Elevated Translation Initiation Factor eIF4E is an Attractive Therapeutic Target in Multiple Myeloma

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Running title: Elevated eIF4E is therapeutic target in Myeloma

Keywords: Multiple myeloma, eIF4E, Protein synthesis, translation

Financial support: S.Li, J.Fu and S. Lentzsch were supported by NIH grant R01CA175313, C.L and MYM were supported by NIH grant R01HL93716. U.H was supported by NIH grant R01MH096702.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.
Abstract

eIF4E is the key regulator of protein translation and critical for translation. The oncogenic potential of tumorigenesis which is highly contingent on cap-dependent eIF4E also arises from the critical role in the nuclear export and cytosolic translation of oncogenic transcripts. Inhibition of Exportin1 (XPO1) which is the major nuclear export protein for eIF4E-bound oncoprotein-mRNAs results in decreased tumor cell growth \textit{in vitro} and \textit{vivo} suggesting that eIF4E is critical in multiple myeloma (MM). Indeed we found that eIF4E is overexpressed in myeloma cell lines and primary myeloma cells compared to normal plasma cells. While stable overexpression of eIF4E in MM cells significantly increases tumorigenesis, knockdown of eIF4E impairs MM tumor progression in human xenograft mice model. Using a tet-on inducible eIF4E-knockdown system, eIF4E-downregulation, blocks MM tumor growth \textit{in vivo} correlating with decreased eIF4E expression. Further overexpression and knockdown of eIF4E revealed that eIF4E regulates translation of mRNAs with highly complex 5'-untranslated regions such as c-MYC and C/EBPβ and subsequently proliferation in MM cells, but not in non-malignant bone marrow stromal cells. Since many transcription factors that are critical for MM proliferation exhibit a higher dependency on protein translation, eIF4E is an ideal and selective tool to target MM cell growth.
Introduction

eIF4E plays a central role in protein synthesis and has a key role in the control of cell growth, proliferation, differentiation, and metabolism in eukaryotic cells(1). It recognizes and binds to the 7-methylguanosine cap in the 5' untranslated regions (5'UTRs) of mRNAs, transporting these mRNAs to the eIF4F translation initiation complex, which includes eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A. An increase in eIF4E level or activity does not lead to increased rates of global translation, but instead results in increased translation of mRNAs with highly complex 5’-untranslated regions(2). Several genes including MYC, Cyclin D1, CCAAT/enhancer-binding protein beta (C/EBPβ) and vascular endothelial growth factor (VEGF) involved in tumorigenesis are regulated at the translational level by eIF4E(3-6). Further eIF4E competitive inhibitors, such as XPO1 inhibitors, abrogate its pro-survival function by decreasing export and translation of target mRNAs(7). In AML the XPO1 inhibitor KPT-330 (Karyopharm Therapeutics) induced decreased levels of proteins derived from capped mRNAs associated with eIF4E mRNA, which are dependent upon XPO1 for nuclear export further suggesting a critical role for eIF4E(8). Overexpression and/or activation of eIF4E has been also associated with tumor formation and progression including lymphoma, and cancers of the breast, colon, lung and prostate(9), but the role of eIF4E in multiple myeloma is largely unknown.

Multiple myeloma (MM) is a plasma cell disorder, associated with an accumulation of monoclonal terminally differentiated plasma cells within the bone marrow, and usually the presence of a monoclonal immunoglobulin(10). In 2012, approximately 21,700 new cases were diagnosed in the US and estimated 10,710 deaths occurred from this disease(11). Even with the introduction of novel and more potent treatment regimens for MM, the disease remains an incurable plasma cell malignancy(12) and novel treatments especially for relapsed/ refractory patients are urgently needed. In MM, ribavirin (RBV) is a physical mimic of the m7G cap, and thus blocks eIF4E resulting in a potentially effective anti-cancer agent. Combination of RBV and
velcade showed synergistic anti MM effect (13). 4EGI-1 behaves as a functional 4E-BP1 mimetic inhibiting the interaction between eIF4E and eIF4G and decreases the expression of eIF4E regulated proteins in myeloma cells (14). More recently Attar-Schneider at al investigated by high throughput assay of mRNA microarrays the significance of eIF4E/eIF4GI silencing to transcription factors, microRNAs and phenotype. They showed that different imprints for eIF4E and eIF4GI affect the expression of cellular proteome, transcription factors, microRNAs, and phenotype in MM (15).

Taken together, since tumor cell growth is more contingent on cap-dependent protein translation than normal tissues(16) the role of eIF4E in tumorigenesis and cancer progression has generated increasing interest as a therapeutic target in cancer(13, 16-21). Frequent mutations in genes involved in mRNA translation resulting in increased protein translation support the role of translational control in the pathogenesis of multiple myeloma(22). Here we provide novel insights into the role of the translation initiation factor eIF4E in MM tumor growth. By overexpressing and knockdown of eIF4E as well as using an inducible eIF4E-shRNA in human MM xenograft mouse models we found that expression of critical transcription factors and subsequently MM tumor growth is highly dependent on eIF4E expression. Therefore direct or functional inhibition of eIF4E by competitive selective inhibitors of nuclear export (XPO1 inhibitors) such as KPT-330, abrogate its pro-survival function by decreasing export and translation of target mRNAs and represent a promising approach.

**Materials and Methods**

**Chemicals and antibodies**

Cell culture media, sera and penicillin-streptomycin were purchased from Gibco BRL (Gaithersburg, MD). Antibodies and inhibitors were purchased from the following vendors: ant-
C/EBPβ (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-IRF4, anti-c-MYC and anti-εIF4E antibody from Cell Signaling (Beverly, MA); Pomalidomide, anti-β-Actin antibody, DMSO from Sigma-Aldrich (St. Louis, MO), anti-I-MYC antibody from Abcam Inc. (Cambridge, MA).

**Cell culture and cell selection**

MM cell lines RPMI-8226, MM.1S, H929 and U266 were purchased from the American Type Culture Collection (ATCC) in the last five years. OPM2 was provided by Dr. Klaus Podar (Dana Farber Cancer Institute, Boston, MA) in 2002. Cells were tested for mycoplasma and shown to be contamination free and authentication was not performed. The stocks of the cell lines were stored frozen in liquid nitrogen, cell are thawed at need and maintained for no more than 3 months. The MM cell lines MM.1S, RPMI-8226, U266, H929 and OPM2 were cultured in RPMI-1640 plus 10% FBS and 100 U/mL penicillin/streptomycin at 37°C and 5% CO₂ as described before (23). Human MM cells were isolated from patient bone marrow samples as described before (24). Mononuclear cells were isolated by Ficoll (Invitrogen, Carlsbad, CA) followed by magnetic separation using CD138⁺ antibody-specific micro beads according to the manufacturer’s protocol (Miltenyi Biotech, Auburn, CA). The negative population was considered as CD138⁻. The purity of the myeloma cells, as assessed by hCD138⁺/hCD45⁺ staining.

Normal plasma cells were obtained from pilot vials of mobilized peripheral blood from healthy donors. In brief, mononuclear cells were isolated by Ficoll (Invitrogen, Carlsbad, CA) followed by magnetic separation using CD138⁺ antibody-specific micro beads (Miltenyi Biotech, Auburn, CA). All samples were obtained after informed consent was given. All studies were approved by the Institutional Review Board of Columbia University Medical Center, New York.
Human bone marrow stromal cells (BMSCs) were isolated from patient bone marrow samples as described previously (25). Briefly, isolated mononuclear cells were cultured with 10% (vol/vol) heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 2 mM glutamine at 37°C and 5% CO2 overnight. Non-adherent cells were removed and adherent cells were cultured for expanding for 7–10 days. The purity of the BMSCs, as assessed by hCD29+/hCD90+/hCD45- staining.

**Cell proliferation assays**

Briefly, U266, RPMI-8226 or primary stromal cells (5x10^4/well) were incubated in 96-well for 72h. The WST-1 reagent (Clontech, Mountain View, CA) was added to react for 3 h. Measurement of absorbance of the samples at 450 nm (reference wavelength 690 nm) against the background control was performed using the Synergy HT Multi-Detection Microplate Reader (Biotek Instruments, Inc., Winooski, VT).

**SDS-PAGE and Western Blot Analysis**

Briefly, protein was extracted from cells using RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The blots were incubated with the appropriate antibodies to detect the protein level of interest and the immune complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) as described before(26).

**Quantitative Real-time PCR analysis**
For the determination of mRNA levels of eIF4E and c-MYC, total RNAs were isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Total RNA was converted into cDNA using Superscript III RT (Invitrogen). Quantitative RT-PCR was performed as described before(23). The following primer sets were used:

eIF4E: 5’-ACAAGTCAGTCTGAAACCATGAAC-3’ and 5’-CTTCATCCTCTCGGACCACTCTCC -3’;
c-MYC: 5’-TCTCCGTCTCAGGATTCTCT-3’ and 5’-TCTGACCTTTTGCCAGGAGC-3’;
β-actin: 5’-GGACTTCGAGCAAGAGATGG-3’ and 5’-AGCACTGTGTTGGCGTACAG-3’

Transduction of pCDH-GFP and pCDH-GFP-eIF4E

Expression vectors for the full-length wild-type eIF4E were generated by inserting the respective coding regions into pCDH-GFP vector. In brief, full-length human eIF4E cDNA was cut out from pHA-eIF4E (Addgene, Cambridge, MA)(27) by HindIII/XhoI double digestion followed by filling-in of 5’ overhangs by DNA polymerase I large (Klenow) fragment to form blunt ends. Similarly, pCDH-CMV-MCS-EF1-COPGFP (System Biosciences, Mountain View, CA) was digested by EcoRI followed by filling-in of 5’ overhangs then ligated with eIF4E fragment by T4 ligase (NEB, Ipswich, MA). Lentivirus were packaged and concentrated by PEG-it (System Biosciences, Mountain View, CA) from 293TN cells supernatant after co-transfection of pCDH-CMV-eIF4E-EF1-COPGFP with pPACKH1 packaging plasmids (System Biosciences, Mountain View, CA). Myeloma cells (2×10^6) were transduced with an empty vector lentiviral control pCDH-GFP(EV) or pCDH-GFP-eIF4E construct. The efficiency of virus transduction was determined by GFP co-expression. The transduction rate of U266 and RPMI-8266 cells was greater than 70% and 90%, respectively at 72 hours after transduction.
Transduced cells were selected by Influx cell sorter (BD Bioscience, San Jose, CA) and analyzed by western blotting or cell proliferation assay.

**eIF4E shRNA knockdown assay**

Lentiviral shRNA were used to knockdown eIF4E expression in MM cells. In brief, lentiviral pLKO.1-sh-eIF4E (Sigma, St. Louis, MO) target to the sequence as follows: 5’-ACTCTGTAAATAGTTCAGTA-3’. The shRNA stable transfectants were obtained by puromycin (5 μg/ml) selection and analyzed by western blotting or cell proliferation assay. For tet-on inducible eIF4E knockdown MM cells, tet-on-pLKO-puro (Addgene, Cambridge, MA)(28) was used to generate inducible sh-eIF4E lentiviral construct with the same target sequence as above and the correct constructs were confirmed by DNA sequencing. The viruses containing empty vector sequences were used as a control. RPMI-8266 or U266 cells were incubated with lentivirus particle and polybrene 8 μg/ml for 16h, and then washed with media. Transduced MM cells were maintained in 10% tetracycline-free FBS (Clontech, Mountain View, CA). Cells were selected for 3 days in puromycin (5μg/ml). The efficiency of virus transduction was determined by puromycin-resistance and the transduction rate of U266 or RPMI-8266 was greater than 60% or 70%, respectively. Selected cells were treated with or without doxycycline (200 ng/ml) for 3 days to induce knockdown eIF4E.

**Cell Cycle assays**

MM Cells (1×10⁶ cells/ml) were cultured for 72 hours at 37°C, harvested, and washed with ice-cold PBS, fixed with 70% ethanol for 1 h at 4°C, and pretreated with RNase (Worthington, Lakewood, NJ) for 30 min at 37°C. Cells were stained with PI (20μg/mL) (Sigma Aldrich).
Analyses were performed on a BD FACSCalibur flow cytometer and analyzed using ModFit LT2.0 and Cellquest software (BD Biosciences).

**Clonogenic assay**

5x10³ myeloma cells per well were seeded in a 24-well plate in methycellulose medium (Stem Cell Technologies). 1.1% methylcellulose was diluted to a final concentration of 1% by the addition of cell suspension. Colonies were stained with crystal violet (Sigma Aldrich) after 14 days and colony numbers were counted using a Leica DM IL LED inverted phase contrast microscope.

**Human MM Xenograft Mouse Model**

Female SCID Beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg-J</sup>/Crl) mice were purchased from Charles River Laboratories (Wilmington, MA) at age 6-8 weeks with a weight between 20-25 g. All animal procedures were approved by the IACUC of Columbia University, New York. Sample size was chosen accordingly to our experience(29). For human tumor xenograft studies, U266 myeloma cells (5x10⁶) in 100 µL PBS together with an equal volume of Matrigel basement membrane matrix (BD Biosciences) were injected subcutaneously (s.c.). Mice were weighed twice weekly and observed daily for diarrhea or any changes in behavior and condition. Tumor sizes were measured twice weekly in a no blinded manner. After mice were sacrificed, the tumors were excised, weighed and taken pictures. Tumor weights are reported as mean ± SEM (n = 5). CT-tet-on shRNA or eIF4E-tet-on shRNA cells were injected subcutaneously into SCID/bg mice. 11 days after implantation, animals were randomized to receive either vehicle (5% sucrose) or doxycycline (1mg/ml in 5% sucrose) via drinking water for duration of study.
Statistical analyses

Each experiment was repeated at least 3 times, and all quantitative data are presented as mean ± SEM. Statistical differences were determined by Student’s t-test (two tailed). The results were considered statistically significant if P <0.05.

Results

eIF4E is highly expressed in primary myeloma cells and myeloma cell lines

We examined eIF4E expression in normal plasma cells, primary CD138⁺MM cells, MM cell lines and CD138⁻ mononucleated cells. Analysis of eIF4E protein and mRNA levels by western blotting and real-time PCR revealed significant (p<0.01) higher expression in primary CD138⁺ MM cells and MM cell lines compared to normal plasma cells and CD138⁻ cells (Figure 1A, B). This was confirmed by patient bone marrow (BM) cells immunofluorescence staining showing higher expression of eIF4E in primary MM cells (red arrow) compared to CD138⁻ MNCs (green arrow) (Figure 1C).

Knock-down eIF4E selectively inhibits growth of multiple myeloma but not of bone marrow stromal cells

To determine whether eIF4E protein is required for MM cell growth, we used shRNA to knock down eIF4E in MM cells by lentiviral mediated transduction. The human MM cell lines RPMI-8226 and U266 were infected with shRNAs lentivirus generated from pLKO.1-puro vectors containing either control non-targeting shRNA (CT-shRNA) or eIF4E targeting shRNA (eIF4E-shRNA), and stably transfected cells were obtained after puromycin selection. eIF4E-shRNA but
not the CT-shRNA led to significant reduction of eIF4E expression at protein (Figure 2A) and mRNA (Figure 2B) levels. Silencing of eIF4E in myeloma cells significantly inhibited cell growth (66% in RPMI-8226, 76% in U266, P<0.01)(Figure 2C). In accordance with that, cell cycle analysis revealed that knockdown of eIF4E decreased the cell numbers in S-phase with concomitant increase of cells in G0/G1 growth arrest (63% vs 75% in U266 cells) (37% vs 50% in RPMI-8226) (Figure 2D). Importantly, eIF4E knockdown resulted in significant reduction (P<0.001) of clonogenic tumor growth reflected by decreased colony numbers (mean±SD: CT-shRNA 27.6±4.2 vs eIF4E-shRNA 5.3±3.4) in U266 and (CT-shRNA 21.3±1.2 vs eIF4E-shRNA 3.7±1.2) in RPMI-8226 cells (Figure 2E). Next we established an inducible eIF4E knockdown in our MM cells. We stably infected U266 and RPMI-8226 with shRNA lentivirus generated from a robust inducible knockdown vector pLKO-tet-On. Doxycycline-induced eIF4E-tet-on-shRNA expression resulted in significant decrease of eIF4E protein (Figure 2F) and significantly inhibited (RPMI by 72% and U266 by 46%, P<0.01) cell growth (Figure 2G). To determine whether eIF4E is specifically critical for MM cells growth, we tested its role in primary stromal cells from MM patients, which exhibit a low eIF4E expression (Supplementary Figure 1). Primary stromal cells were similarly infected with eIF4E-shRNA or CT-shRNA lentivirus and stably transfected cells were obtained after puromycin selection (Figure 2H). Interestingly, silencing of eIF4E in stromal cells did not affect proliferation (Figure 2I), indicating that eIF4E is specifically critical for malignant cells growth in MM cells.

**Ectopic overexpression of eIF4E significantly increases multiple myeloma cell growth in vitro**

To examine the effects of overexpression of eIF4E on MM, we transduced RPMI-8226 and U266 cells with lentiviral particles encoding human eIF4E with GFP as selection marker. eIF4E overexpression (OE) was confirmed by western blotting and RT-PCR (Figure 3A, B).
Overexpression of eIF4E resulted in significant (p<0.001) increase of MM cell growth compared to EV control cells (Figure 3C). Cell cycle analysis revealed decreased population of U266 cells in G0/G1 (62% vs 49%) and RPMI-8226 cells in G0/G1 (40% vs 31%) (Figure 3D), and without significant effects on cell apoptosis in U266 (Supplementary Figure 2). Overexpression of eIF4E further led to the significant increase (p=0.004) of clonogenic MM tumor growth with expansion of clonogenic colony numbers (22.3 ± 2.5 vs 40.3 ± 2.1 in U266) and (23± 1 vs 39.3 ± 5.7 in RPMI-8226)(Figure 3E).

**eIF4E is critical for multiple myeloma tumorigenesis *in vivo***

To determine the role of high expression of eIF4E in MM tumor growth *in vivo*, we generated subcutaneous MM xenografts in severe combined immunodeficient x beige (SCID/bg) mice using the U266-CT-shRNA and U266-eIF4E-shRNA cells. Mice injected with U266-eIF4E-shRNA cells showed a significant (P<0.001) and up to 90% decreased tumor size after 23 days (Figure 4A). To determine whether overexpressed eIF4E induces MM tumor growth in vivo, we injected EV-U266 or eIF4E-OE-U266 cells subcutaneously. In contrast to EV-U266 tumors, animals bearing eIF4E-OE-U266 xenografts showed a significant increase (p<0.001) of tumor growth (180%) after 13 days (Figure 4B). Next we wanted to explore whether the further growth of already established tumors depends on eIF4E by using a tet-on inducible knockdown system. We generated subcutaneous MM xenografts in SCID/bg mice using the inducible U266-tet-CT-shRNA and U266-tet-eIF4E-shRNA cells. Doxycycline or vehicle treatment was started after the tumor was established on day 11. In contrast to vehicle-treated U266-tet-on-eIF4E-shRNA or doxycycline-treated U266-control-tet-on-shRNA tumors, doxycycline treated animals bearing U266-tet-eIF4E-shRNA xenografts showed a significant inhibition (P<0.001) of tumor growth by 80% after 19 days. The inhibition of tumor growth correlated with the doxycycline-induced eIF4E knockdown, further confirming the critical role of eIF4E in MM tumorigenesis (Figure 4C).
Immunohistochemical staining of tumors confirmed the decrease of eIF4E expression in doxycycline-treated mice bearing U266-tet-eIF4E-shRNA tumors compared with tumors of vehicle-treated or non-doxycyclin treated mice (Figure 4D).

**eIF4E regulates C/EBPβ, MYC and IRF4 critical for MM cell proliferation**

In multiple myeloma, several transcription factors such as c-MYC, IRF4 and C/EBPβ are essential for malignant tumor growth and involved in tumorigenesis(30-32). The expression of c-MYC and C/EBPβ are highly regulated at the translational level(2, 6, 33). c-MYC is activated in MM cells(30, 34) and targeting MYC by short hairpin RNA induces cell death in myeloma cell lines(31). We analyzed whether eIF4E regulates C/EBPβ, c-MYC and IRF4 all critical for MM cell proliferation. Indeed as shown by western blotting eIF4E KD and OE in MM cells upregulated and downregulated expression of eIF4E, respectively (Figure 5A). The regulation at the translational level is further supported by quantitative RT-PCR showing that c-MYC mRNA level is not affected by eIF4E knockdown when c-MYC protein level is downregulated (Figure 5B).

**Discussion**

It is known that the expression of proteins such as c-MYC, Cyclin D1, C/EBPβ and VEGF are regulated at the translational level by eIF4E (3-6), Since these factors are critically involved in MM cell growth and survival, eIF4E might be an attractive target for anti-myeloma treatment(35). Indeed we found significantly elevated eIF4E levels in myeloma cell lines including H929, U266, MM.1S, RPMI 8226, OPM2 and primary myeloma cells compared to CD138+ cells on both protein and mRNA levels. In contrast normal plasma cells, which usually do not actively proliferate and exhibited limited protein translation activity showed undetectable
eIF4E protein levels (Figure 1) suggesting that eIF4E might be a specific target to inhibit protein translation in multiple myeloma. In accordance with that we found that eIF4E knockdown inhibits MM cell growth and decreases colony formation in U266 and RPMI-8226 cells. In contrast knockdown of eIF4E in BMSCs did not affect proliferation suggesting that non-malignant cells are less dependent on protein translation. Interestingly Attar-Schneider et al reported that bone marrow mesenchymal stem cells from patients promoted eIF4E/eIF4GI expression in MM cells, causing elevated translational activity and enhanced expression of oncogenic signals, and result in increase of proliferation and death(36). On the other hand, introduction of ectopic eIF4E significantly increased the in vitro growth as well colony formation of multiple myeloma cells. The role of eIF4E in MM tumorigenesis was confirmed in a human MM xenograft mouse model showing that knockdown of eIF4E inhibits MM tumor growth while overexpression of eIF4E accelerated tumor progression. In addition we established an inducible shRNA human MM xenograft mouse model in which eIF4E knockdown could be precisely turned-on or -off upon doxycycline treatment or withdrawn. In this model the tumor growth curves separate with knockdown of eIF4E reflecting a significant tumor growth inhibition associated with knockdown of eIF4E. As expected eIF4E did not significantly affect apoptosis since protein translation is primarily required for cell proliferation. Taken together, these results strongly suggest that eIF4E is essential for the maintenance of the transformed phenotype of myeloma cells both in vitro and in vivo.

Prior and current studies of the effects of eIF4E on proteins regulating cell growth and cell cycle revealed that c-MYC, IRF4 and C/EBPβ protein expression level correlates with eIF4E expressions in MM cells (23, 32). In contrast, normal plasma and CD138⁻ cells showed very low or no detectable expression levels of eIF4E as well as c-MYC, IRF4 and C/EBPβ. Knockdown and introduction of ectopic eIF4E resulted in down and up regulation of MYC, IRF4 and C/EBPβ suggesting that those factors depend on eIF4E-mediated protein translation (Figure 5A). In
multiple myeloma, MYC, IRF4 and C/EBPβ are essential for malignant tumor growth. c-MYC is activated in multiple myeloma cells (30, 34) and targeting MYC by short hairpin RNA induces cell death in myeloma cell lines (31). Further MYC overexpression in tumorigenesis is linked to increased eIF4E activity and upregulated protein synthesis (33, 37). IRF4 was identified as an oncogene associated with the chromosomal translocation t(6;14) (p25;q32) (38) controlling MM survival (31) and overexpression of IRF4 is an adverse prognostic survival marker (31, 39). IRF4 is a target of lenalidomide in MM (23, 40). Interestingly, in myeloma, IRF4 and c-MYC mutually reinforce the expression of each other (31). Taken together, these data suggest that high expression or at least a critical threshold of eIF4E is required for protein translation of transcription factors c-MYC, IRF4 and C/EBPβ and therefore for malignant growth of multiple myeloma cells. The use of inhibitors that directly target the translation initiation complex eIF4F show a promise effects in MM (13-16). Further eIF4E also exhibits oncogenic potential that arises from its critical roles in the nuclear export and cytosolic translation of oncogenic transcripts. Therefore approaches using eIF4E nuclear pore complex inhibitors such as KPT-330 present a promising approach. In conclusion, our studies show that high or at least a critical threshold level of eIF4E is required for myeloma cell growth in order to maintain a sufficient protein translation of cap-dependent transcription factors. This results in a higher sensitivity and dependence of MM cells on protein translation in contrast to non-malignant cells. Therefore protein translation provides an ideal selective therapeutic target allowing the simultaneous and selective reduction of numerous potent growth and survival factors critical for malignant growth.
References


Figure legends

Figure 1. eIF4E is highly expressed in primary MM cells and MM cell lines compared to normal plasma cells and CD138⁻ MNCs

Normal plasma cells (PC 1, 2, 3), primary MM cells (MM 1, 2, 3), MM cell lines (H929, RPMI-8226, U266, OPM2 and MM.1S) and CD138⁻ cells were analyzed for (A) eIF4E protein levels by western blotting of whole cell extracts using β-actin as a loading control and (B) eIF4E RNA levels by RT-PCR relative to non-malignant plasma cell (PC1). (C) Mononucleated bone marrow cells of myeloma patients (BM1 and BM2) were stained with immunofluorescent antibodies for expression of eIF4E (green staining), CD138⁻ (red staining) and nuclear (purple staining) and analyzed using ZEISS, Axiovert 40 CFL fluorescence microscope.

Figure 2. Knockdown of eIF4E selectively inhibits growth of multiple myeloma but not of bone marrow stromal cells

RPMI-8226 or U266 cell lines were infected with either control (CT)-shRNA or eIF4E-shRNA lentiviral particles. After 3 days, cells were selected by puromycin (5µg/ml) for 5 days and analyzed (A) for eIF4E by western blotting and β-actin as loading control, and (B) by RT-PCR to confirm knockdown of eIF4E (C) Both CT-shRNA and eIF4E-shRNA-MM cells were cultured for 2d and proliferation were measured by WST-1 reagent assay. (D) CT- and eIF4E-shRNA cells were analyzed by PI staining for cell cycle analysis. (E) CT-and eIF4E-shRNA-MM cells were seeded in a 24-well plate in methycellulose medium and colony numbers were counted using a Leica DM IL LED inverted phase contrast microscope after 14 days. (F) RPMI-8226 or U266 cell lines were infected with either CT-tet-on-shRNA or eIF4E-tet-on-shRNA lentiviral particles as described above. Selected cells were treated with or without doxycycline (200 ng/ml) for 3 days to induce knockdown eIF4E. Cell lysates were analyzed for eIF4E by western blotting and (G) after 5 days culture proliferation was measured using WST-1 reagent assay.
CT-shRNA and eIF4E-shRNA stromal cells were generated by lentiviral infection. Three days after infection, cells were selected by puromycin (1µg/ml) for 5 days. Cell lysates were analyzed by western blotting for eIF4E expression. CT- and eIF4E-shRNA stromal cells were cultured for 2 days and proliferation was measured by WST-1 reagent assay.

Figure 3. Ectopic overexpression of eIF4E significantly increases MM cell growth in vitro

RPMI-8226 or U266 cells were infected with either empty vector PCDH-GFP or PCDH-eIF4E lentiviral particles. Three days after infection, GFP+ cells were selected by cell sorter (BD Influx). Cell lysates were analyzed by western blotting to compare the levels of eIF4E. β-actin expression was probed for loading control. EV and eIF4E cells were used for mRNA extraction and reverse transcription. eIF4E mRNA levels were compared by real-time PCR. EV and eIF4E cells were seeded in 96 well plates for 2d culture and DNA synthesis was measured by ³H-thymidine incorporation. EV and eIF4E cells were analyzed by PI staining for cell cycle analysis and seeded in a 24-well plate in methycellulose medium. Colonies were stained with crystal violet (Sigma Aldrich) after 14 days and colony numbers were counted using a Leica DM IL LED inverted phase contrast microscope.

Figure 4. eIF4E is critical for MM tumorigenesis in vivo

Subcutaneous tumor growth was measured by using calipers and calculated with the volume formula: 0.5×long diameter ×short diameter². Each bar represents the mean ± SEM (n = 5). After CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg/J</sup>/Crl mice were sacrificed, the tumors were excised, weighed, and pictures were taken. Tumor weights are reported as mean ± SEM (n = 5). * indicated significance with P < 0.05. Control (CT)-shRNA-U266 and eIF4E-shRNA-U266 cells were injected subcutaneously into SCID/bg mice. Mice were sacrificed after 24 days. EV-U266 cells and eIF4E-OE-U266 cells were injected subcutaneously into SCID/bg mice. Mice were
sacrificed after 14 day. (C) CT-tet-on shRNA or eIF4E-tet-on shRNA cells were injected subcutaneously into SCID/bg mice. 11 days after implantation, animals were randomized to receive either vehicle (5% sucrose) or doxycycline (1mg/ml in 5% sucrose) via drinking water for duration of study. Mice were sacrificed after 22 day. (D) Tumors harvested at the end of the study were fixed in formalin and subsequently processed for immunohistochemical staining for eIF4E. Images (x630 magnification) were acquired using Leica DMI 6000B microscope.

Figure 5. eIF4E regulates C/EBPβ, MYC and IRF4 critical for MM cell proliferation

(A) Control (CT)-shRNA, eIF4E-shRNA, empty vector PCDH-GFP and PCDH-eIF4E U266 or RPMI-8266 cells were analyzed by western blotting to compare the levels of eIF4E, c-MYC, IRF4, and C/EBPβ. β-actin expression was probed for loading control. (B) c-MYC mRNA levels of Control (CT)-shRNA, eIF4E-shRNA, empty vector PCDH-GFP and PCDH-eIF4E RPMI-8266 cells were compared by real-time PCR.
Figure 1. (A) Western blot analysis showing the expression of eIF4E and β-actin in normal plasma cells, primary MM cells, and MM cell lines. (B) Bar graph depicting the relative eIF4E mRNA levels in normal plasma cells, primary MM cells, and MM cell lines. (C) Fluorescence microscopy images of BM 1 and BM 2 showing the localization of eIF4E, CD138, and nuclear proteins in primary myeloma cells.
Figure 2.

A. eIF4E, β-Actin

B. RNA levels relative control

C. % Change of Proliferation

D. % cell numbers

E. Colony Numbers

F. eIF4E, β-Actin

G. % Change of Proliferation

H. Stromal cell #1, Stromal cell #2

I. % Change of Proliferation
Figure 3.

A. [Image showing protein expression levels with eIF4E and β-Actin as references for RPMI-8226 and U266 samples withEmpty Vector and eIF4E-OE conditions.]

B. [Graph showing mRNA levels relative to control with * indicating significance for RPMI-8226 and U266 samples comparing Empty Vector and eIF4E-OE conditions.]

C. [Graph showing % Change of Proliferation with * indicating significance for RPMI-8226 and U266 samples comparing Empty Vector and eIF4E-OE conditions.]

D. [Bar graph showing % cell numbers with G0/G1, S, and Sub-G1 phases for RPMI-8226 and U266 samples comparing Empty Vector and eIF4E-OE conditions.]

E. [Bar graph showing Colony Numbers with * indicating significance for RPMI-8226 and U266 samples comparing Empty Vector and eIF4E-OE conditions.]
Figure 4.

A. Tumor Volume (mm$^3$) vs. Day for CT-shRNA and elf4E-shRNA.

B. Tumor Volume (mm$^3$) vs. Day for Empty Vector and elf4E-OE.

C. Tumor Volume (mm$^3$) vs. Day for CT tet-on-shRNA, elf4E tet-on-shRNA, CT tet-on-shRNA DOX, elf4E tet-on-shRNA DOX, DOX.

D. Immunohistochemistry images for Vehicle and DOX.
Figure 5.

A.

RPMI-8226

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<tr>
<th>Protein</th>
<th>Empty Vector</th>
<th>elf4E-OE</th>
<th>CT-siRNA</th>
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<td>C/EBP β</td>
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<td>IRF-4</td>
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<tr>
<td>Actin</td>
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U266

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<th>CT-siRNA</th>
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</thead>
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<tr>
<td>I-MYC</td>
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<td>C/EBP β</td>
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<tr>
<td>Actin</td>
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B.

RPMI-8228

c-MYC mRNA levels relative to control

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<th>Condition</th>
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<th>CT-siRNA</th>
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<tr>
<td>elf4E-OE</td>
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<td>CT-siRNA</td>
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<tr>
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Molecular Cancer Therapeutics

Elevated Translation Initiation Factor eIF4E is an Attractive Therapeutic Target in Multiple Myeloma

Shirong Li, Jing Fu, Caisheng Lu, et al.

Mol Cancer Ther Published OnlineFirst March 3, 2016.

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doi:10.1158/1535-7163.MCT-15-0798

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