British Journal of Cancer (2012) 106, 1660–1667

www.bjcancer.com

The cap-translation inhibitor 4EGI-1 induces apoptosis in multiple myeloma through Noxa induction

G Descamps[1], P Gomez-Bougie[1,2], J Tamburini[3,4], A Green[3], D Bouscary[3,4], S Maïga[1], P Moreau[2], S Le Gouill[1,2], C Pellat-Deceunynck[1] and M Amiot*[,1]

[1]Inserm, U892, Univ Nantes, CNRS, UMR 6299, Nantes, F-44000, France; [2]Service d'Hématologie Clinique, Centre Hospitalier Universitaire de Nantes, Nantes 44093, France; [3]Institut Cochin, Université Paris Descartes, CNRS UMR 8104, Inserm U1016, 22 rue Méchain, Paris 75013, France; [4]Service d'Hématologie Clinique, AP-HP, Hôpital Cochin, 22 rue Méchain, Paris 75013, France

BACKGROUND: Cancer cells are frequently addicted to deregulated oncogenic protein translation. The small molecule 4EG-I selectively inhibits the cap-dependent translation of mRNAs. As multiple myeloma is an incurable disease that requires new therapeutic approaches, we investigated whether targeting the translation initiation pathway could be a target for myeloma therapy.

METHODS: Six myeloma cell lines and primary samples were included in this study. The 4EGI-1 effect was determined by AnnexinV staining and caspase activation. Modification of Bcl-2 protein expression was analysed, and the significance of modified proteins was analysed by knock-down experiments.

RESULTS: We demonstrated that 4EGI-1 impaired the assembly of the eIF4F complex and decreased the expression of the eIF4E-regulated proteins in myeloma cells. Furthermore, we showed that 4EGI-1 induced strong apoptosis in five out of six myeloma cell lines. Apoptosis is associated with the activation of the intrinsic mitochondrial pathway. The 4EGI-1 triggered Noxa induction only in cells undergoing apoptosis through endoplasmic reticulum (ER) stress. Furthermore, Noxa silencing prevented myeloma cells from 4EGI-1-induced apoptosis. Finally, Noxa induction led to a disruption of Mcl-1/Bim complexes in parallel to the generation of 'Mcl-1-free Noxa'.

CONCLUSION: Our results suggested that the use of inhibitors that directly target the translation initiation complex eIF4F could represent a potential novel approach for multiple myeloma therapy.

British Journal of Cancer (2012) **106,** 1660–1667. doi:10.1038/bjc.2012.139 www.bjcancer.com
Published online 17 April 2012

Keywords: cap-dependent translation; eIF4F complex; 4EGI-1; Noxa; multiple myeloma

Multiple myeloma (MM) is a plasma cell malignancy characterised by the accumulation of malignant plasma cells in the bone marrow. Despite recent advances in the treatment of MM, including proteasome inhibitors (bortezomib) and immunomodulatory agents (thalidomide and lenalidomide), this disease remains largely incurable with a median survival of 6 years. Patients invariably relapse, and therefore, alternative treatment strategies are urgently needed.

Dysregulation of protein synthesis has an important role in oncogenesis. The role of the 5′ cap mRNA-binding protein eukaryotic translation initiation factor (eIF)4E (eIF4E) in tumourigenesis and tumour progression has been well documented, and aberrant expression of eIF4E results in malignant transformation and increased cancer susceptibility (Lazaris-Karatzas *et al*, 1990; Ruggero *et al*, 2004). The expression and function of eIF4E are regulated at multiple levels, including inhibitory interactions with 4-EBP proteins. The 4EBP1 protein prevents the assembly of eIF4F by competing with eIF4G to bind to eIF4E. Elevated levels of eIF4E stimulate the translation of a subset of mRNAs termed 'weak mRNAs', which are involved in critical cellular processes implicated in cell proliferation, escape to apoptosis and angiogenesis. These weak mRNAs have long 5′-untranslated regions and high G/C content, resulting in complex hairpin structures that rely on an increased eIF4E activity to be translated. The overexpression of eIF4E in NIH 3T3 cells results in an increase in cyclin D1 translation, demonstrating that cyclin D1 is a putative target of eIF4E (Rosenwald *et al*, 1993; De Benedetti and Graff, 2004). C-myc is also one of the weak mRNA oncogene (Rosenwald *et al*, 1993), and its expression is effectively increased by eIF4E overexpression (Saito *et al*, 1983). Therefore, targeting eIF4E may be a rational anticancer therapy. Antisense oligonucleotide (ASO)-mediated eIF4E regulation in cancer cells has a limited effect on total protein synthesis, but strongly affects eIF4E-regulated proteins and induces apoptosis (Graff *et al*, 2007). Notably, eIF4E–ASO strongly reduced eIF4E levels in human tumour xenografts, which significantly decreased tumour growth without eliciting cytotoxicity in normal tissues (Graff *et al*, 2007, 2008). In addition to eIF4E–ASO, other strategies have been developed to inhibit mRNA cap-dependent translation. The small molecule 4EGI-1 pharmacologically mimics 4E-BP function and inhibits mRNA cap-dependent translation by competing for eIF4E/eIF4G interaction (Moerke *et al*, 2007). It was previously reported that 4EGI-1 inhibits the cellular expression of oncogenic proteins encoded by weak mRNAs and exhibits cytotoxic activity in cancer cell lines (Moerke *et al*, 2007; Fan *et al*, 2010).

In the present study, we investigated the *in vitro* activity of the cap-dependent translation inhibitor 4EGI-1 and its potential mechanism of action in both myeloma and primary myeloma cells, and we showed that 4EGI-1 effectively kills MM cells through Noxa induction.

*Correspondence: Dr M Amiot; E-mail: martine.amiot@inserm.fr
Revised 20 February 2012; accepted 21 March 2012; published online 17 April 2012

MATERIALS AND METHODS

Human myeloma cell lines and primary samples

L363, LP-1, OPM-2 and NCI-H929 human myeloma cell lines (HMCLs) were purchased from DSMZ (Braunschweig, Germany). The U266 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The XG-6 cell line has been previously established in our laboratory and is cultured in the presence of 3 ng/ml r-IL-6 (Novartis, Basel, Switzerland). The HMCLs were maintained in RPMI-1640 medium supplemented with 5% FCS, 2 mM glutamine and 5×10^{-5} M 2-βME. Primary myeloma cells were obtained from four relapsed patients treated in our institution. Plasma cells were purified using CD138-immunomagnetic beads. In all cases, the purity of the plasma cells was higher than 90%, as assessed by morphology. Peripheral blood lymphocytes (PBL) from healthy donors were obtained after Ficoll Hypaque density gradient.

Reagents and monoclonal antibodies

The 4EGI-1 was purchased from Chembridge (San Diego, CA, USA). z-VAD-fmk and caspase inhibitor were purchased from Promega (Charbonnières, France) and Calbiochem (St Quentin en Yvelines, France), respectively. The following antibodies were used: eIF4E, eIF4G, c-myc, cleaved caspase-9 (Cell Signaling Technology, Ozyme, Saint Quentin Yvelines, France); Bax mAb (Immunotech, Beckman Coulter, Marseille, France); Bak, Puma and PARP1 (Calbiochem, Merk); Bcl-x_L (BD Biosciences, Le Pont de Claix, France); Noxa (Alexis Biochemicals, Enzo Life Sciences, Villeurbanne, France); caspase-3, caspase-9, cyclinD1, Mcl-1, ATF-4 (Santa Cruz Biotechnology, Le Perray en Yvelines, France); cyclin D2 (Abcam, Paris, France); survivin (R&D Systems, Lille, France); Mcl-1 (Neomarkers, Thermo Scientific, Ilkirch, France); Bcl-2 (Dako, Trappes, France); Bim and actin (Millipore, Saint Quentin en Yvelines, France).

Detection of apoptotic cells and Bax activation

Cell death was quantified either by AnnexinV staining (Beckman Coulter) or by quantification of cells with a subG1 DNA content. A total of 2×10^5 cells was cultured alone or with 4EGI-1 for 18 h and then labelled with Annexin V-FITC, according to the manufacturer's instructions. Quantification of cells with a subG1 DNA content was determined by cell cycle analysis using the Modfit LT as previously described (Descamps *et al*, 2006). Flow cytometric analysis was then performed on a FACSCalibur using the Cell Quest software (Becton Dickinson, San Jose, CA, USA). Bax activation was performed according to published protocol (Gomez-Bougie *et al*, 2004).

7-Methyl-guanosine cap affinity assay

A total of 5×10^6 cells was lysed in 300 μl lysis buffer containing 1% digitonin. Cell lysates were clarified by centrifugation (11 000 **g**, 30 min, 4 °C), and supernatants were incubated with 7m-GTP-Sepharose beads (GE Healthcare, Chalfont St Giles, UK) at 4 °C for 2 h with constant shaking. Beads were washed three times with $1 \times$ PBS and denatured, and the supernatants were separated by SDS–PAGE for western blot analysis.

Small interfering RNA

siNoxa (N8) and control non-targeted siRNAs were synthesised by Ambion (Life Technologies, St Aubin, France). siPuma, siBax and siBak were purchased from Thermo Scientific. Cells were plated at 1×10^6 cells per well in a six-well plates. After 24 h, 100 pmol siRNA was transfected into the cells using Lipofectamine RNAiMAX (Invitrogen, Life Technologies, St Aubin, France) reagent according to the manufacturer's instructions. After transfection, the cells were incubated for 72 h and subjected to various analyses.

Quantitative real-time PCR

Quantitative PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, St Aubin, France) and the MX4000 instrument (Stratagene, Massy, France). TaqMan gene expression assays for Noxa (also called PMAIP1; Hs00560402_m1) and RPL37a (Hs01102345_m1) were from Applied Biosystems. The following thermal cycling parameters were used: 50 °C for 2 min for optimal AmpErase UNG activity and then 40 cycles at 95 °C for 30 s and 60 °C for 1 min. To control the specificity of the amplified product, a melting curve analysis was performed. The amplification of non-specific product was not observed. Amplification of the housekeeping gene *RPL37a* was conducted for each sample as an endogenous control.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed according to published protocols (Gomez-Bougie *et al*, 2007).

RESULTS

4EGI-1 impaired the translation initiating complex eIF4F in HMCLs

Because 4EGI-1 mimics 4E-BP function by competing for eIF4E/eIF4G interaction, we first examined the expression of these proteins in HMCLs by immunoblotting. Whereas the level of expression of eIF4E was comparable between the different cell lines, except weaker in XG-6, the eIF4G level of expression is very heterogeneous from highly expressed in LP-1, intermediate in L363, OPM-2 and NCI-H929, to very low in both XG-6 and U266 (Figure 1A). We further examined whether 4EGI-1 inhibited the interaction between eIF4E and eIF4G in myeloma cells by pull-down assays using 7m-GTP sepharose beads that mimic the cap structure of mRNAs. As shown in Figure 1B, incubation with 4EGI-1 strongly decreased the amount of eIF4G bound to eIF4E in every cell lines tested, suggesting that the translation-initiating complex eIF4F assembly is impaired. We then examined the impact of 4EGI-1 on the expression of cyclin D, c-myc and survivin proteins, which are well known to be regulated through cap-dependent translation initiation mechanisms (De Benedetti and Graff, 2004; Graff *et al*, 2007; Konicek *et al*, 2008). The downregulation of both c-myc and survivin was observed in all cell lines (Figure 1C). Because myeloma cells are always characterised by a dysregulation of at least one cyclin D (Bergsagel *et al*, 2005), mainly cyclin D1 or D2, the modifications of the expression of these two proteins were analysed in the different cell lines. Upon 4EGI-1 treatment, we always found a downregulation of cyclin D1 or cyclin D2, and both when they were co-expressed (Figure 1C). Altogether, these results indicate that 4EGI-1 reduced the expression of cell cycle and survival proteins regulated by a cap-dependent translation mechanism.

4EGI-1 induced apoptosis of both HMCLs and primary myeloma cells

We further determined the effects of 4EGI-1 on myeloma cell line survival after exposure for 18 h. Six HMCLs were treated with various concentrations of 4EGI-1, and cell viability was assessed by AnnexinV staining. As presented in Figure 2A, 4EGI-1 induced a dose-dependent cytotoxic effect for five of the six HMCLs tested, whereas L363, OPM-2 and XG-6 were the most sensitive cell lines. The percentage of apoptotic cells ranged from 30–78% at 50 μM 4EGI-1. U266 was resistant to 4EGI-1 at the doses and time point

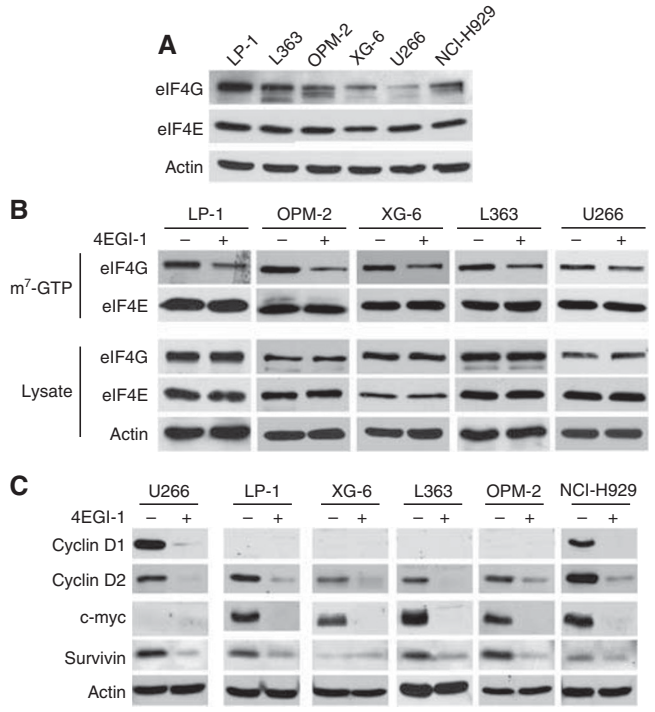

Figure 1 4EGI-1 disrupted eIF4E and eIF4G interaction, and inhibited cap-dependent protein expression. (**A**) eIF4E and eIF4G expression in HMCLs. Equivalent amounts of cell lysates were separated by SDS–PAGE and then immunoblotted with the indicated antibodies. (**B**) 4EGI-1 disrupts eIF4E and eIF4G interaction in myeloma cell lines. LP-1, L363, OPM-2, U266 and XG-6 cells were treated with 50 μM 4EGI-1 for 6 h and then lysed. Supernatants were clarified, and m^7 GTP pull-down assays were performed. Immunoblotting were performed using anti-eIF4E and anti-eIF4G antibodies. (**C**) 4EGI-1 inhibits cyclin D, c-myc and survivin expression. Cell lines were treated with 50 μM 4EGI-1 for 18 h. Equivalent amounts of cell lysates were separated by SDS–PAGE and then immunoblotted with the indicated antibodies. Actin was used as a loading control.

investigated. In a similar manner, 4EGI-1 induced apoptosis in two purified myeloma cell samples, and the percentage of apoptotic cells at 50 μM 4EGI-1 was 30 and 42% (Figure 2A). In contrast, PBLs from healthy donors were resistant to 4EGI-1 (Figure 2A). To determine whether the intrinsic mitochondrial pathway mediates 4EGI-1-induced apoptosis, we next investigated the activation of caspase-9, and of downstream effector caspase-3 as well as PARP-1 cleavage (Figure 2B). Cleavage of PARP-1, caspase-9 and caspase-3 was observed in every apoptosis-sensitive cell line. In contrast, the 4EGI-1-resistant U266 cell line exhibited neither PARP-1 nor caspase cleavage. To assess the involvement of caspases, we evaluated the sensitivity to 4EGI-1 in the presence of z-VAD-fmk or caspase-9 inhibitor. The pan-caspase inhibitor significantly reduced the 4EGI-1 induced apoptosis (50% inhibition), the caspase-9 inhibitor was as potent as the pan-caspase inhibitor showing the involvement of the intrinsic pathway (Figure 2C). To confirm the importance of this pathway, Bax activation was determined by flow cytometric analysis using an anti-Bax conformational antibody. We further demonstrated that 4EGI-1 triggered Bax activation in both LP-1 and NCI-H929 cells (Figure 3A). Altogether, these results indicated that 4EGI-1-induced apoptosis is associated with the activation of the mitochondrial pathway. We then investigated the respective role of the Bak and Bax effector proteins in 4EGI-1-induced apoptosis by silencing Bax or Bak alone or together in LP-1 cells (Figure 3B). The silencing of either Bax or Bak did not alter apoptosis induced by 4EGI-1, whereas the silencing of both effector proteins strongly reduced apoptosis in LP-1 cells (Figure 3C). This result confirmed that 4EGI-1 induced-apoptosis mainly occurs through the mitochondrial pathway, in which Bax and Bak have a redundant role.

4EGI-1 affected the Bcl-2 protein expression network and upregulated Noxa expression that has a major role in 4EGI-1-induced apoptosis

To decipher the events that trigger apoptosis through the mitochondrial pathway, Bcl-2 family protein expression was analysed by western blot analysis after 4EGI-1 treatment. Among these anti-apoptotic proteins, the cellular expression of both Mcl-1 and Bcl-x_L was decreased, whereas Bcl-2 expression was not affected by 4EGI-1 (Figure 4A). These changes were consistently observed in every cell line studied. The opposite regulation of the BH3-only proteins was observed in response to 4EGI-1 treatment; although Bim EL expression decreased, Bim L and Bim S were not affected, and both Puma and Noxa expression increased (Figure 4A). Puma was upregulated in every cell line that showed endogenous expression of Puma, whereas Noxa was only strongly increased in myeloma cell lines that underwent 4EGI-1-induced apoptosis (Figure 4A). Indeed, Noxa induction was observed in all cell lines except U266, which was not sensitive to 4EGI-1-induced apoptosis. We next focused our study on Noxa and Puma, because these two proteins are essential initiators of apoptosis. To determine whether 4EGI-1-induced apoptosis was dependent on Noxa or Puma induction, we knocked down Noxa or Puma expression by siRNA (siNoxa or siPuma, respectively). As shown in Figure 4B, the complete downregulation of Noxa expression in LP-1 cells caused a significant refractory effect on cell death, with a 40% reduction in the number of apoptotic cells (Figure 4B). A similar result was also observed in OMP-2 cells (Figure 4B). In contrast, whereas siPuma induced a highly significant decrease in Puma expression, its impact on apoptosis was not significant. Altogether, these results seemed to indicate that Noxa induction has a major role in 4EGI-1-induced apoptosis. To confirm Noxa induction in primary myeloma cells, a sufficient number of myeloma cells has been purified from two patients to assess Noxa expression by immunoblotting. As illustrated in Figure 4C, 4EGI-1 induced both a decrease of Mcl-1 associated with an induction of Noxa in primary myeloma cells. To better characterise the role of Noxa in 4EGI-1-induced apoptosis, we performed a kinetic analysis in LP-1 cells that showed that Noxa induction occurred as soon as 3 h post-treatment, and was associated with caspase-3 cleavage (Figure 4D). Finally, we determined whether the increase in Noxa expression occurs at the transcriptional level. By real-time PCR, we observed a 3.8-fold increase in Noxa mRNA in LP-1 cells (Figure 4E). In contrast, Noxa mRNA did not significantly increase in the 4EGI-1-resistant U266 cell line. Thus, these results suggest that Noxa transcription mediated in part the pro-apoptotic effect of 4EGI-1 in MM. To begin to unravel the mechanism of Noxa induction, we investigated whether Noxa could be due to an ER stress. Because ATF-4 is a well-known downstream target of the PERK branch of the ER stress (Walter and Ron, 2011), but also acts as a direct transcriptional activator of Noxa (Armstrong *et al*, 2010), we investigated whether ATF-4 was upregulated under 4EGI-1 treatment. Of interest, we found an upregulation of ATF-4 in OPM-2 cells in parallel to Noxa induction, whereas ATF-4 was not induced in U266 (Figure 4F). These results suggest the involvement of ATF-4 upregulation in Noxa transcriptional induction.

The induction of Noxa led to a disruption of Mcl-1/Bim complexes and the generation of 'Mcl-1-free Noxa'

Because Noxa specifically binds to Mcl-1 (Chen *et al*, 2005), we investigated the dynamic of Mcl-1 complexes to further

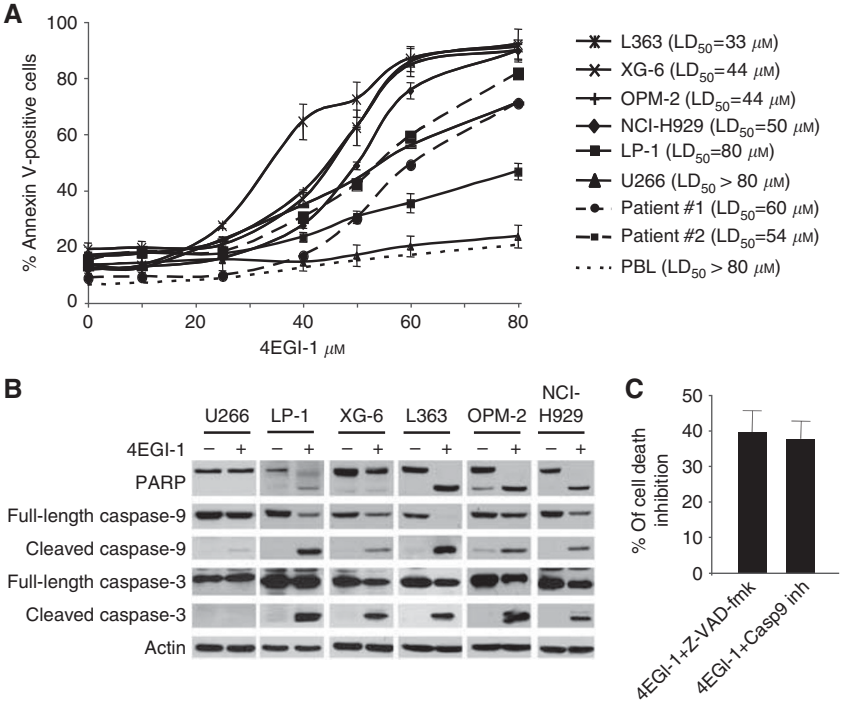

Figure 2 4EGI-1-induced apoptosis involved the intrinsic pathway. (**A**) Primary myeloma cells, HMCLs and PBL were treated with the given concentration of 4EGI-1 for 18 h in culture conditions. Cell death was then assessed by AnnexinV staining. (**B**) Myeloma cell lines were treated with 50 μM 4EGI-1 for 18 h in culture conditions. Equivalent amounts of cell lysates were separated by SDS–PAGE and then immunoblotted with PARP-1, anti-caspase-3 and anti-caspase-9 antibodies. Actin was used as a loading control. (**C**) Cells were pre-incubated with 20 μM caspase inhibitors (z-VAD-fmk or caspase-9 inhibitor) for 4 h before to be treated by 4EGI-1 for 18 h. Cell death was expressed as the percentage of cells with subG1 DNA content.

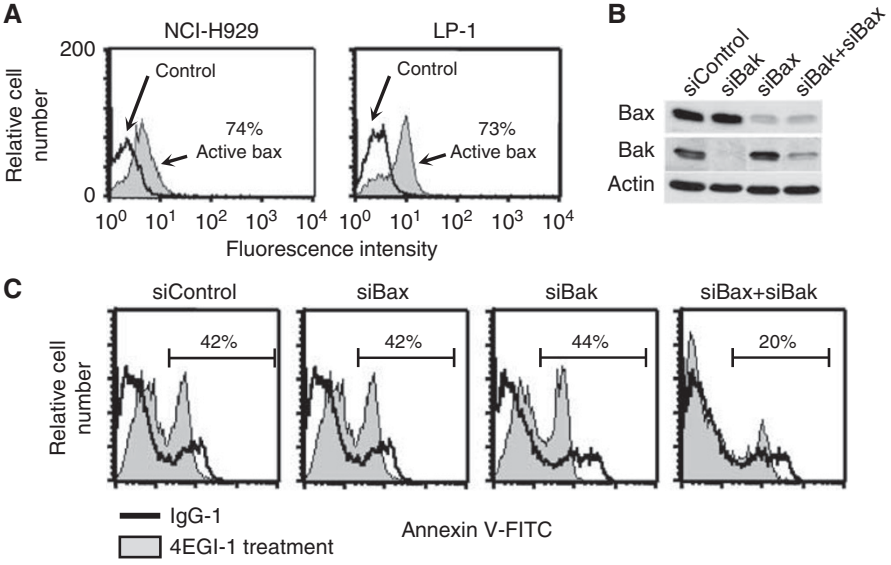

Figure 3 4EGI-1 induced Bax activation, and simultaneous knockdown of Bak and Bax inhibited 4EGI-1-induced apoptosis. (**A**) Cell lines were treated with 50 μM 4EGI-1 for 18 h in culture conditions. Cells were then quantified for Bax activation using the conformation-dependent anti-Bax antibody (6A7). (**B**) LP-1 cells were transfected with the indicated siRNA for 72 h before being treated with 50 μM 4EGI-1 for 18 h. Equivalent amounts of cell lysates were separated by SDS–PAGE and then immunoblotted with the indicated antibodies. Actin was used as a loading control. (**C**) Cell death was assessed by Annexin V staining in LP1 cells.

determine the role of Noxa in 4EGI-1 induced apoptosis. As shown in Mcl-1 immunoprecipitates (Figure 5A, lane 2, 8 and 14), we found that Mcl-1 was associated with Bim, Puma and Noxa, and the abundance of the various Mcl-1 complexes depended on the relative quantities of each pro-apoptotic protein (Bim, Puma and Noxa) in the different cell lines. Notably, LP-1 cells do not express Bim, which resulted in a high prevalence of Mcl-1/Puma complexes in these cells (Figure 5A, lane 8). The increased expression of both Puma and Noxa upon 4EGI-1 treatment lead to an increase in both Mcl-1/Puma and Mcl-1/Noxa complexes, as shown in Mcl-1 immunoprecipitates (Figure 5A, lane 5, 11 and 17). Notably, whereas all Noxa was complexed to

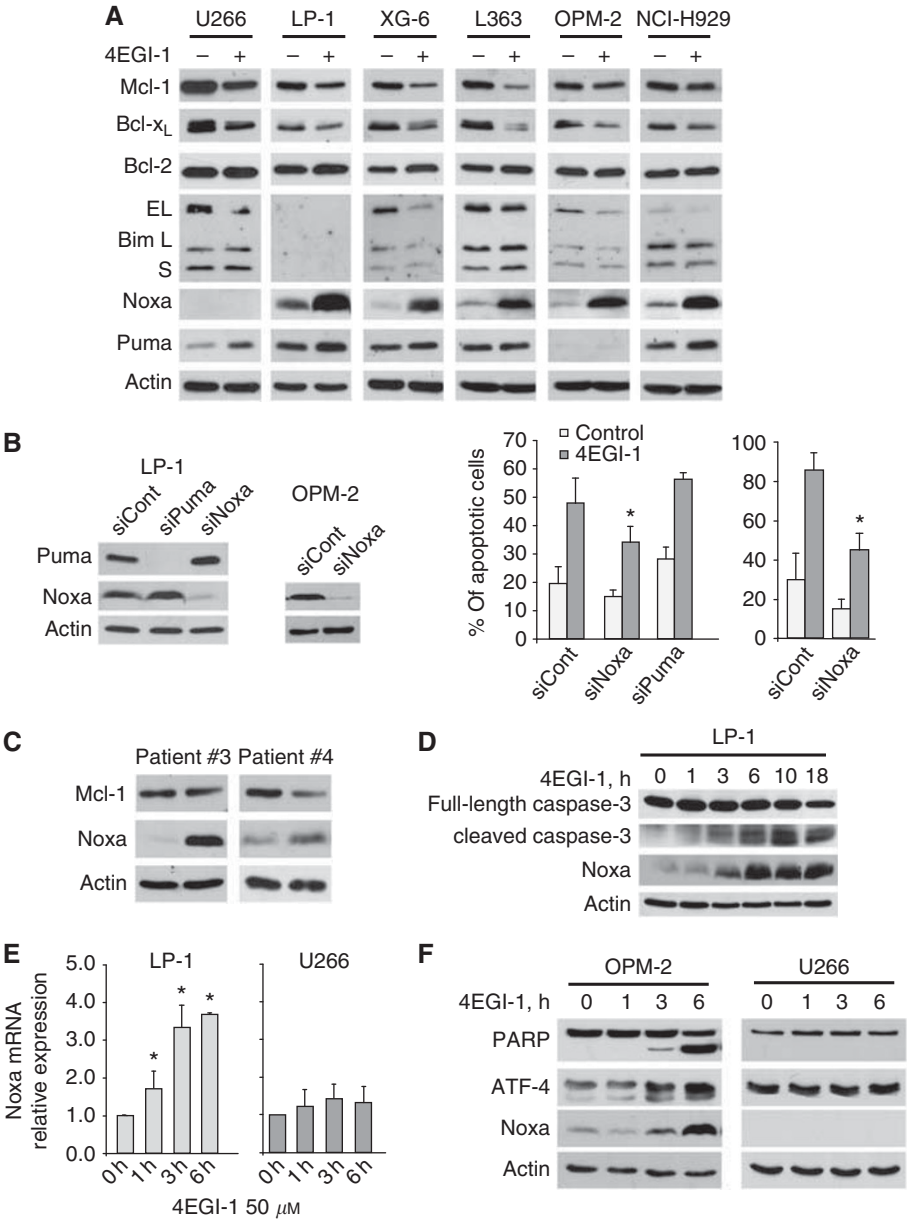

Figure 4 4EGI-1 modulated Bcl-2 protein family expression. (**A**) Cell lines were treated with 50 μM 4EGI-1 for 18 h, and equivalent amounts of cell lysates were immunoblotted with the indicated antibodies. (**B**) LP-1 and OPM-2 cells were transfected with the indicated siRNA for 72 h and then cultured alone or with 50 μM 4EGI-1 for 18 h. Cell extracts were immunoblotted for the indicated proteins. Cell death was assessed by Annexin V staining, and the mean ± s.d. of at least three experiments is presented. (Student's *t*-test, $^*P < 0.05$). (**C**) Purified myeloma cells (2×10^6 cells) were treated with 50 μM 4EGI-1 for 18 h, and equivalent amounts of cell lysates were immunoblotted with the indicated antibodies. (**D**) LP-1 cells were treated with 50 μM 4EGI-1 for 1, 3, 6, 10 or 18 h. Equivalent amounts of cell lysates were analysed by immunoblotting with anti-caspase-3 and anti-Noxa antibodies. Actin was used as a loading control. (**E**) Noxa mRNA was measured by quantitative PCR in LP-1 and U266 cells treated with 50 μM 4EGI-1 at the indicated times. The mean ± s.d. of three experiments is expressed as a ratio to RPL37a mRNA. Statistics were done using Student's *t*-test ($^*P < 0.05$) (**F**) OPM-2 and U266 cells were treated with 50 μM 4EGI-1 for 1, 3 or 6 h. Equivalent amounts of cell lysates were analysed by immunoblotting with anti-PARP-1, anti-ATF-4 and anti-Noxa antibodies. Actin was used as a loading control.

Mcl-1 in viable cells, a large amount of Noxa was 'Mcl-1-free' in response to 4EGI-1 treatment in all cell lines tested. Noxa 'Mcl-1-free' was evidenced by its detection only in the supernatants of Mcl-1 immunoprecipitates treated by 4EGI-1 (Figure 5A, lane 6, 12 and 18), contrary to untreated samples (Figure 5A, lane 3, 9 and 15). In both NCI-H929 and OPM-2 cells, we found that Noxa upregulation led to a disruption of Mcl-1/Bim complexes, particularly the complexes including Bim L and S isoforms (Figure 5A, lane 5 and 17). Finally, we also investigated the Bak/Mcl-1 complex, because the disruption of this complex was reported as important under some stimuli (Willis *et al*, 2005; Chen *et al*, 2007). However, the intensity of Mcl-1/Bak complexes was similar in Mcl-1 immunoprecipitates treated or not by 4EGI-1, indicating that these complexes were not affected upon 4EGI-1 treatment (Figure 5A). Because it was recently demonstrated that 'Mcl-1-free' Noxa could bind to Bcl-x_L in response to some stimuli, we next investigated whether this interaction occurred in response to 4EGI-1 treatment (Lopez *et al*, 2010; Zhang *et al*, 2011). Bcl-x_L was immunoprecipitated from lysates that were previously depleted of Mcl-1. As shown in Figure 5B, Bcl-x_L was not

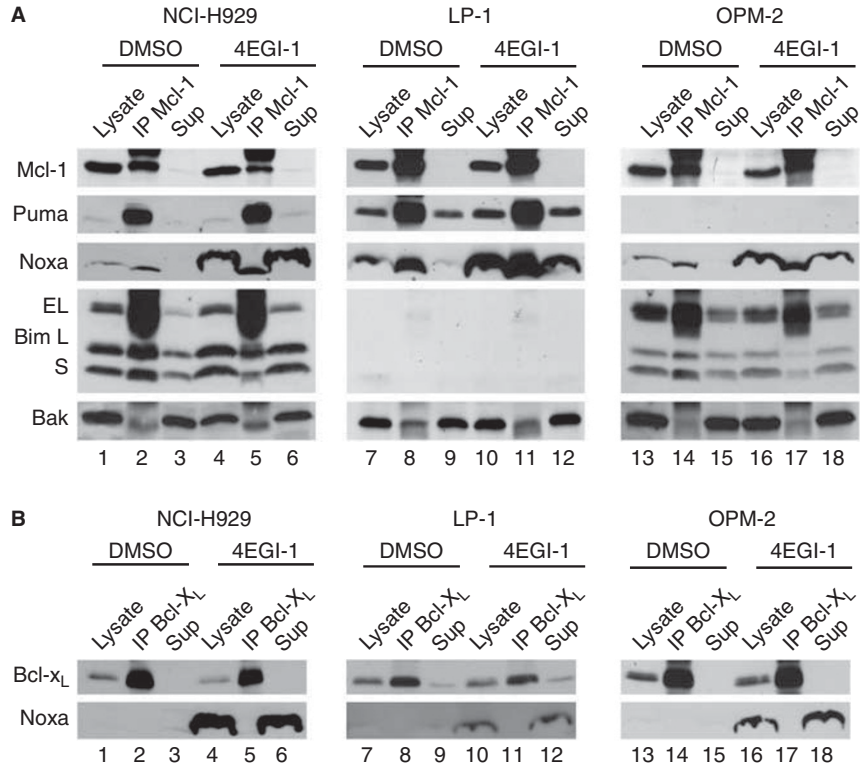

Figure 5 'Mcl-1/Bcl-x_L-free' Noxa is involved in 4EGI-1 induced-apoptosis. (**A**) 'Mcl-1-free' Noxa is detected in Mcl-1 immunoprecipitate supernatant. NCI-H929, LP-1 and OPM-2 cells were cultured with or without 50 μM 4EGI-1 for 18 h. Cell extracts were immunoprecipitated with an anti-Mcl-1 antibody. Immunoblotting were then performed on both immunoprecipitates and immunoprecipitate supernatant using the indicated antibodies. (**B**) 'Mcl-1/Bcl-x_L free' Noxa is detected in Bcl-x_L immunoprecipitate supernatant previously depleted of Mcl-1. Bcl-x_L was then immunoprecipitated from Mcl-1-depleted lysates, and immunoprecipitates and supernatants were analysed for the presence of Noxa.

associated with Noxa, whereas free Noxa was still detected as evidenced in the supernatant of Bcl-x_L immunoprecipitates (Figure 5B, lane 6, 12 and 18). Altogether, these results suggest that 'Mcl-1/Bcl-x_L-free' Noxa could also induce the activation of Bax or Bak.

DISCUSSION

Elevated eIF4E function in tumours preferentially and disproportionately influences the expression of potent regulators of cell growth and survival proteins that are involved in malignancy and is thereby an attractive new anticancer therapeutic target. The delivery of eIF4E-specific ASOs in human tumour xenografts causes a selective and significant reduction in eIF4E expression that results in the inhibition of tumour growth (Graff *et al*, 2007, 2008). The eIF4E/eIF4G interaction is inhibited by 4EGI-1, and it is therefore an alternative to use eIF4E-specific ASOs. In the present study, we demonstrated that 4EGI-1 inhibits the eIF4E/eIF4G interaction and the subsequent translation of eIF4E-regulated proteins in myeloma cells. Indeed, 4EGI-1 treatment suppressed the expression of survivin, c-myc, and cyclin D1 and D2, which are well known as cap-dependent proteins. Of note, whereas the expression of eIF4E appears homogenous among the different cell lines studied, the expression of eIF4G is very heterogeneous. Thus, in this particular cell type, we could hypothesise that eIF4G, but not eIF4E, is the limiting component of the assembly of eIF4F complex. We further demonstrated that 4EGI-1 efficiently kills five out of six myeloma cell lines; only the U266 cell line is resistant to 4EGI-1-induced cell death. This result is consistent with previous studies that show that 4EGI-1 induced apoptosis (Moerke *et al*, 2007; Tamburini *et al*, 2009; Fan *et al*, 2010). We showed that cell death occurs mainly through the mitochondrial pathway. Indeed, both caspase-9 and Bax were activated in response to 4EGI-1 treatment, and cell death was significantly inhibited by the caspase-9 inhibitor. Furthermore, Bak and Bax knockdown simultaneously lead to a total inhibition of apoptosis, which confirmed the involvement of the mitochondrial pathway. Moreover, the knockdown of either Bak or Bax had no effect on apoptosis, indicating that both proteins have a redundant role in 4EGI-1-induced apoptosis.

Given the central role of Bcl-2 family proteins as the major regulators of the intrinsic mitochondrial pathway in response to diverse stress signals, we investigated the relative expression of the Bcl-2 family members in response to 4EGI-1 treatment. We found that Mcl-1 and Bcl-x_L were consistently downregulated by 4EGI-1 in every cell line tested, whereas Bcl-2 expression was not affected. In agreement with these results, several studies have reported that Mcl-1 expression is controlled by eIF4E phosphorylation (Wendel *et al*, 2007; Hsieh *et al*, 2010). Whereas a significant downregulation of Mcl-1 and Bcl-x_L survival proteins was also observed in U266 cells following 4EGI-1 treatment, this cell line did not undergo apoptosis, which indicates that the reduction in expression of these anti-apoptotic proteins was not sufficient for determining a commitment to cell death. In addition to these changes in the anti-apoptotic protein levels, we observed that 4EGI-1 triggers the upregulation of Noxa and Puma expression. The upregulation of Puma was modest, but was found in all cell lines that endogenously expressed Puma. In contrast, Noxa upregulation was more impressive than that of Puma and was consistently associated with the induction of apoptosis. Indeed, the 4EGI-1-resistant cell line U266 did not present any induction of Noxa expression. Notably, we previously reported that Noxa induction could be detected in U266 cells under bortezomib

treatment, indicating that the absence of Noxa induction is not due to a deletion of the gene (Gomez-Bougie *et al*, 2007). The absence of Noxa induction was also found in PBLs (data not shown) that were resistant to 4EGI-1. Because both Noxa and Puma were upregulated in both p53 wt (NCI-H929 and XG-6) and p53-mutated cell lines (LP-1, OPM-2 and L363; Moreaux *et al*, 2011), we can exclude the possibility that Noxa and Puma upregulation is due to a p53 responses. The siRNA-mediated knockdowns were carried out to determine the specific involvement of Puma and Noxa in 4EGI-1-induced apoptosis. We demonstrated that Noxa, but not Puma, was critical for the induction of apoptosis. Thus, we can hypothesise that Puma has a role in amplifying apoptosis in this system. Furthermore, 4EGI-1 increased Noxa mRNA levels, suggesting that this regulation occurs at the transcriptional level. Different hypotheses can be proposed to explain the increase in Noxa translation. Noxa could be translated in an IRES-mediated manner, as it was previously demonstrated for Bmf isoforms (Grespi *et al*, 2010). Indeed, it has been shown that Bmf isoforms could be activated in response to stress that is associated with the reprogramming of the translation machinery from cap-dependent to cap-independent translation (Grespi *et al*, 2010). It is also possible that 4EGI-1 could induce off-target effects as previously reported. 4EGI-1 was shown to induce ER stress-related proteins independently of its inhibitory action of cap-dependent mRNA translation (Fan *et al*, 2010). The ER stress results in the unfolded protein response due to the activation of different stress sensors (Walter and Ron, 2011). Among them, transcriptional control regulated by PERK leads to the upregulation of the transcriptional factor ATF-4, which is known as a direct transcriptional activator of Noxa (Wang *et al*, 2009; Armstrong *et al*, 2010). Of interest, we demonstrated that Noxa was induced in concert with ATF-4 upregulation in cells undergoing apoptosis under 4EGI-1 treatment, whereas both ATF-4 upregulation and Noxa induction were not observed in U266-resistant cells to 4EGI-1-induced apoptosis. Consistent with the study of Fan *et al* (2010), these results strongly suggested that the ER stress is involved in Noxa induction, but further investigations will be necessary to elucidate the mechanism of resistance of U266 to ER stress. To address how Noxa induction activates apoptosis, we therefore have investigated the interaction of Noxa with its major binding partner Mcl-1 (Chen *et al*, 2005). In viable myeloma cells, we found that Noxa was fully complexed with Mcl-1, suggesting that Noxa was kept in check by Mcl-1. Upon 4EGI-1 treatment, despite the decrease of total Mcl-1, the Mcl-1/Noxa complexes were significantly increased and a large amount of Noxa appeared to be 'Mcl-1-free'. In viable myeloma cells, we have previously shown that both Mcl-1 and Bcl-2 sequester Bim, but only the disruption of the Mcl-1/Bim complex seems to be related to the induction of apoptosis (Gomez-Bougie *et al*, 2004, 2007). In the present study, we showed that 4EGI-1 also induced a disruption of the Mcl-1/Bim complex, indicating that Bim release could trigger Bax/Bak activation. Of note, BimS isoform release was the most important, this isoform was described as being the most potent inductor of apoptosis (O'Connor *et al*, 1998). In LP-1 cells lacking Bim expression, Mcl-1 sequesters both Puma and Noxa, and both Mcl-1/Puma and Mcl-1/Noxa complexes were increased upon 4EGI-1 treatment, indicating that the release of an activator BH3 protein is not an absolute requirement for the induction of cell death. Thus, in LP-1 cells, we hypothesise that Noxa can directly activate Bax or Bak. This last result is in agreement with the recent study showing that BH3 domains other than those of Bim and Bid can directly activate Bak or Bax (Du *et al*, 2011). Indeed, Du *et al* (2011) showed that there are both weak and strong direct activators, and they classified Noxa as an intermediate activator that may significantly contribute to apoptosis induction. In conclusion, our study demonstrates that 4EGI-1 leads to the inhibition of several oncogenic and survival proteins that are deregulated in myeloma cells, and to the increase in Noxa and Puma BH3-only proteins. Altogether, these modifications act in concert to induce robust apoptosis. Notably, among all of the changes, Noxa induction appears to have a crucial role in the induction of the apoptotic programme. Taken together with the notion that malignant cells are preferentially susceptible to the inhibition of cap-dependent translation, our study suggests that inhibitors of the translation could become a very attractive and potentially effective therapy in MM.

ACKNOWLEDGEMENTS

This study was supported by the Ligue Régionale contre le Cancer Grand-Ouest (2010) and Actions Cancer 44.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Armstrong JL, Flockhart R, Veal GJ, Lovat PE, Redfern CPF (2010) Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in Neuroectodermal tumor cells. *J Biol Chem* **285**: 6091–6100

Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy Jr J (2005) Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood* **106**: 296–303

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17**: 393–403

Chen S, Dai Y, Harada H, Dent P, Grant S (2007) Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res* **67**: 782–791

De Benedetti A, Graff JR (2004) eIF-4E expression and its role in malignancies and metastases. *Oncogene* **23**: 3189–3199

Descamps G, Wuillème-Toumi S, Trichet V, Venot C, Debussche L, Hercend T, Collette M, Robillard N, Bataille R, Amiot M (2006) CD45neg but not CD45pos human myeloma cells are sensitive to the inhibition of IGF-1 signaling by a murine anti-IGF-1R monoclonal antibody, mAVE1642. *J Immunol* **177**: 4218–4223

Du H, Wolf J, Schafer B, Moldoveanu T, Chipuk EJ, Kuwana T (2011) BH3 domains other than Bim and Bid can directly activate Bax/Bak. *J Biol Chem* **286**: 491–501

Fan S, Li Y, Yue P, Khuri FR, Sun SY (2010) The eIF4E/eIF4G interaction inhibitor 4EGI-1 augments TRAIL-mediated apoptosis through c-FLIP down-regulation and DR5 induction independent of inhibition of cap-dependent protein translation. *Neoplasia* **12**: 346–356

Gomez-Bougie P, Bataille R, Amiot M (2004) The imbalance between Bim and Mcl-1 expression controls the survival of human myeloma cells. *Eur J Immunol* **34**: 3156–3164

Gomez-Bougie P, Wuillème-Toumi S, Ménoret E, Trichet V, Robillard N, Philippe M, Bataille R, Amiot M (2007) Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res* **67**: 5418–5424

Graff JR, Konicek BW, Carter JH, Marcusson EG (2008) Targeting the eukaryotic translation initiation factor 4E for cancer therapy. *Cancer Res* **68**: 631–634

Graff JR, Konicek BW, Vincent TM, Lynch RL, Monteith D, Weir SN, Schwier P, Capen A, Goode RL, Dowless MS, Chen Y, Zhang H, Sissons S, Cox K, McNulty AM, Parsons SH, Wang T, Sams L, Geeganage S, Douglass LE, Neubauer BL, Dean NM, Blanchard K, Shou J, Stancato LF, Carter JH, Marcusson EG (2007) Therapeutic suppression of translation initiation factor eIF4E expression reduces tumor growth without toxicity. *J Clin Invest* **117**: 2638–2648

Grespi F, Soratroi C, Krumschnabel G, Sohm B, Ploner C, Geley S, Hengst L, Häcker G, Villunger A (2010) BH3-only protein Bmf mediates apoptosis

upon inhibition of CAP-dependent protein synthesis. *Cell Death Differ* **17**: 1672–1683

Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR, Meyuhas O, Shokat KM, Ruggero D (2010) Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. *Cancer Cell* **17**: 249–261

Konicek BW, Dumstorf CA, Graff JR (2008) Targeting the eIF4F translation initiation complex for cancer therapy. *Cell Cycle* **7**: 2466–2471

Lazaris-Karatzas A, Montine KS, Sonenberg N (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature* **345**: 544–547

Lopez H, Zhang L, George NM, Liu X, Pang X, Evans JJ, Targy NM, Luo X (2010) Perturbation of the Bcl-2 network and an induced Noxa/Bcl-xL interaction trigger mitochondrial dysfunction after DNA damage. *J Biol Chem* **285**: 15016–15026

Moerke NJ, Aktas H, Chen H, Cantel S, Reibarkh MY, Fahmy A, Gross JD, Degterev A, Yuan J, Chorev M, Halperin JA, Wagner G (2007) Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G. *Cell* **128**: 257–267

Moreaux J, Klein B, Bataille R, Descamps G, Maïga S, Hose D, Goldschmidt H, Jauch A, Rème T, Jourdan M, Amiot M, Pellat-Deceunynck C (2011) A high-risk signature for patients with multiple myeloma established from the molecular classification of human myeloma cell lines. *Haematologica* **96**: 574–582

O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC (1998) Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* **17**: 384–395

Rosenwald IB, Lazaris-Karatzas A, Sonenberg N, Schmidt EV (1993) Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. *Mol Cell Biol* **13**: 7358–7363

Ruggero D, Montanaro L, Ma L, Xu W, Londei P, Cordon-Cardo C, Pandolfi PP (2004) The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. *Nat Med* **10**: 484–486

Saito H, Hayday AC, Wiman K, Hayward WS, Tonegawa S (1983) Activation of the c-myc gene by translocation: a model for translational control. *Proc Natl Acad Sci USA* **80**: 7476–7480

Tamburini J, Green AS, Bardet V, Chapuis N, Park S, Willems L, Uzunov M, Ifrah N, Dreyfus F, Lacombe C, Mayeux P, Bouscary D (2009) Protein synthesis is resistant to rapamycin and constitutes a promising therapeutic target in acute myeloid leukemia. *Blood* **114**: 1618–1627

Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic régulation. *Science* **334**: 1081–1086

Wang Q, Mora-Jensen H, Weniger MA, Perez-Galan P, Wolford C, Hai T, Ron D, Chen W, Trenkle W, Wiestner A, Ye Y (2009) ERAD inhibitors integrate ER stress with an epigenetic mechanism to activate BH3-only protein NOXA in cancer cells. *Proc Natl Acad Sci USA* **106**: 2200–2205

Wendel HG, Silva RL, Malina A, Mills JR, Zhu H, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Teruya-Feldstein J, Pelletier J, Lowe SW (2007) Dissecting eIF4E action in tumorigenesis. *Genes Dev* **21**: 3232–3237

Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DC (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19**: 1294–1305

Zhang L, Lopez H, George NM, Liu X, Pang X, Luo X (2011) Selective involvement of BH3-only proteins and differential targets of Noxa in diverse apoptotic pathways. *Cell Death Differ* **18**: 864–873