAF reduced XBP1s and GRP78 and inhibited ERK activation in both MM200 and Mel-RM cells (Figures S6a-c and S7a-c) (James *et al.*, 2012). Taken together, the above results suggest that constitutive activation of MEK/ERK singling triggers ER stress in melanoma cells, and that induction of ER stress by PLX4720 and

dabrafenib in wild-type BRAF melanoma cells is associated with enhanced activation of ERK rather than caused by off-target effects of the inhibitors.

Activation of MEK/ERK signaling is critical for sustaining *de novo* protein production in melanoma cells

Activation of the MEK/ERK pathway promotes protein synthesis that is often enhanced in cancer cells and may represent an underlying mechanism of chronic ER stress by uncoupling the ER protein folding load with the ER protein folding capacity (De Benedetti and Graff, 2004; Silvera *et al.*, 2010). Indeed, the magnitude of nascent protein production was significantly higher in melanoma cells than melanocytes (Figure 3a), which was however reduced by U0126 in both $BRAF^{V600E}$ (Mel-RMu and MM200) and wild-type BRAF (Mel-RM) melanoma cells (Figure 3b). Similarly, PLX4720 reduced protein synthesis in Mel-RMu and MM200 cells, but increased the synthesis in Mel-RM cells (Figure 3c). Thus, constitutive activation of MEK/ERK plays an important role in maintaining high levels of protein production in melanoma cells. Of note, neither U0126 nor PLX4720 inhibits protein synthesis in melanocytes (Figures 3b and c).

MEK/ERK-mediated activation of protein synthesis involves phosphorylation of the translation initiator eIF4E (Hou *et al.*, 2012; Phillips and Blaydes, 2008). Consistent with this, the basal levels of phosphorylated eIF4E were generally higher in melanoma cells than melanocytes (Figure S8), which were rapidly reduced by PLX4720 in $BRAF^{V600E}$, and by U0126 in both $BRAF^{V600E}$ and wild-type BRAF, melanoma cells (Figures 3d-g), suggesting that the increased protein production in melanoma cells is coupled with phosphorylation of eIF4E mediated by MEK/ERK.

MEK/ERK signaling triggers chronic ER stress through increased protein production in melanoma cells

To examine whether increased protein synthesis mediated by the MEK/ERK pathway causes ER stress, we treated MM200 and Mel-RM cells with the small molecule 4EGI-1, an inhibitor of eIF4E (Fan *et al.*, 2010; Moerke *et al.*, 2007). 4EGI-1 at a concentration (10μM) that did not induce noticeable cell death caused partial, but significant, inhibition of protein synthesis (Figure 4a). This was associated with downregulation of XBP1s and GRP78 (Figures 4b and c), recapitulating, at least in part, the effects of inhibition of MEK/ERK on protein synthesis and activation of the UPR (Figures 1-3). In addition, 4EGI-1 abolished enhancement in protein synthesis and upregulation of XBP1s and GRP78 triggered by PLX4720 in Mel-RM cells (Figure S9a-c). These results suggest that MEK/ERK-induced activation of the UPR is mediated by eIF4E.

We confirmed the role of enhanced protein synthesis in MEK/ERK-induced ER stress by knocking down eIF4E with siRNA (Figure 4d), which significantly inhibited *de novo* protein synthesis and reduced XBP1s and GRP78 transcript expression in MM200 and Mel-RM cells (Figure 4e, 4f, and S10a), in line with the role of eIF4E and increased protein synthesis in induction of ER stress. In support, introduction of a construct expressing the phosphomimetic S209D (serine-to-aspartic acid) eIF4E mutant into Mel-RM cells caused an increase in nascent protein synthesis and GRP78 and XBP1s expression and abolished reduction in protein synthesis and inhibition of XBP1s and GRP78 induced by U0126 (Figures 4g-i and S10b).

Oncogenic BRAF activates eIF4E and the UPR in melanocytes

To further confirm that activation of MEK/ERK signaling induces ER stress in melanocytic cells, we infected HEM-1455 human melanocytes with a lentiviral construct expressing $BRAF^{V600E}$. Enforced expression of $BRAF^{V600E}$ caused activation of ERK, which was associated with induction of phosphorylation of eIF4E (Figures 5a and b), increased protein synthesis, albeit moderately, and activation of IRE1 and ATF6 signaling of the UPR (Figures 5c-e). In contrast, overexpression of wild-type BRAF did not increase ERK activation, consistent with previous reports (Scurr *et al.*, 2010), nor did it cause activation of IRE1 and ATF6 signaling (Figure S11). Intriguingly, the

expression levels of phosphorylated eIF2α and ATF4 were also increased moderately by ectopic expression of $BRAF^{V600E}$ in melanocytes (Figure 5a). Regardless, these data not only confirm the role of activation of MEK/ERK in causing chronic ER stress, but also suggest that occurrence of ER stress is an early event in the pathogenesis of melanoma. Indeed, $BRAF^{V600E}$ is found in the majority of nevi (Michaloglou *et al.*, 2005; Pollock *et al.*, 2003; Taube *et al.*, 2009), and the UPR is activated at early stages of melanoma initiation by $HRAS^{G12V}$ (Denoyelle *et al.*, 2006). Interestingly, oncogenic BRAF induces senescence in melanocytes (Dhomen *et al.*, 2009; Scurr *et al.*, 2010), but once the senescence barrier is overcome, it drives melanomagenesis (Dhomen *et al.*, 2009; Scurr *et al.*, 2010). By analogy, induction of ER stress by oncogenic activation of MEK/ERK signaling in melanocytic cells may also set a barrier for melanoma initiation. It is conceivable that only those cells that can survive chronic ER stress triggered by MEK/ERK may acquire malignant phenotypes.

XBP1 and GRP78 promote proliferation of melanoma cells

We examined the functional consequence of constitutive activation of IRE1 and ATF6 signaling in melanoma cells. Although siRNA knockdown of XBP1 or GRP78 did not trigger noticeable cell death, it inhibited proliferation in MM200 and Mel-RM cells (Figures 6a, 6b, and S12), However, knockdown of XBP1 or GRP78 did not impact on inhibition of proliferation caused by PLX4720 in MM200 cells or by U0126 in MM200 and Mel-RM cells (Figures 6c and 6d), in line with downregulation of XBP1 and GRP78 by inhibition of MEK/ERK signaling (Figures 1a-d and 2a-c). These results suggest that XBP1 and GRP78 activated by MEK/ERK signaling primarily affect proliferation of melanoma cells under steady-state conditions. Of note, both PLX4720 and U0126 primarily exert inhibitory effects on cell proliferation at the concentrations (3μM and 10μM, respectively) used in this study, although they can induce cell death when used at higher concentrations in a proportion of melanoma cell lines (Jiang *et al.*, 2011; Wang *et al.*, 2007). The effect of XBP1 on melanoma cell proliferation was also shown by treatment of MM200 and Mel-RM cells with salicylaldehyde that inhibits the IRE1 endonuclease activity thus blocking generation of XBP1s (Figure 6e) (Volkmann *et al.*, 2011). Intriguingly, overexpression of XBP1 or GRP78 did not

protect sensitive melanoma cells from PLX4720- or U0126-induced inhibition of proliferation (Figure S13 and S14), suggesting that XBP1 or GRP78 at levels above those driven by MEK/ERK signaling does not provide further proliferative advantage to melanoma cells under steady-state conditions.

Activation of IRE1/XBP1 signaling contributes to survival of melanoma cells undergoing acute ER stress

Induction of ER stress that potentially leads to cell death by MEK/ERK signaling seems paradoxical as its activation has been well established to be protective against cellular stress in melanoma cells (Hersey *et al.*, 2006; Jiang *et al.*, 2007). Nevertheless, MEK/ERK-mediated induction of GRP78 plays an important role in protecting melanoma cells from apoptosis induced by pharmacological ER stress (Jiang *et al.*, 2007). Similarly, IRE1/XBP1 signaling also contributed to survival of melanoma cells undergoing pharmacological ER stress, in that treatment with salicylaldehyde or siRNA knockdown of XBP1 enhanced apoptosis of MM200 and Mel-RM cells in response to the ER stress inducers tunicamycin (TM) or thapsigargin (TG) (Figures S15a-c). Therefore, chronic activation of IRE1 and ATF6 signaling not only promotes melanoma cell proliferation, but also protects melanoma cells from apoptosis undergoing acute ER stress, which conceivably plays a role in resistance of melanoma cells to therapeutic agents that induce ER stress (Jiang *et al.*, 2009a).

Since downregulation of PP2A activity is an important protective mechanism against pharmacological ER stress in melanoma cells (Tay *et al.*, 2012), we examined whether constitutive activation of MEK/ERK signaling that causes chronic ER stress plays a role in regulation of PP2A activity. PLX4720 did not impinge on the phosphatase activity of PP2A in MM200 cells (Figure S16a), indicating that relief of melanoma cells from MEK/ERK-triggered chronic ER stress is unable to enhance PP2A activity conceivably due to attenuation of feedback regulation of PP2A by ERK (Garcia *et al.*, 2002; Letourneux *et al.*, 2006). Nevertheless, it reduced PP2A activity in Mel-RM cells (Figure S16b), consistent with downregulation of PP2A activity by induction of ER stress (Tay *et al.*, 2012). It seems likely that while acute ER stress downregulates PP2A activity, constitutive

activation of MEK/ERK plays a role in keeping PP2A activity in check to a minimal level. PP2A is known to have a tumour suppressive role in many types of cancer (Janssens *et al.*, 2005; Kalev and Sablina, 2011).

Introduction of exogenous oncogenic BRAF has been reported to inhibit melanoma cell growth by inducing autophagy (Maddodi *et al.*, 2010). However, the mechanism(s) involved remains unknown. Our results suggest that this may be associated with induction of ER stress by activation of MEK/ERK signaling, in that ER stress is known to trigger autophagy, which is nevertheless believed to play a protective role in cells undergoing ER stress (Li *et al.*, 2008). Since melanoma cells carrying endogenous oncogenic BRAF are additive to its signaling for survival and growth (Hoeflich *et al.*, 2006), it is unlikely that autophagy, if any, resulting from constitutive chronic ER stress that is pro-proliferative inhibits $BRAF^{V600E}$ melanoma cell growth.

Although we have clearly demonstrated that constitutive activation of MEK/ERK signaling is not only a mechanism of adaptation to ER stress, but also a source of chronic ER stress in melanoma cells, it remains puzzling how MEK/ERK signaling spares the PERK branch, the main pathway that mediates ER stress-induced apoptosis (Boyce and Yuan, 2006; Puthalakath *et al.*, 2007; Xu *et al.*, 2005). It is believed that there is no ER stress inducer that can selectively elicits any particular signaling pathway of the UPR (Lin *et al.*, 2007; Walter and Ron, 2011). Indeed, activation of MEK/ERK signaling by ectopic expression of $BRAF^{V600E}$ resulted in activation of all three branches of the UPR in melanocytes. Since IRE1 and ATF6 activities are progressively attenuated, whereas PERK signaling is maintained and thus triggers apoptosis in many types of cells undergoing prolonged ER stress (Lin *et al.*, 2007), it is possible that activation of MEK/ERK exerts a role in sustaining IRE1 and ATF6 activities, rather than selectively activating the pathways in melanoma cells. In addition, while induction of ER stress by MEK/ERK signaling is resolved, other elements such as increased glycolysis and cellular acidosis are still able to trigger ER stress in melanoma cells (Liu *et al.*, 2009; Ma and Hendershot, 2004; Zhuang *et al.*, 2010). Under such conditions, IRE1 and ATF6 activities

are attenuated with diminishing of MEK/ERK signaling, whereas persistent ER stress continues driving activation of the PERK pathway.

Another paradox that needs to be clarified is how vemurafenib induces ER stress in both $BRAF^{V600E}$ and wild-type melanoma cells, although our results indicate that the ability of vemurafenib to induce ER stress in melanoma cells may be independent of its effect on activation of MEK/ERK signaling. This is of particular importance as it suggests that the therapeutic efficacy of this clinically available BRAF inhibitor may be determined not only by its inhibitory effect on MEK/ERK but also by its ability to induce ER stress. Further studies using animal models and melanoma samples from patients before and after treatment with the inhibitor are clearly warranted.

Materials and Methods

Cell Lines

Human melanoma cell lines Mel-RMu, MM200, Mel-CV, IgR3, A2058 and SkMel-28 ($BRAF^{V600E}$) and Mel-RM, Mel-JD, ME4405 and ME1007 ($BRAF^{WT}$) and human melanocyte lines HEMn-MP and HEMn-DP were obtained and cultured as described previously (Jiang *et al.*, 2010; Ye *et al.*, 2013).

Antibodies and other reagents.

Antibodies and reagents used are listed in Table S1 & S2.

Apoptosis

Quantitation of apoptotic cells was carried out by measurement of sub-G1 DNA content as described elsewhere (Jiang *et al.*, 2011).

Western Blot analysis

Western blot analysis was carried out as described previously (Jiang *et al.*, 2011). Labeled bands were detected by Luminata Crescendo Western HRP substrate (Millipore, Billerica, MA), and images were captured and the intensity of the bands was quantitated with ImageReader LAS-4000 (Fujifilm Corporation, Tokyo, Japan).

Detection of spliced XBP1 mRNA (XBP1s)

Detection of XBP1s was carried out as previously described (Jiang *et al.*, 2008).

Lentiviral Gene Transduction and DNA Constructs

The lentiviral vector *pCDH-CMV-MCS-EF1-copGFP* containing Myc-tagged $BRAF^{V600E}$ or $BRAF^{WT}$ was used to produce lentiviruses and to transduce human melanocytes as described previously (Haferkamp *et al.*, 2009). Overexpression of $BRAF^{V600E}$ and $BRAF^{WT}$ was confirmed by western blot using Myc tag and BRAF antibody, respectively.

siRNA knockdown

Transfection of siRNA siGENOME SMARTpools (Table S4) was carried out as described previously (Jiang *et al.*, 2011).

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

This work was supported by the NSW State Cancer Council, Cancer Institute NSW, and National Health and Medical Research Council (NHMRC), Australia. X. D. Zhang is supported by a senior research fellowship of NHMRC.

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Legends to Figures

Figure 1 - PLX4720 inhibits activation of the IRE1 and ATF6 pathways of the UPR in $BRAF^{V600E}$ melanoma cells.

a. RT-PCR analysis showing that PLX4720 (3μM) alters the expression of XBP1s mRNA (n=3).

b & c. qPCR analysis showing that PLX4720 (3μM) alters the expression of XBP1s (b) and GRP78 (c) mRNA (n=3, mean±SEM; Student's *t*-test; *p<0.05).

d, e, & f. Whole cell lysates from cells with or without PLX4720 (3μM) treatment for indicated periods were subjected to Western blot analysis (n=3).

Figure 2 - Inhibition of MEK/ERK reduces activation of the UPR in melanoma cells.

a. RT-PCR analysis showing that U0126 (20μM) alters the expression of XBP1s mRNA (n=3).

b. qPCR analysis showing that U0126 (20μM) alters the expression of GRP78 mRNA (n=3, mean±SEM; Student's *t*-test; *p<0.05).

c. Whole cell lysates from cells transfected with ERK1 and ERK2 siRNA were subjected to Western blot analysis (n=3).

d. RT-PCR analysis showing that ERK1 and ERK2 siRNA transfection alters the expression of XBP1s. (n=3).

e. qPCR analysis showing that ERK1 and ERK2 siRNA transfection alters the expression of GRP78 mRNA. (n=3, mean±SEM; Student's *t*-test; *p<0.05).

Figure 3 - Activation of MEK/ERK signaling is critical for sustaining *de novo* protein production in melanoma cells

a. Click-iT® protein synthesis assay showing magnitude of nascent protein synthesis (n=3, mean±SEM; Student's *t*-test; *p<0.05).

b & c. Click-iT® protein synthesis assay showing magnitude of nascent protein synthesis in cells treated with or without U0126 (20μM) (b) and in cells treated with or without PLX4720 (3μM) (c) for 16 hours (n=3, mean±SEM; Student's *t*-test; *p<0.05).

d & f. Western blot analysis for total lysates treated with or without PLX4720 (3μM) (d) or with or without U0126 (20μM) (f) for indicated periods (n=3).

e & g. Quantitation of peIF4E as shown in d (e) and f (g) by normalizing to total eIF4E (n=3, mean±SEM; Student's *t*-test; *p<0.05).

Figure 4 – Activation of MEK/ERK triggers ER stress in melanoma cells.

a. Click-iT® protein synthesis assay showing magnitude of nascent protein synthesis in cells treated with or without 4EGI-1 (10μM) for 12 hours (n=3, mean±SEM; Student's *t*-test; *p<0.05).

b & c. qPCR analysis of XBP1s(b) and GRP78(c) mRNA expression after 4EGI-1 (10μM) treatment (n=3, mean±SEM; Student's *t*-test; *p<0.05).

d & g. Western blot analysis for total lysates transfected with eIF4E siRNA(d) and phosphomimetic S209D eIF4E mutant constructs(g) (n=3).

e & f. qPCR analysis showing XBP1s(e) and GRP78(f) mRNA expression after eIF4E knockdown (n=3, mean±SEM; Student's *t*-test; *p<0.05).

h & i. qPCR analysis showing XBP1s(h) and GRP78(i) mRNA expression in $eIF4E^{S209D}$ transected cells with or without U0126 (20μM) (n=3, mean±SEM; Student's *t*-test; *p<0.05).

Figure 5 - Oncogenic BRAF activates eIF4E and the UPR in melanocytes.

a. Whole cell lysates of HEM1455 melanocytes transduced with pCDH-empty vector or pCDH-BRAF^{V600E}-myc were subjected Western blot analysis (n=3).

b. Quantitation of peIF4E as shown in **a** by normalizing to total eIF4E (n=3, mean±SEM; Student's *t*-test; *p<0.05).

c. RT-PCR analysis showing that transduction of HEM1455 with either pCDH-empty vector or pCDH-BRAF^{V600E}-myc alters the expression of XBP1s mRNA (n=3).

d. Click-iT® protein synthesis assay showing magnitude of nascent protein synthesis in HEM1455 transduced with either pCDH-empty vector or pCDH-BRAF^{V600E}-myc (n=3).

e. qPCR analysis showing changes of GRP78 mRNA in HEM1455 transduced with either pCDH-empty vector or pCDH-BRAF^{V600E}-myc (n=3).

Figure 6 - XBP1 and GRP78 promote proliferation of melanoma cells

a. (Left) Whole cell lysates from cells transfected with the control or GRP78 siRNA were subjected to Western blot analysis (n=3). (Right) BrdU incorporation analysis in cells transfected with the control or GRP78 siRNA.

b. (Left) qPCR analysis validating XBP1 knockdown efficiency. (Right) BrdU incorporation analysis in cells transfected with the control or XBP1 siRNA.

c & d. BrdU incorporation analysis in (c) MM200 or (d) Mel-RM cells transfected with the control, GRP78 (left) or XBP1 (right) siRNA followed by PLX4720 (3μM) or U0126 (20μM) treatment for 48 hours (n=3, mean±SEM; Student's *t*-test; *p<0.05).

e. (Left) qPCR analysis of XBP1s and (Right) BrdU incorporation analysis in cells treated with or without Salicylaldehyde (60μM).