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Colorectal cancer cells with the BRAF^{V600E} mutation are addicted to the ERK1/2 pathway for growth factor-independent survival and repression of BIM

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Abstract

The RAF-mitogen-activated protein kinase kinase 1/2-extracellular signal-regulated kinase 1/2 (RAF-MEK1/2-ERK1/2) pathway is activated in many human tumours and can protect cells against growth factor deprivation; however, most such studies have relied upon overexpression of RAF or MEK constructs that are not found in tumours. Here we show that expression of the endogenous *BRAF^{V600E}* allele in mouse embryonic fibroblasts from conditional knock-in transgenic mice activates ERK1/2, represses the BH3-only protein BIM and protects cells from growth factor withdrawal. Human colorectal cancer (CRC) cell lines harbouring *BRAF^{V600E}* are growth factor independent for the activation of ERK1/2 and survival. However, treatment with the MEK1/2 inhibitors U0126, PD184352 or the novel clinical candidate AZD6244 (ARRY-142886) overcomes growth factor independence, causing CRC cell death. BIM is de-phosphorylated and upregulated following MEK1/2 inhibition in all CRC cell lines studied and knockdown of BIM reduces cell death, indicating that repression of BIM is a major part of the ability of BRAF^{V600E} to confer growth factor-independent survival. We conclude that a single endogenous *BRAF^{V600E}* allele is sufficient to repress BIM and prevent death arising from growth factor withdrawal, and CRC cells with BRAF^{V600E} mutations are addicted to the ERK1/2 pathway for repression of BIM and growth factor-independent survival.

Keywords

apoptosis; BIM; BRAF; colorectal cancer; ERK1/2

Introduction

Most normal cells are dependent upon growth factors for their survival and proliferation, whereas cancer cells are growth factor independent and can evade apoptosis (Hanahan and Weinberg, 2000). Apoptosis following withdrawal of growth factors is regulated by the BCL-2 protein family in which the pro-apoptotic BAX and BAK proteins are restrained by their interaction with the antiapoptotic BCL-2 family members, such as BCL-2 and MCL-1; this balance is disrupted by the binding of BH3-only proteins to BCL-2 or MCL-1, thereby

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releasing BAX or BAK to promote cell death (Cory *et al.*, 2003; Chen *et al.*, 2005; Willis *et al.*, 2007). BIM is a BH3-only protein that promotes cell death following withdrawal of survival factors (Bouillet *et al.*, 1999; Dijkers *et al.*, 2000; Ewings *et al.*, 2007a). Survival factors repress BIM expression by reducing its transcription (Dijkers *et al.*, 2000; Gilley *et al.*, 2003). In addition, the most abundant splice variant, BIM_{EL}, is phosphorylated by extracellular signal-regulated kinase 1/2 (ERK1/2), preventing it from binding to its target pro-survival BCL-2 proteins (Ewings *et al.*, 2007a; Ewings *et al.*, 2007b) and promoting its proteasomal turnover (Ley *et al.*, 2003; Luciano *et al.*, 2003; Weston *et al.*, 2003; Ley *et al.*, 2004; Marani *et al.*, 2004; Ley *et al.*, 2005).

The RAF-mitogen-activated protein kinase kinase 1/2-ERK1/2 (RAF-MEK-ERK1/2) signalling pathway is activated in human tumours because of activating mutations in RAS or BRAF. $BRAF^{V600E}$ is found in 10-25% of human colorectal cancers (CRC) (Davies *et al.*, 2002; Mercer and Pritchard, 2003), early in the adenoma to carcinoma sequence (Rajagopalan *et al.*, 2002; Yuen *et al.*, 2002), and cancer cells harbouring $BRAF^{V600E}$ typically exhibit high ERK1/2 activity. Although tumours accumulate numerous mutations, they evolve to be dependent on a specific oncogene(s) to maintain the malignant phenotype; a phenomenon termed ‘oncogene addiction’ (Weinstein, 2002). Several studies have shown that the activation of ERK1/2 can inhibit BIM expression and prevent cell death arising from growth factor withdrawal (Ley *et al.*, 2003; Luciano *et al.*, 2003; Weston *et al.*, 2003; Marani *et al.*, 2004). However, these studies have typically involved the overexpression of RAF or MEK mutants or the use of conditional kinases that are not found in human tumours and may not faithfully mimic the effects of $BRAF^{V600E}$.

Here we show that conditional activation of a single $BRAF^{V600E}$ allele in primary mouse embryonic fibroblasts (MEFs) from transgenic conditional knock-in mice is sufficient to repress BIM expression and protect against growth factor withdrawal. CRC cell lines with $BRAF^{V600E}$ fail to upregulate BIM or undergo apoptosis following growth factor withdrawal unless the ERK1/2 pathway is also inhibited; furthermore, death arising from inhibition of the ERK1/2 pathway is substantially reduced by the use of short hairpin RNA or small interfering RNA (siRNA) to BIM. These data suggest that CRC cells with $BRAF^{V600E}$ are addicted to the ERK1/2 pathway for the repression of BIM and growth factor-independent survival.

Results

Expression of a single endogenous $BRAF^{V600E}$ allele is sufficient to repress BIM and protect cells from growth factor withdrawal

$Braf^{LSL\text{-}V600E}$ mice conditionally express a knock-in mutation of $Braf^{V600E}$ (Mercer *et al.*, 2005); Cre recombinase activity is required to induce recombination of the Lox-STOP-Lox cassette and allow expression of $BRaf^{V600E}$ (Figure 1a). We used the CreER™ system in which Cre is expressed as a fusion protein with a tamoxifen-responsive version of the hormone-binding domain of the oestrogen receptor (Hayashi and McMahon, 2002). Primary MEFs derived from $Braf^{+/LSL\text{-}V600E}$;*CreER*™ double heterozygote embryos were treated over a time course with 4-HT (4-hydroxytamoxifen). The CreER™ protein was stabilized and localized to the nucleus within 5 h (Figure 1 b), but full recombination of the $Braf^{LSL\text{-}V600E}$ allele was not achieved until 96 h (Figure 1c). These studies show for the first time the efficient regulation of a floxed allele by the CreER™ protein and establish the conditions for maximal induction of the $Braf^{V600E}$ allele by 4-HT.

Primary MEFs derived from either $Braf^{+/+}$;*CreER*™ or $Braf^{+/LSL\text{-}V600E}$;*CreER*™ embryos underwent apoptosis following serum starvation. Inclusion of 4-HT to induce $Braf^{V600E}$ expression strongly inhibited this apoptotic response in $Braf^{+/LSL\text{-}V600E}$;*CreER*™ MEFs but

not in *Braf*$^{+/+}$;*CreER*™ MEFs as measured by three different apoptosis assays (Figure 2a and Supplementary Figure S1). This protective effect of 4-HT was due to BRAF^{V600E}-dependent activation of the MEK-ERK1/2 pathway as it was reversed by the MEK inhibitor U0126 (Figure 2b). 4-HT treatment was able to partially reverse the loss of mitochondrial membrane potential (MMP) arising from serum withdrawal in *Braf*$^{+/LSL\text{-}V600E}$;*CreER*™ MEFs but not in *Braf*$^{+/+}$;*CreER*™ MEFs (Figure 2c).

BCL-2 proteins control the loss of mitochondrial membrane potential, and BIM has been implicated in the death of MEFs following the loss of growth factors (Ewings *et al.*, 2007a). Indeed, when *Braf*$^{+/LSL\text{-}V600E}$;*CreER*™ MEFs were serum starved, there was a striking increase in BIM expression, predominantly the BIM$_{EL}$ isoform, which was completely prevented by the inclusion of 4-HT (Figure 3a). Serum withdrawal from *Braf*$^{+/LSL\text{-}V600E}$;*CreER*™ MEFs caused only a modest increase in BIM mRNA levels, as judged by (quantitative) reverse transcription PCR (Q)RT-PCR), and 4-HT treatment did not reverse this (Figure 3b). However, the expression of Braf^{V600E} did cause the MEK-dependent hyperphosphorylation of BIM$_{EL}$, and the Braf^{V600E}-dependent downregulation of BIM$_{EL}$ was reversed by the inclusion of the proteasome inhibitor MG132 (Figure 3c); furthermore, MG132 potentiated the induction of BIM$_{EL}$ upon serum withdrawal. These results indicate that the expression of a single Braf^{V600E} allele is sufficient to repress BIM expression and it does so largely by promoting the phosphorylation and proteasome-dependent turnover of BIM$_{EL}$ rather than by repressing *BIM* transcription.

Growth factor-independent survival in colorectal cancer cells with the BRAF^{V600E} mutation is reversed by the inhibition of MEK1/2

Initial experiments revealed that COLO205 cells fail to increase caspase/DEVDase activity (Figures 4a and b) or die (Figure 4c) following serum withdrawal. Similar results were observed in three other BRAF^{V600E}-positive CRC cell lines (J Wickenden and S Cook, unpublished observations). In contrast, when COLO205 cells were serum starved in the presence of U0126, caspase activation was strikingly enhanced and accelerated (Figure 4a) and there was a large increase in the number of dead cells (Figure 4c); this was also seen in HT29, LS411 and CO115 cells (summarized in Figure 4d). U0126 also induced some death in cells maintained in fetal bovine serum (FBS) in some instances (for example, COLO205 cells, Figures 4a and c). The effect of U0126 was dose dependent; half-maximal cell death being induced by 300 n_M−1 μ_M U0126 (Figure 4e). In addition, the effect of U0126 was replicated by PD184352, a more selective MEK1/2 inhibitor (Figure 4f).

Although death arising from MEK inhibition was inhibited by the caspase inhibitor zVAD.fmk in HT29 cells, death of COLO205 (Figure 4f) and LS411 cells (Supplementary Figure S2A) was largely caspase independent (Figure 4f). zVAD.fmk was fully competent to inhibit caspase activity in COLO205 cells (Supplementary Figure S2B) and had no off-target effects on de-phosphorylation of ERK1/2 or expression of BIM (Supplementary Figure S2C). This indicates that although caspases are activated during MEK inhibitor-induced cell death in COLO205 and LS411 cells, death can proceed through an alternative pathway if caspase activation is blocked.

Inhibition of MEK1/2 results in de-phosphorylation and accumulation of BIM protein

BIM is rate determining for cell death following withdrawal of survival factors (Bouillet *et al.*, 1999; Ewings *et al.*, 2007a). BIM$_{EL}$ was the major BIM isoform present in COLO205 and HT29 cells maintained in 10% FBS and migrated as a diffuse hyperphosphorylated series of bands (Figures 5a and b). Although serum starvation inactivates ERK1/2 and promotes BIM expression in fibroblasts (Weston *et al.*, 2003; Ewings *et al.*, 2007a), it did not inactivate ERK1/2 and caused little increase in BIM expression in CRC cells (Figures 5a

and b). However, the addition of U0126 to cells (whether in 10% FBS or serum starved) resulted in the rapid de-phosphorylation of ERK1/2 and BIM_{EL} and an increase in BIM_{EL} levels. U0126 did not affect the antiapoptotic BCL-2 proteins, except at 24 h when decreased levels of BCL-2 and MCL-1 were observed (Figure 5a). BIM expression is also repressed by the phosphatidylinositol 3-hydroxykinase (PI3K)-dependent protein kinase B (PKB) pathway (Dijkers *et al.*, 2000; Gilley *et al.*, 2003), but U0126 did not influence PKB phosphorylation (Figure 5a). Furthermore, serum starvation caused de-phosphorylation of PKB but had little effect on BIM levels (Figure 5a); similar results were observed in LS411 and CO115 cells (Supplementary Figure S3A and B) despite the very strong constitutive activation of PKB in CO115 cells (Supplementary Figure S3C). These data demonstrate that $BRAF^{V600E}$-positive CRC cells are growth factor independent for ERK1/2 activation and repression of BIM, this can be reversed by the administration of an MEK1/2 inhibitor and the ERK1/2 pathway is the dominant pathway for the repression of BIM in these cells.

AZD6244 (ARRY-142886) is a potent and selective MEK1/2 inhibitor that is undergoing pre-clinical and clinical evaluation (Wallace *et al.*, 2006; Davies *et al.*, 2007) and which does not inhibit the ERK5 pathway (Balmanno *et al.*, submitted). AZD6244 was able to cause a substantial increase in the basal expression of BIM, predominantly BIM_{EL}, even in COLO205 cells maintained in FBS (Figure 5c). Treatment of HT29 cells with AZD6244 overcame growth factor-independent survival, resulting in substantial cell death (Figure 5d), and also caused the de-phosphorylation of BIM_{EL} and enhanced its expression upon serum withdrawal (Figure 5e). Thus, the MEK1/2-specific clinical candidate, AZD6244, replicated our results with U0126.

Reduced BIM expression protects HT29 cells against death induced by U0126 and AZD6244

Growth factor withdrawal-induced cell death requires *de novo* protein synthesis in some systems (Weston *et al.*, 2003). Indeed, cycloheximide protected cells from caspase activation arising from MEK inhibition (Supplementary Figure S4A), exacerbated the reduction in MCL-1 and also reduced both basal and induced expression of BIM_{EL} (Supplementary Figure S4B). As BIM expression decreases and cells are protected under these conditions, we considered that BIM might contribute to MEK inhibitor-induced cell death. To test this, we used RNA interference to reduce BIM expression. HT29 cells formed the focus of the RNAi experiments as their death was largely caspase dependent (Figure 4f), and BIM is primarily involved in the activation of caspase-dependent death pathways.

To assess the function of BIM, we used lentivirus (Wiznerowicz and Trono, 2003) to deliver short hairpin RNA against BIM to HT29 cells. BIM protein expression was reduced by 50-60% though it was not possible to fully prevent the increase in BIM_{EL} following treatment with serum-free medium (SF) with U0126 (SF + U0126) (Supplementary Figure S5A); other members of the BCL-2 family were unaffected. Overexposure of the BIM blot revealed knockdown of smaller BIM splice variants. A control virus expressing the same sequence but with four mismatches (BIM-MM) did not affect BIM protein levels. Treatment with SF + U0126 in WT cells increased the fraction of cells with sub-G1 DNA and this was significantly reduced in the BIM RNAi cells, whereas the BIM mismatch sequence had no effect (Supplementary Figure S5B). As a control, cisplatin-induced cell death was unaffected by the short hairpin RNAs used.

This partial reduction in cell death could reflect partial knockdown of BIM, a partial function for BIM or adaptation during the selection of virally infected cells. To address this, we used siRNA oligos to knock down human BIM transiently (Figure 6a). Knockdown of BIM was complete under these conditions (Figure 6b) and reduced death arising from the combination of serum withdrawal and U0126 or AZD6244 by 60% (Figure 6c). The

corresponding siRNA oligos from mouse BIM, used as a control (Figure 6a), failed to reduce BIM expression and had no effect on cell death (Figures 6b and c). Thus, BIM contributes in large part to cell death arising when HT29 cells are serum starved in the presence of U0126 or AZD6244.

$BRAF^{V600E}$ provides a constitutive MEK-dependent signal for BIM_{EL} degradation

Transcription of BIM is repressed by the PI3K-dependent regulation of FOXO-3A (Dijkers *et al.*, 2000; Gilley *et al.*, 2003). However, the ERK1/2 pathway can also repress BIM mRNA levels in fibroblasts (Weston *et al.*, 2003) and epithelial cells (Reginato *et al.*, 2005). When HT29 cells were deprived of growth factors, we found that BIM mRNA expression increased after withdrawal of growth factors but this was not enhanced further by MEK inhibition (Supplementary Figure S5C). Together, these data indicate a relatively minor function for the ERK1/2 pathway in repressing BIM mRNA levels in HT29 cells.

BIM_{EL}, the most abundant form of BIM in all four cell lines, undergoes proteasomal degradation after phosphorylation by ERK1/2 (Ley *et al.*, 2003; Luciano *et al.*, 2003; Ley *et al.*, 2004; Marani *et al.*, 2004); consequently, we examined the turnover of BIM_{EL} in COLO205 and HT29 cells. Cells were serum starved in the presence of U0126 for 18 h to increase the level of BIM protein. Cells were then washed to remove U0126 and subjected to an emetine chase in SF media, with or without fresh U0126 (Figure 7a). In both cell lines, ERK1/2 was rapidly re-activated in fresh SF medium, resulting in the rapid phosphorylation and degradation of BIM_{EL}; U0126 reversed all of these effects (Figure 7b). These results indicate that there is a strong, constitutive, MEK-dependent signal for degradation of BIM_{EL} in CRC cells harbouring $BRAF^{V600E}$.

ERK1/2-dependent phosphorylation of BIM_{EL} also inhibits its binding to pro-survival BCL-2 proteins such as MCL-1 (Ewings *et al.*, 2007a; Ewings *et al.*, 2007b). To investigate if $BRAF^{V600E}$ signalling could regulate BIM_{EL}−MCL-1 complexes, COLO205 and HT29 cells were treated with SF + U0126 for 18 h to induce BIM expression and the formation of BIM_{EL}−MCL-1 complexes; cells were then washed to remove U0126 and placed in fresh SF media (Figure 7c). Reactivation of ERK1/2 was complete within 30 min in COLO205 cells or 10 min in HT29 (Figure 7d). In both cases, as the ERK1/2 was reactivated, BIM_{EL} became phosphorylated and the amount of BIM_{EL} recovered in MCL-1 IPs was reduced. In both cell lines, this dissociation of BIM_{EL} from MCL-1 preceded any decrease in total BIM_{EL}. For example, loss of BIM_{EL} from MCL-1 IPs occurred within 30 min in COLO205 cells at which point total BIM_{EL} levels were unchanged; in HT29 cells, loss of BIM_{EL} from MCL-1 IPs occurred within 10 min and again total BIM_{EL} levels were unchanged (Figure 7d). Indeed, the dissociation of BIM_{EL} from the MCL-1−BIM_{EL} complex is not due to ERK-dependent ubiquitination or proteasomal degradation of BIM (Ewings *et al.*, 2007a). Study of the BCL-X_L−BIM complex showed a similar pattern of dissociation as seen with MCL-1 (data not shown). Thus, the constitutive activation of the ERK1/2 pathway by $BRAF^{V600E}$ in CRC cells promotes dissociation of BIM_{EL} from its pro-survival target proteins and its proteasomal degradation.

Discussion

Growth factor-independent cell proliferation requires that cancer cells evade growth factor withdrawal-induced cell death; indeed, these are both hallmarks of cancer cells (Hanahan and Weinberg, 2000). Presumably, tumour cells must evolve mechanisms to repress or tolerate BIM. Several studies have shown that activation of ERK1/2 can block BIM expression and prevent cell death arising from growth factor withdrawal but these have usually involved ectopic overexpression of RAF or MEK mutants that are not found in human tumours, raising concerns about their physiological relevance. Here we have studied

MEFs from knock-in transgenic mice that exhibit conditional expression of a single *Braf*^{V600E} allele and CRC cells harbouring a single *BRAF*^{V600E} allele; in both cases, these mutant oncoproteins are expressed from their endogenous promoters rather than being overexpressed.

The importance of the Lox-STOP-Lox system is best exemplified by studies of genetically engineered mice with *K-ras*^{G12D} alleles (Frese and Tuveson, 2007). Although conditional overexpression of ectopic *K-ras*^{G12D} promotes proliferation and tumour initiation in several tissues, these models do not always faithfully reproduce the development of human cancers with *KRAS*^{G12D} mutations due to supraphysiological RAS signalling. In contrast, the expression of endogenous *K-ras*^{G12D} alleles by crossing K-ras$^{+/LSL\text{-}G12D}$ mice with appropriate cre transgenic mice provides exquisite temporal and spatial control over oncogene expression. In the case of the lung and pancreas, this leads to the development of benign adenomas and eventually adenocarcinomas with many of the histopathological and molecular characteristics of human non-small-cell lung carcinoma and pancreatic ductal adenocarcinoma, respectively (Jackson *et al.*, 2001; Hingorani *et al.*, 2003; Tuveson *et al.*, 2004). These studies highlight the advantage of expressing oncogenes from endogenous alleles and are helping to define the relative importance of various RAS effector pathways in tumour development. An important advance in our study was the use of primary MEFs derived from *Braf*$^{+/LSL\text{-}V600E}$;*CreER*™ mice (Mercer *et al.*, 2005). Treatment of these MEFs with 4-HT allowed full recombination of the *Braf*$^{+/LSL\text{-}V600E}$ allele, the first time this has been demonstrated with the CreER™ system for any floxed allele. Significantly, expression of a single *Braf*^{V600E} allele and resultant activation of the endogenous ERK1/2 pathway protected against growth factor withdrawal and completely blocked the otherwise substantial increase in BIM expression. The fact that this was observed in primary MEFs indicates that this pathway alone is sufficient to repress BIM expression and cell death.

Prompted by these results, we also examined CRC cell lines harbouring a single *BRAF*^{V600E} allele and exhibiting a strong constitutive activation of ERK1/2. We found that (i) these cells were growth factor independent for survival; (ii) inhibition of the ERK1/2 pathway promoted cell death and this was substantially dependent upon BIM and (iii) constitutive ERK1/2 signalling is responsible for repressing BIM expression and function. The ‘oncogene addiction’ hypothesis posits that tumours evolve an unusual dependence upon certain oncogenes and the signalling pathways they control to maintain the malignant phenotype; for example, CRC cells with mutations in *KRAS* are addicted to the mutant KRAS oncoprotein (Shirasawa *et al.*, 1993). We found that CRC cells with BRAF^{V600E} were growth factor independent for cell survival and this could be overcome by any of the three distinct MEK inhibitors (U0126, PD184352 or AZD6244). Indeed, in some cases, MEK inhibition alone could induce cell death in the presence of FBS, indicating the degree to which these cells have evolved an extreme dependency upon the ERK1/2 pathway for survival. This is all the more remarkable as some of these CRC cell lines also harbour *PIK3CA* mutations (HT29) and/or exhibit strong basal PKB activation (CO115 cells) (Supplementary Figure S3B), a pathway that promotes cell survival. This underlines the extent to which these cells are addicted to the ERK1/2 pathway for growth factor-independent survival and suggests that inhibition of ERK1/2 signalling may be particularly effective in killing BRAF^{V600E}-positive CRC cells. Similar conclusions have been drawn in BRAF^{V600E}-positive melanoma (Karasarides *et al.*, 2004).

There is a prominent function for increased BIM expression in death arising from growth factor withdrawal in MEFs (Ewings *et al.*, 2007a). We found that the expression of endogenous Braf^{V600E} was sufficient to block BIM expression in *Braf*$^{+/LSL\text{-}V600E}$;*CreER*™ MEFs (Figure 3a). Similarly, all the four BRAF^{V600E}-positive CRC cell lines failed to increase BIM expression unless serum starvation was combined with MEK inhibition;

indeed, in some cases, the administration of U0126 or AZD6244 to cells in complete medium was sufficient to increase BIM expression, indicating that these cells are addicted to the ERK1/2 pathway for repression of BIM, even when they are exposed to growth factor-rich FBS, which activates the PI3K-PKB pathway, possess PIK3CA mutations or exhibit strong basal PKB activity, such as CO115 cells (Supplementary Figure S3B).

BIM was specifically implicated in death arising from MEK inhibition by the use of a BIM-specific short hairpin RNA and two different BIM-specific siRNAs, which reduced cell death by at least 60%. However, whereas BIM is involved in death arising from MEK inhibition in these CRC cells, it may not be the only regulator. For example, inhibition of MEK in the presence of FBS caused de-phosphorylation of BIM_{EL} and some increase in BIM expression but only a modest increase in cell death. This may indicate that there is a crucial threshold level of BIM required for cell death that is only achieved upon serum withdrawal and MEK inhibition or that other important regulators are also induced by serum withdrawal and MEK inhibition. In addition, even when the siRNA-mediated knockdown of BIM was complete (Figure 6b), this did not completely prevent cell death (Figure 6c), again suggesting that other regulators are operating in parallel; likely candidates might include BAD, which is regulated by both the ERK1/2-RSK and PKB pathways (Datta *et al.*, 1997; Eisenmann *et al.*, 2003; Boisvert-Adamo and Aplin, 2008).

We observed little evidence of regulation of BIM mRNA levels by the ERK1/2 pathway in either MEFs or CRC cells. Furthermore, in both cell systems, BIM_{EL} was by far the most abundant isoform and was certainly the major isoform that was dynamically regulated by MEK inhibition. Among the canonical splice forms, BIM_{EL} is unique in being subject to extensive multisite phosphorylation by ERK1/2, which targets it for polyubiquitination and proteasomal degradation. Indeed, the downregulation of BIM_{EL} in MEFs was reversed by MG132 (Figure 3c), and both COLO205 and HT29 cells exhibited a strong constitutive MEK-dependent signal for BIM_{EL} degradation (Figure 7b). Although growth factor independent for ERK1/2 activity, the CRC cells remained growth factor dependent for PKB activation (Figure 5a), so inactivation of the PI3K-PKB pathway upon serum withdrawal may contribute to increases in *BIM* mRNA levels, perhaps by the activation of FOXO3A (Dijkers *et al.*, 2000; Gilley *et al.*, 2003). However, the fact that serum withdrawal alone caused little or no increase in BIM protein expression in 4-HT-treated MEFs or CRC cells suggests that any mature BIM_{EL} that is expressed following serum withdrawal is rapidly phosphorylated by ERK1/2 and thereby degraded. Thus, ERK1/2-dependent turnover of BIM_{EL} appears to be the dominant signal responsible for restraining BIM expression in both MEFs and CRC cells with $BRAF^{V600E}$.

BAX and BAK are thought to be kept in check by the pro-survival BCL-2 proteins until liberated by binding of the BH3-only proteins to the pro-survival proteins (Chen *et al.*, 2005; Willis *et al.*, 2007). In addition to protein turnover, ERK1/2-dependent phosphorylation of BIM_{EL} can prevent its binding to pro-survival BCL-2 proteins (Ewings *et al.*, 2007a; Ewings *et al.*, 2007b), whereas withdrawal of growth factors and/or inhibition of ERK1/2 promotes the association of BIM_{EL} with MCL-1. Here we have demonstrated that this is also the case in CRC cells where washout of U0126 and reactivation of ERK1/2 resulted in the phosphorylation of BIM_{EL} and its dissociation from MCL-1. Thus, the constitutive activation of ERK1/2 observed in COLO205 and HT29 cells drives dissociation of BIM_{EL} from MCL-1 and subsequent BIM_{EL} turnover.

The ability of BIM to promote apoptosis and its expression following withdrawal of growth factors suggest that BIM has some of the credentials of a tumour suppressor protein. Indeed, the loss of one *Bim* allele accelerates Myc-induced leukaemia in the mouse (Egle *et al.*, 2004), and disruption of the *BIM* locus has been observed in various haematological

malignancies, most notably in 17% of mantle cell lymphomas (Tagawa *et al.*, 2005). In contrast, the disruption of the *BIM* gene seems to be rare in solid tumours and yet many tumour cell lines exhibit very low levels of BIM, suggesting that alternative mechanisms are employed to repress BIM. These may include epigenetic mechanisms in renal cell carcinoma (Zantl *et al.*, 2007), but activation of signalling pathways downstream of oncogenes is emerging as one such mechanism. Mutant forms of EGFR can repress BIM expression in lung cancer, and this is overcome by EGFR inhibitors that are in clinical use (Costa *et al.*, 2007; Cragg *et al.*, 2007; Gong *et al.*, 2007). Our demonstration that BIM is repressed in an ERK1/2-dependent manner in CRCs harbouring $BRAF^{V600E}$ suggests that increased expression of BIM may contribute to cell death in response to inhibitors of the BRAF–MEK–ERK1/2 pathway such as AZD6244. Finally, it is important to note that the inhibition of the ERK1/2 pathway appears to initiate parallel caspase-dependent and caspase-independent cell death pathways in COLO205 and LS411 cells. Future studies should seek to investigate the pathway of caspase-independent cell death in these cells as it may be important in the response of CRC cells to ERK1/2 pathway inhibitors.

Materials and methods

Cells and cell lines

Primary MEFs were prepared from E14.5 embryos as described earlier (Hüser *et al.*, 2001). All studies were performed on MEFs following immediate isolation from the embryo. MEFs and the CRC cell lines, COLO205, HT29 and CO115, were maintained in Dulbecco's Modified Eagle's high-glucose Medium (DMEM, Invitrogen, Carlsbad, CA, USA). The LS411 cell line was maintained in RPMI (Invitrogen). Both media were supplemented with 10% FBS (Invitrogen), 20 mM L-glutamine (Invitrogen) and 100 U streptomycin (Invitrogen). All four CRC lines harbour the $BRAF^{V600E}$ mutation and have wild-type KRAS and mutant p53.

Antibodies

The following antibodies were used for western blotting: BCL-2 (Santa Cruz; 7382); BCL-X_L (Cell Signalling Technology, Danvers, MA, USA; 2762); BIM (Chemicon, Temecula, CA, USA; AB17003); BIM (rat, for analysis of immunoprecipitates) (Calbiochem/Novabiochem, San Diego, CA, USA; AM53); MCL-1 (Santa Cruz, CA, USA; sc-819); phospho-Akt/PKB (Cell Signalling Technologies; 9271); Akt/PKB (Cell Signalling Technologies; 9272); phospho-ERK1/2 (Cell Signalling Technologies; 9106); and ERK1 (BD Biosciences, San Jose, CA, USA; 61003). The ERK2 antibody was a gift from Professor Chris Marshall (ICR, London).

Immunoprecipitation and western blot analysis

At the required times, cells were lysed in Triton-Glycerol (TG) lysis buffer and analysed by western blot as described earlier (Weston *et al.*, 2003; Todd *et al.*, 2004). For immunoprecipitation, cell extracts were normalized for equal protein loading and incubated at 4 °C with MCL-1 antibody (sc-819) bound to protein A sepharose beads (Sigma, St Louis, MO, USA).

Apoptosis assays

Cell extracts were assayed for DEVDase activity as described earlier (Weston *et al.*, 2003). The proportion of cells with hypo-diploid (sub-G1) DNA was assessed by propidium iodide staining and flow cytometry (Weston *et al.*, 2003; Todd *et al.*, 2004). The annexin V assay was performed as described earlier (Hüser *et al.*, 2001). Changes in mitochondrial membrane potential were examined by flow cytometric analysis of cells stained with

tetramethylrhodamine ethyl ester (Molecular Probes, Eugene, OR, USA) (Scaduto and Grotyohann, 1999), a cell-permeable dye accumulating in mitochondria with unaltered membrane potential. Cells were harvested by trypsinization and 5×10^5 cells were incubated with 20 nM tetramethylrhodamine ethyl ester for 20 min at 37 °C followed by the analysis with FACScan (20 000 cells per sample). The fluorescence intensity of tetramethylrhodamine ethyl ester was monitored at 582 nm (FL-2).

Quantitative reverse transcription PCR

Total RNA was extracted from cells using TRI-Reagent (Sigma) and reverse transcription PCR was performed according to the protocol supplied with the TaqMan Reverse Transcription reagents (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was determined to be the most stable housekeeping gene using the GeNorm protocol (Vandesompele *et al.*, 2002) and the following primers were used (5′-TGCACCACCAACTGCTTAGC; 3′-GGCATGGACTGTGGTCATGAG). Primers for human BIM were 5′-ACCTTCTGATGTAAGTTCTGAGTGTGA and 3′-GGATTACCTGTGGCTCTGTCTG. The primers for mouse BIM were 5′-GTCCTCCAGTGGGTATTTCT and 3′-CAGATCTTCAGGTTCCTCCT. Quantitative PCR was performed using SYBR Green (Applied Biosystems, Foster City, CA, USA) in a Chromo-4 Thermal Cycler analysed with Opticon Software (BioRad, Hercules, CA, USA).

siRNA sequences and RNAi

For transient RNAi, the following oligos were used: human BIM1 GACCGAGAAGGTAGACAATT; human BIM2 GCAACCTTCTGATGTAAGT; mouse Bim1 GGAGGAACCTGAAGATCTG; the mouse Bim sequence has four mismatches when compared with the human BIM and served as a specificity control. HT29 cells were plated the day before transfection at 2×10^5 per well (six-well tray) in pen/strep-free medium. Briefly, 500 pmol of each human Bim siRNA was mixed with Optimem media (1000 pmol for control mouse siRNA), and an equivalent volume of Optimem was combined with Lipofectamine 2000 and incubated for 5 min. Both siRNA and Lipofectamine 2000 were combined, mixed well and incubated for 20 min. Following incubation, siRNA/Lipofectamine complexes were added dropwise to cell cultures. Transfection medium was aspirated after 24 h and individual drug treatments were initiated for a further 30 h incubation.

Statistical analysis

Results were analysed for statistical significance using analysis of variance with a Tukey's post-test for parametric data and Kruskal-Wallis test for non-parametric data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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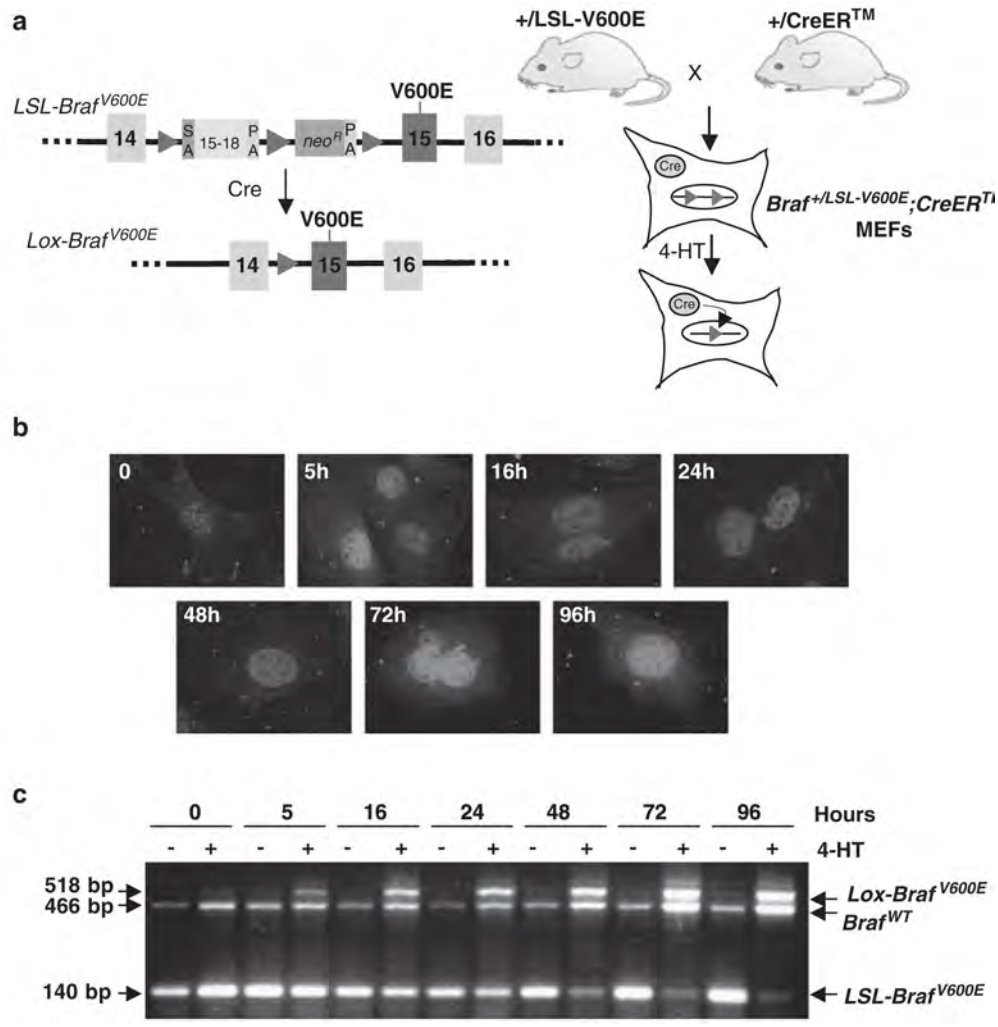

Figure 1.

Regulation of endogenous BRAF^{V600E} expression using CreER™. (**a**) Induction of ^{V600E}BRaf by CreER™. Conversion of the *LSL-Braf^{V600E}* allele expressing ^{WT}BRaf to the *Lox-Braf^{V600E}* allele expressing ^{V600E}BRaf is shown. This allele is described in full in Mercer *et al.* (2005). Mice heterozygous for *Braf$^{LSL-V600E}$* and *CreER*™ alleles were intercrossed and MEFs were obtained from resulting E14.5 embryos. To induce activation of Cre, and thus LSL recombination, MEFs were treated with 4-HT. (**b**) Immunofluorescence of Cre. *Braf$^{+/LSL-V600E}$;CreER*™ MEFs were treated over a time course of up to 96 h with 4-HT and immunostained with a Cre antibody (green), counterstained with DAPI (blue) and the images merged. 4-HT induces a rapid translocation of the CreER™ protein within 5 h, and it remains here throughout the time course. (**c**) PCR detection of *LSL-Braf^{V600E}* recombination. *Braf$^{+/LSL-V600E}$*;CreER™ MEFs were treated with 4-HT or carrier control for the time course indicated, genomic DNA was isolated and subjected to PCR with the primers described in Mercer *et al.* (2005). Recombination is first detected within 5 h of 4-HT treatment and continues to increase in level throughout the time course. By 96 h, recombination is almost complete as indicated by the virtual absence of the *LSL-Braf^{V600E}* allele and the high level of the *Lox-Braf^{V600E}* allele. DAPI, 4′,6-diamidino-2-phenylindole; MEFs, mouse embryonic fibroblasts.

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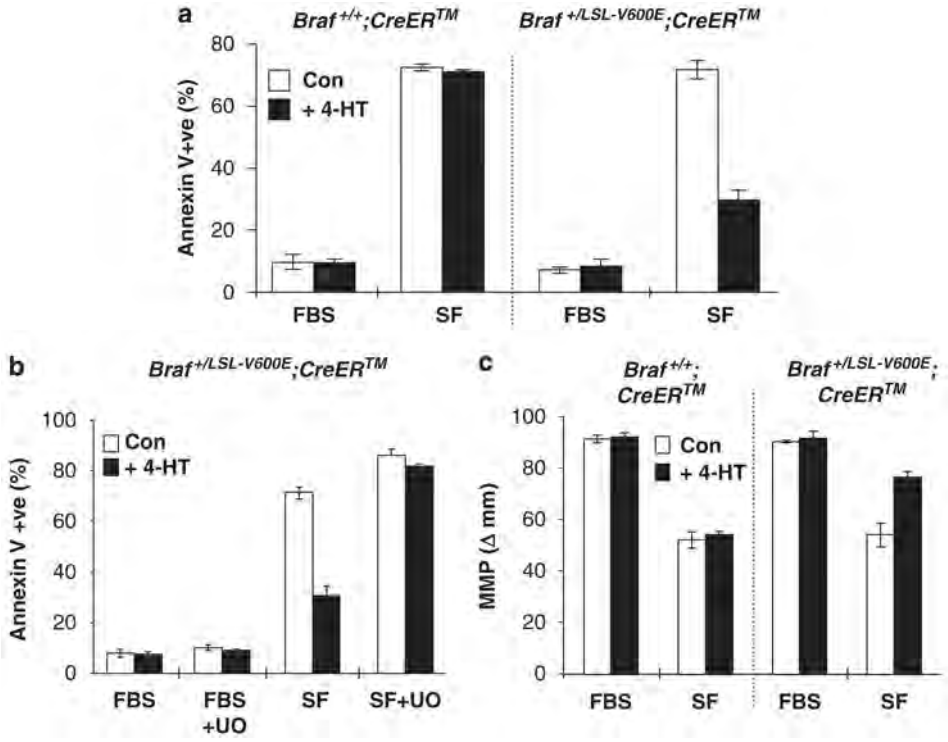

Figure 2.

Conditional expression of a single endogenous $BRAF^{V600E}$ allele protects MEFs from growth factor withdrawal. (**a**) $Braf^{LSL\text{-}V600E}$ MEFs and control $Braf^{+/+}$ MEFs containing the CreER™ allele were treated with 4-HT or carrier control for 96 h after which they were treated for 48 h in the presence (FBS) or absence (SF) of growth factors. The cells were subjected to an annexin V assay. Results are the mean ± s.e.m. pooled from at least three experiments. (**b**) $Braf^{LSL\text{-}V600E}$ MEFs containing the CreER™ allele were treated with 4-HT or carrier control for 96 h after which they were treated for 48 h in the presence (FBS) or absence (SF) of growth factors, with or without 10 μ_M U0126 and subjected to an annexin V assay. Results are the mean ± s.e.m. pooled from at least three experiments. (**c**) $Braf^{LSL\text{-}V600E}$ MEFs and control $Braf^{+/+}$ MEFs containing the CreER™ allele were treated with 4-HT or carrier control for 96 h, they were then treated for 48 h in the presence (FBS) or absence (SF) of growth factors after which MMPwas measured. Results are the mean ± s.e.m. pooled from at least three experiments. FBS, fetal bovine serum; MEFs, mouse embryonic fibroblasts.

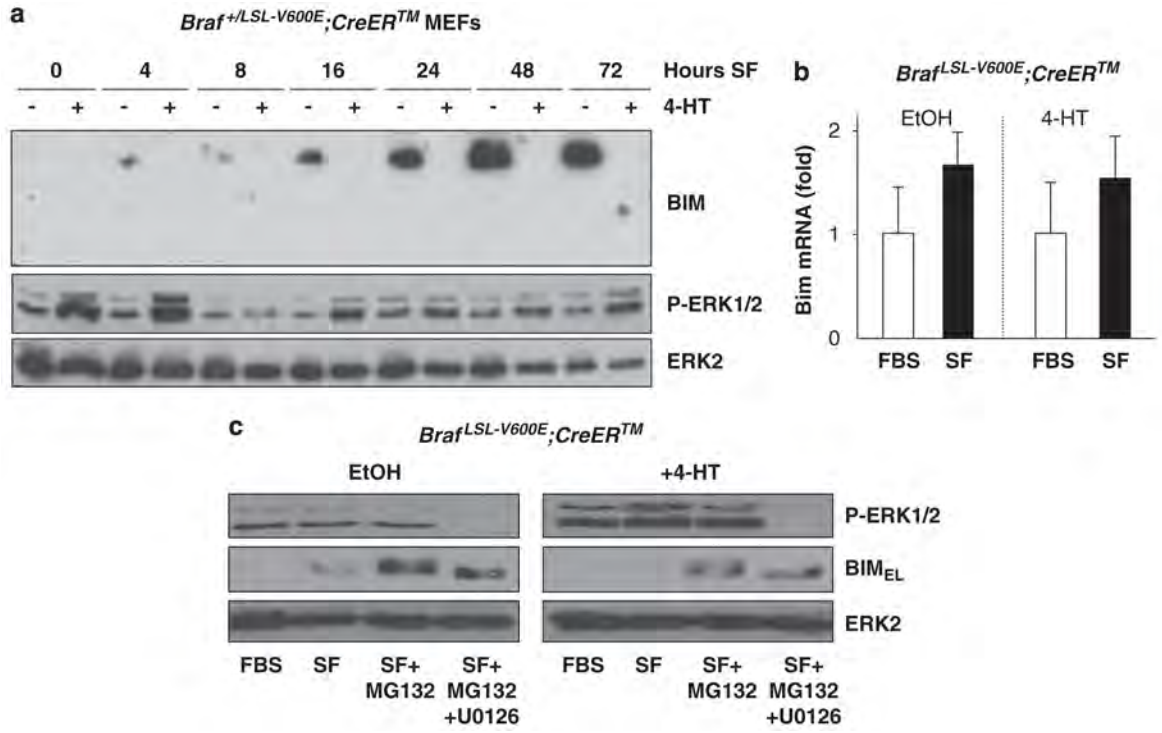

Figure 3.

Expression of a single endogenous BRAF^{V600E} allele inhibits the expression of BIM$_{EL}$ following growth factor withdrawal. (**a**) *Braf*$^{LSL\text{-}V600E}$ MEFs containing the CreER™ allele were treated with (+) or without (−) 4-HT for 96 h after which they were grown in the absence of growth factors (SF) over a time course of up to 72 h. Protein lysates were prepared at each time point, and western blots prepared and analysed with antibodies for BIM, phospho-ERK and ERK2. (**b**) *Braf*$^{LSL\text{-}V600E}$ MEFs containing the CreER™ allele were treated with or without 4-HT for 96 h after which they were treated for 48 h in the presence (FBS) or absence (SF) of growth factors. BIM mRNA levels were normalized to those of GAPDH, and the result is the mean ± s.e.m. of three experiments. (**c**) *Braf*$^{LSL\text{-}V600E}$ MEFs containing the CreER™ allele were treated with or without 4-HT for 96 h after which they were treated for 48 h in the presence (FBS) of growth factors, in the absence (SF) of growth factors, in SF with 30 μM MG132 or in SF with 30 μM MG132 and 10 μM U0126. Protein lysates were prepared and subjected to western blot analysis with the antibodies indicated as described in Materials and methods. ERK2, extracellular signal-regulated kinase 2; FBS, fetal bovine serum; MEFs, mouse embryonic fibroblasts.

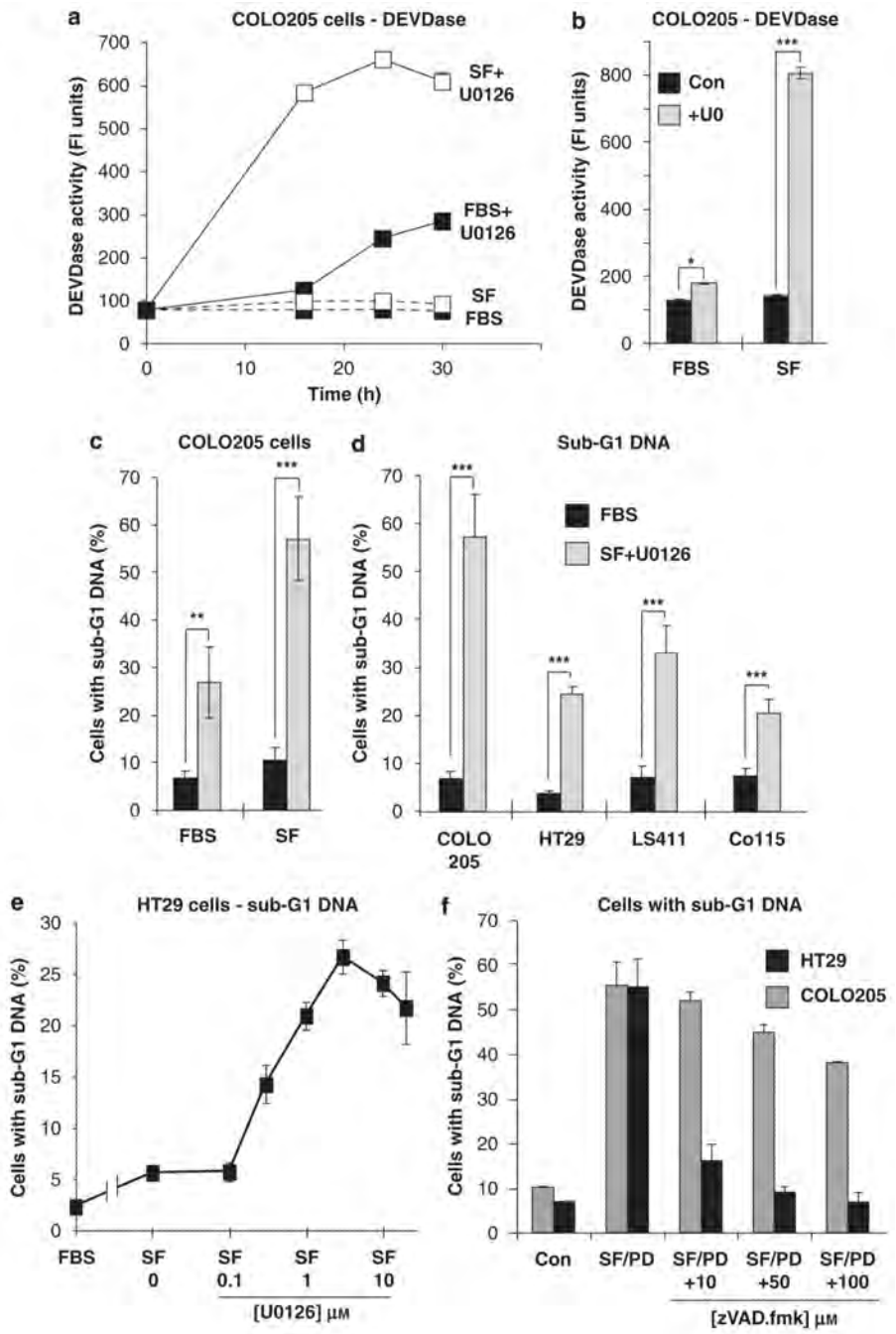

Figure 4.

Inhibition of MEK overcomes growth factor-independent survival in CRC cells with $BRAF^{V600E}$. (**a** and **b**) COLO205 cells were serum starved (SF) or placed in fresh FBS-containing medium (FBS) with or without 20 μM U0126 as indicated. Cells were then assayed for DEVDase activity and each data point represents (**a**) the mean ± range of duplicate dishes of cells or (**b**) the mean ± s.e.m. pooled from at least three experiments performed in duplicate. (**c**) COLO205 cells were treated 30 h in the presence (FBS) or absence (SF) of serum, with or without 20 μM U0126 and analysed for the percentage of cells with sub-G1 DNA. (**d**) The indicated cell lines were treated for 30 h in the presence

(FBS) or serum starved with 20 μM U0126 and analysed for the percentage of cells with sub-G1 DNA. (**e**) HT29 cells were maintained in FBS or serum starved in the presence of increasing concentrations of U0126 for 30 h and analysed for the percentage of cells with sub-G1 DNA. (**f**) HT29 and COLO205 cells were maintained in FBS or serum starved with 2 μM PD184352 in the presence of increasing doses of zVAD.fmk for 30 h before being analysed for the percentage of cells with sub-G1 DNA. In (**a**), (**e**) and (**f**), results are mean ± s.d. from a single experiment performed with biological triplicates; similar results were obtained in two further experiments. In (**b-d**), results are the mean ± s.e.m. pooled from at least three experiments performed in duplicate ± s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$. CRC, colorectal cancer; FBS, fetal bovine serum.

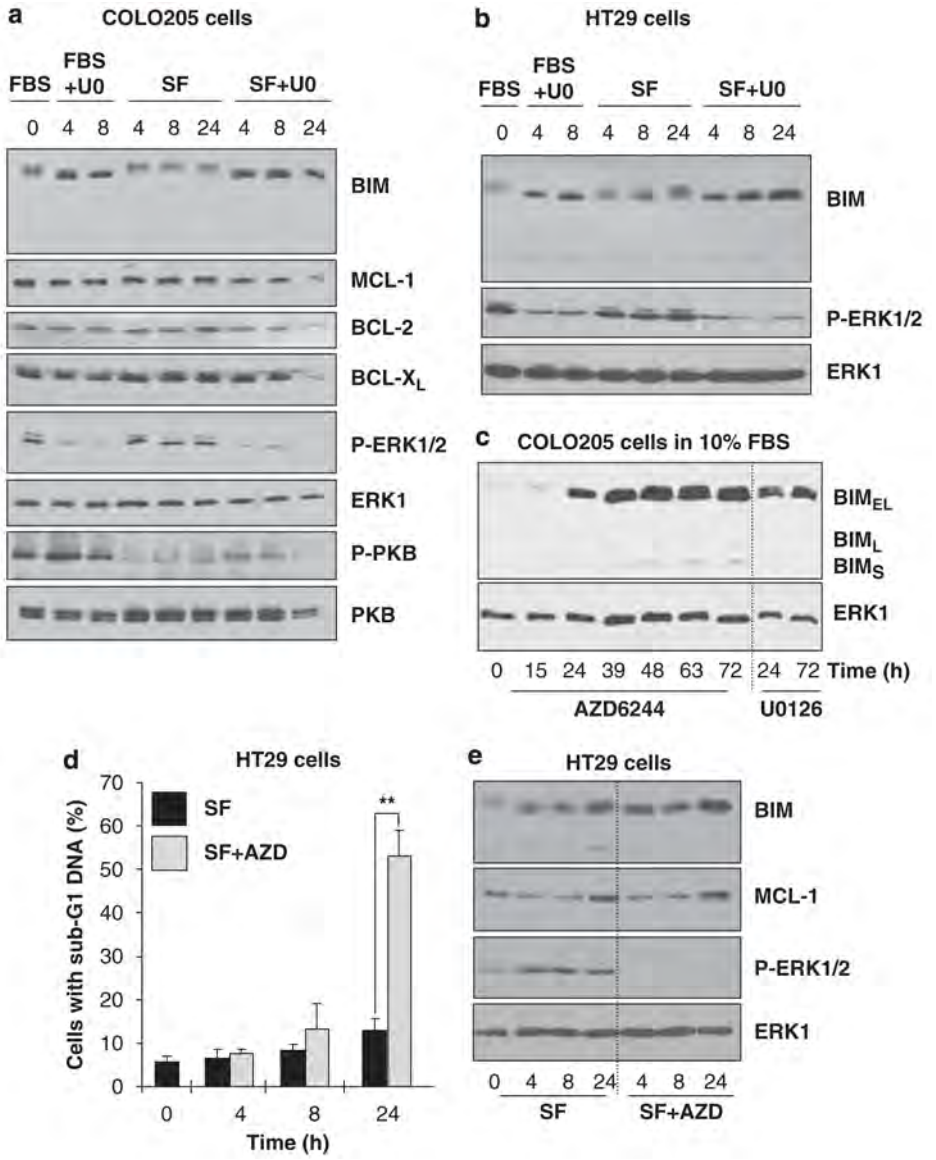

Figure 5.

MEK inhibition promotes de-phosphorylation and increased expression of BIM_{EL} in CRC cells with $BRAF^{V600E}$. COLO205 cells (**a**) and HT29 cells (**b**) were maintained in FBS or serum starved (SF) with or without 20 μM U0126 before lysis and western blotting analysis as described in Materials and methods. Results are representative of three independent experiments for each cell line. Similar results were obtained in LS411 and CO115 cells (Supplementary Figure S4). (**c**) COLO205 cells maintained in FBS were treated with 2 μM AZD6244 or 10 μM U0126 for the indicated times and probed for BIM expression by western blot; ERK1 was used as a loading control. (**d** and **e**) HT29 cells were serum starved with or without 2 μM AZD6244 for indicated times. (**d**) The percentage of cells with sub-G1 DNA was determined by flow cytometry and data shown are mean ± s.d. of biological triplicates from a single experiment representative of three. ** $P<0.01$. (**e**) Cell lysates were probed for BIM, MCL-1 and P-ERK1/2 by western blot; ERK1 was used as a loading control. Results are representative of three independent experiments. CRC, colorectal cancer; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum.

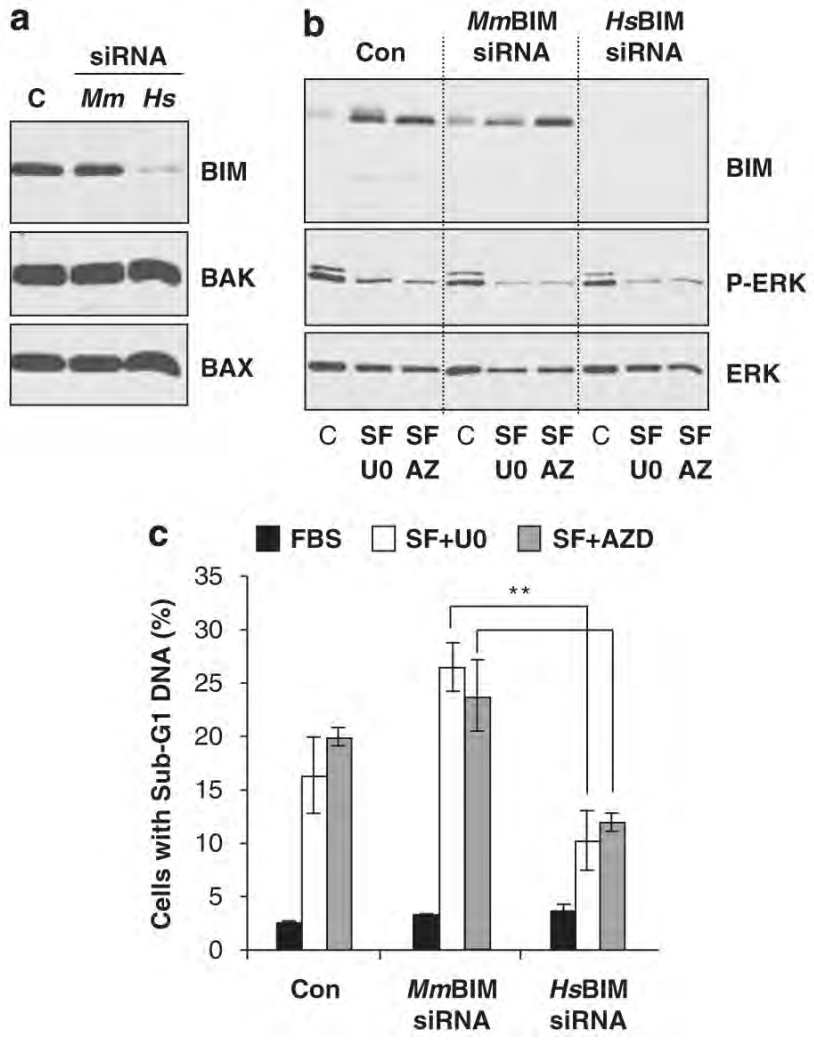

Figure 6.

siRNA-mediated silencing of BIM protects HT29 cells against death induced by MEK inhibition. (**a**) HT29 cells were mock transfected (**c**) or transfected with murine (Mm) or human (Hs) BIM-specific siRNA oligos. The cells were then serum starved in the presence of U0126 to induce BIM. Whole-cell extracts were probed for BIM, BAK and BAX expression. (**b** and **c**) HT29 cells in six-well plates were mock transfected (Con) or transfected with murine (Mm) or human (Hs) BIM-specific siRNA oligos; four wells were transfected in each condition. Cells were then left in complete medium or switched to serum-free medium with 20 μ_M U0126 (SF U0) or 2 μ_M AZD6244 (SF AZ) for 30 h. (**b**) One well of each condition was used to blot for BIM, P-ERK1/2 and ERK1. (**c**) Three wells of each condition were analysed for the percentage of cells with sub-G1 DNA. Data are mean ± s.d. of biological triplicates from a single experiment; similar results were obtained in a total of three experiments. ** P<0.005. ERK1/2, extracellular signal-regulated kinase 1/2; siRNA, small interfering RNA.

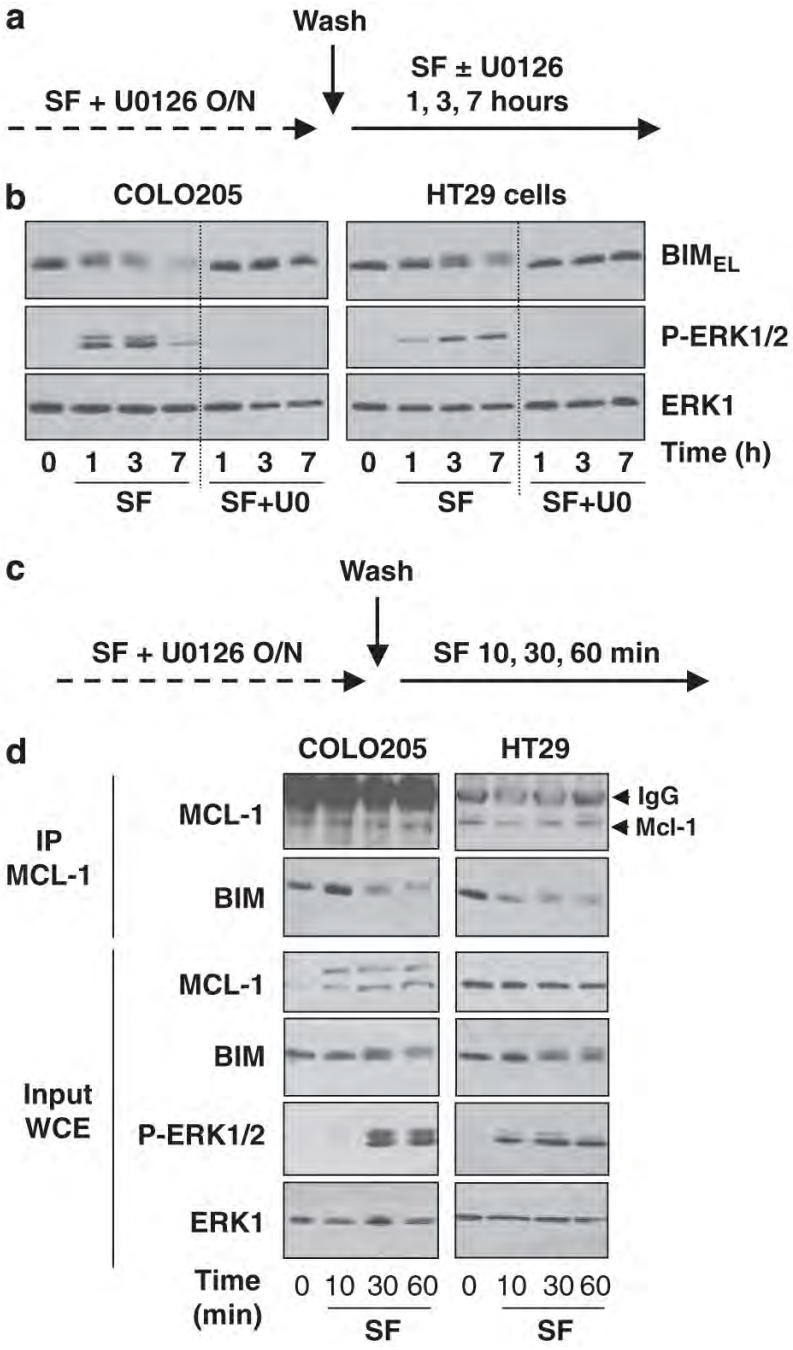

Figure 7.

CRC cells with BRAF^{V600E} exhibit a strong constitutive MEK-dependent degradation of BIM$_{EL}$ and dissociation from MCL-1. (**a**) Schematic representation of treatment regimen for BIM$_{EL}$ turnover. (**b**) COLO205 and HT29 cells were treated overnight with 20 μM U0126 in the absence of growth factors. The cells were pre-treated for 30 min with 10 μM emetine, washed and fresh growth factor-free medium containing emetine with or without U0126 was added for up to 7 h. Cells were harvested for western blotting as described in Materials and methods. Results are representative of three experiments in each case. (**c**) Schematic representation of treatment regimen for BIM$_{EL}$ dissociation from MCL-1. (**d**) COLO205 and

HT29 cells were treated overnight with 20 μ_M U0126 in the absence of growth factors. The cells were washed thoroughly to remove U0126 and fresh serum-free media added for 10, 30 or 60min. Cells were harvested, MCL-1 immunoprecipitated and probed for association with BIM_{EL}. Whole-cell lysates were also probed for MCL-1, BIM, P-ERK1/2 and ERK1. Results are representative of three experiments in each case. CRC, colorectal cancer; ERK1/2, extracellular signal-regulated kinase 1/2.

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