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Impairment of Cytoplasmic eIF6 Activity Restricts Lymphomagenesis and Tumor Progression without Affecting Normal Growth

Annarita Miluzio,[1] Anne Beugnet,[1] Stefano Grosso,[1,2] Daniela Brina,[1] Marilena Mancino,[1] Stefano Campaner,[3] Bruno Amati,[3,4] Ario de Marco,[5,6] and Stefano Biffo[1,2,*]
[1]Histology and Cell Growth, San Raffaele Scientific Institute, 20132 Milan, Italy
[2]DISAV, University of Eastern Piedmont, 15100 Alessandria, Italy
[3]Department of Experimental Oncology, European Institute of Oncology (IEO), IFOM-IEO Campus, 20139 Milan, Italy
[4]Center of Genomic Science of Italian Institute of Technology, IFOM-IEO Campus, Via Adamello 16, 20139 Milan, Italy
[5]Protein Chemistry Unit – Cogentech – IFOM-IEO Campus, 20139 Milan, Italy
[6]University of Nova Gorica (UNG), 5000 Nova Gorica, Slovenia
*Correspondence: biffo.stefano@hsr.it
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SUMMARY

Eukaryotic Initiation Factor 6 (eIF6) controls translation by regulating 80S subunit formation. eIF6 is overexpressed in tumors. Here, we demonstrate that eIF6 inactivation delays tumorigenesis and reduces tumor growth in vivo. eIF6$^{+/-}$ mice resist to Myc-induced lymphomagenesis and have prolonged tumor-free survival and reduced tumor growth. eIF6$^{+/-}$ mice are also protected by p53 loss. Myc-driven lymphomas contain PKCβII and phosphorylated eIF6; eIF6 is phosphorylated by tumor-derived PKCβII, but not by the eIF4F activator mTORC1. Mutation of PKCβII phosphosite of eIF6 reduces tumor growth. Thus, eIF6 is a rate-limiting controller of initiation of translation, able to affect tumorigenesis and tumor growth. Modulation of eIF6 activity, independent from eIF4F complex, may lead to a therapeutical avenue in tumor therapy.

INTRODUCTION

Ribosome biogenesis and translation are the sequential processes that underlie the massive process of protein synthesis (Sonenberg and Hinnebusch, 2009). The increased demand of ribosomes, as detected by enlarged argyrophilic nucleoli, has been demonstrated to be an independent prognostic marker for malignancy (Montanaro et al., 2008). Increased rates of protein synthesis have been associated with cell growth and transformation (Silvera et al., 2010). This said, alterations in the ribosomal machinery have been considered for a long time only a by-product of transformation and tumor growth. This view was challenged in recent years by genetic evidence demonstrating that ribosomal alterations modulate tumorigenesis to an unexpected extent. Unfortunately, general rules are difficult to extrapolate from genetic studies. In some circumstances, insufficiency of either ribosomal proteins or of *trans*-acting factors in ribosome biogenesis results in increased susceptibility to cancer, in spite of reduced growth capability. This is evident in Diamond-Blackfan anemia or Swachmann-Diamond syndrome, where depletion of ribosomal proteins and a *trans*-acting factor in nucleolar biogenesis, respectively, increases the risk of hematological malignancy, in the presence of reduced growth (Narla and Ebert, 2010). It is unclear whether this paradoxical effect is due to reduced immune surveillance or to increased cell autonomous genetic instability. Alternatively, increased cancer susceptibility in the presence of altered ribosomal production may be due to abnormal selection of translated mRNAs, as in the case of dyskeratosis congenita (Yoon et al., 2006). In other cases, reduced growth due to haploinsufficiency of ribosomal proteins limits tumorigenesis, as in the case of rpL24 deficiency in mice, which limits Myc-induced

Significance

The translational machinery is an attractive target for cancer therapy. Initiation factors (IFs) are rate-limiting in translation and regulated by the growth factor signaling pathway. For instance, eIF4F complex is essential for translation of structured mRNAs, is involved in cell cycle progression, and is activated by PI3K-mTOR stimulation. Drugs targeting the mTOR pathway, like rapamycin analogs (rapalogs), are used in cancer treatment and explicate their action impairing eIF4F formation. Unfortunately, many cancer cells are insensitive to rapalogs. We searched for IFs that are rate limiting for translation that are controlled by growth factor activation, but not by mTOR. eIF6 fulfills these conditions. We provide data showing that eIF6 is a potential target for cancer therapy, in alternative to eIF4F.

lymphomagenesis (Barna et al., 2008). In this case, the protective effect of rpL24 deficiency is counterbalanced by skeletal abnormalities, retinal degeneration, retarded growth, and melanocyte deficits (Oliver et al., 2004). In conclusion, the unpredictable results of ribosomal depletion and the difficulty to pharmacologically inhibit ribosomal proteins render the targeting of ribosome biogenesis, likely, unfeasible.

The fine-tuning of gene expression and the use of ribosomes in conditions of demanded growth are coordinated by translational control in the cytoplasm (Sonenberg and Hinnebusch, 2009). Translational control in the cytoplasm is relevant to cancer because it shapes the expression pattern of a given cell through regulation of its protein synthesis rate and the selection of the mRNAs to be translated. In addition, the growth factor signaling pathways, which are always activated in cancer cells, have translation factors as their final effectors (Silvera et al., 2010). Thus, activated translation factors may be a valuable target in cancer therapy. Initiation of translation is the rate-limiting step of protein synthesis and is regulated by initiation factors (IFs) (Jackson et al., 2010).

Initiation of translation requires three sequential steps: (1) formation of 43S preinitiation complex by binding of ternary complex eIF2-GTP-tRNAiMet to the 40S ribosomal subunit; (2) formation of 48S preinitiation complex by binding of 43S subunits to mRNA, assisted by the cap binding complex eIF4F; (3) formation of active 80S ribosome by recruitment of free 60S subunits. Of these three steps, eIF4F formation has attracted a strong interest in cancer biology because of experimental evidence indicating its targetability and its activation by growth factors (Sonenberg, 2008). eIF4F complex controls 48S formation by favoring binding of mRNAs to the 40S and unwinding of their 5′ UTRs, downstream of growth factor activation. Notably, several mRNAs involved in cell cycle progression have structured 5′ UTRs and depend on eIF4F for their efficient translation. Elegant genetic and biochemical studies have shown that eIF4F complex formation is critically dependent from mTOR kinase activity and constitutes an important rate-limiting step in tumor growth. Specifically, the cap binding protein eIF4E in the eIF4F complex is inhibited by 4E-BPs. Blockers of mTOR complex 1 (mTORC1) kinase activity cause dephosphorylation of 4E-BPs, which, in its dephosphorylated form, binds and sequesters the cap binding protein eIF4E (Sonenberg and Hinnebusch, 2009). The net result of mTORC1 inhibitors (such as rapamycin) is the impairment of eIF4F formation (Petroulakis et al., 2006). This mechanism has gathered genetic and biochemical evidence, and is pharmacologically exploited by rapalogs that block mTORC1 or by ATP site mTOR inhibitors (Dowling et al., 2010; Hsieh et al., 2010; Ruggero et al., 2004; Wendel et al., 2004). A drawback of this strategy is the relative insensitivity to rapalogs, as reported in vitro on several cancer cell lines and in patients in vivo. This insensitivity may have several causes including mutations in the Ras pathway (Di Nicolantonio et al., 2010). In line with this, we suggested the presence of rapamycin-insensitive translational regulation stimulated by either protein kinase C (PKC) and Ras activators (Grosso et al., 2008) or by adhesion to extracellular matrix (Gorrini et al., 2005). For this reason, the discovery of targetable initiation factors that act on mTORC1 independent mechanisms can be useful.

Most of the proteins that have been identified in the context of ribosome biogenesis do not have a role in translation and vice versa (Miluzio et al., 2009). Eukaryotic Initiation Factor 6 (eIF6) is an exception because it is necessary for ribosome biogenesis in the nucleolus (Sanvito et al., 1999; Si and Maitra, 1999) and it can act as a translation factor in the cytoplasm (Gandin et al., 2008). eIF6 activity regulates the availability of functional 60S ribosomes. 40S ribosomal subunits can bind 60S subunits in the absence of mRNA generating an inactive 80S complex. eIF6 biochemical activity prevents the association between 40S subunit, not loaded with mRNA, and free 60S subunits. eIF6 activity, by preventing premature 80S formation, therefore allows the presence of a pool of free ribosomes in the cytoplasm (Ceci et al., 2003; Valenzuela et al., 1982). The cytoplasmic activity of eIF6 is peculiar because it is dispensable for translation in vitro (Pestova et al., 1998, 2000). However, in eIF6 haploinsufficient cells, where reduction of cytoplasmic eIF6 occurs, basal protein synthesis is normal, but insulin or phorbol ester-stimulated synthesis is abrogated. We have previously shown that eIF6 is highly expressed in human cancer (Sanvito et al., 2000) and fibroblasts with reduced eIF6 levels cannot be efficiently transformed by oncogenes in vitro (Gandin et al., 2008). Here, we address the question whether eIF6 cytoplasmic expression and activation are important for in vivo tumorigenesis, and whether eIF6 inactivation results in reduced tumor growth.

RESULTS

Delayed Lymphomagenesis by Restriction of Myc-Independent eIF6 Expression

We previously generated eIF6 knockout mice and showed that eIF6 heterozygous (eIF6$^{+/-}$) mice are normal with the exception of reduced fat accumulation (Gandin et al., 2008). To understand how eIF6 affects tumorigenesis and tumor growth in vivo, we selected the Eμ-Myc lymphoma model. In this model, expression of the Myc oncogene in the B cell lineage drives a lethal lymphoma with a median survival of 3 months (Iritani and Eisenman, 1999) and associated with increased translation rate and upregulation of eIF4E (Ruggero et al., 2004). Myc-induced lymphomagenesis is reduced by ribosomal protein rpL24 haploinsufficiency (Barna et al., 2008) and increased by eIF4E overexpression (Ruggero et al., 2004). Thus, this model is ideal for evaluating the relative strength by which eIF6 modulates tumorigenesis in comparison to the other members of the translational machinery.

We crossed eIF6$^{+/-}$ mice with Eμ-Myc mice to generate Eμ-Myc/eIF6$^{+/+}$ and Eμ-Myc/eIF6$^{+/-}$ mice. Myc-induced lymphomagenesis results in a 2-fold increase in eIF6 protein levels, both in wild-type and eIF6$^{+/-}$ mice (Figure 1A), confirming that upregulation of eIF6 is part of the oncogenic process. However, Eμ-Myc/eIF6$^{+/+}$ expressed more eIF6 than Eμ-Myc/eIF6$^{+/-}$ indicating that gene dosage is a strong determinant of eIF6 expression. To establish whether eIF6 upregulation was directly dependent on Myc, we infected primary fibroblasts with an inducible MYC gene. Two-fold overexpression of Myc did not change eIF6 protein levels, whereas it increased eIF4E (Figure 1B). We investigated this further by overexpressing Myc using retroviral infection under the control of LTR elements, which increased Myc mRNA 5-fold, eIF4E mRNA 3-fold, but eIF6 mRNA only around 10% (see Figure S1 available online). These data suggest that, different from other members of the translational machinery

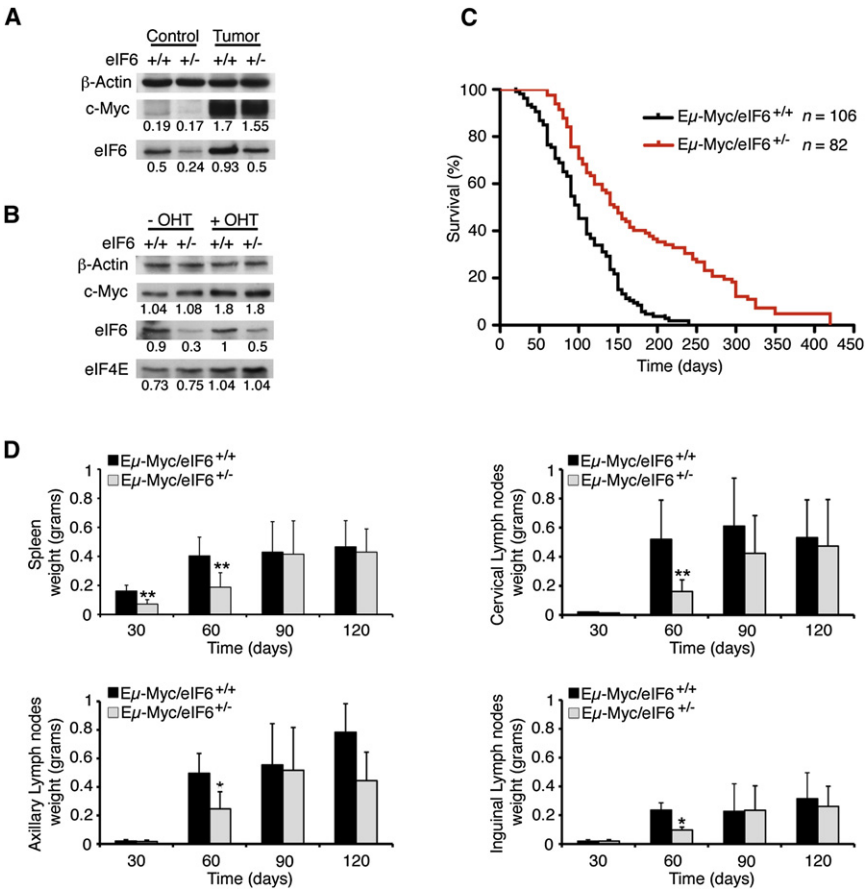

Figure 1. eIF6 Is a Myc-Independent Translation Factor Required for Rapid Myc-Induced Lymphomagenesis

(A) Representative results of western blot analysis on control mice and tumors from Eμ-Myc mice.

(B) Western blot analysis on eIF6$^{+/+}$ and eIF6$^{+/-}$ MEFs expressing MycER, in the absence or presence of OHT. Corresponding densitometric analysis, normalized to actin levels, is indicated.

(C) Kaplan-Meier curves of Eμ-Myc/eIF6$^{+/-}$ mice (n = 82) compared with the Eμ-Myc/eIF6$^{+/+}$ animals (n = 106). ***p < 0.0001.

(D) Weight of spleens and lymph nodes of Eμ-Myc mice. *p < 0.05 and **p < 0.01.

See also Figure S1 and Table S1.

(Ruggero, 2009), eIF6 expression is, at most, weakly regulated by Myc.

Eμ-Myc/eIF6$^{+/-}$ survived longer than Eμ-Myc/eIF6$^{+/+}$ mice (Figure 1C). At 200 days, when virtually all Eμ-Myc/eIF6$^{+/+}$ mice had succumbed to the disease, 40% of Eμ-Myc/eIF6$^{+/-}$ mice were still alive. Maximal survival of Eμ-Myc/eIF6$^{+/-}$ mice was more than 14 months, when mice were sacrificed in the absence of signs of disease. One early sign of lymphomagenesis is the enlargement of the spleen, due to extramedullary hemopoiesis, and of the lymph nodes due to expansion of tumor cells. The spleen of nontransgenics eIF6$^{+/-}$ and eIF6$^{+/+}$ mice were identical, while the spleen of Eμ-Myc/eIF6$^{+/+}$ was grossly enlarged (Table S1). Two-month-old Eμ-Myc/eIF6$^{+/-}$ mice showed little splenomegaly and lymph node enlargement, suggesting a retard in tumorigenesis (Figure 1D). However, the size of lymph nodes and spleen was identical for 3-month-old Eμ-Myc/eIF6$^{+/+}$ and Eμ-Myc/eIF6$^{+/-}$ mice (Figure 1D). In pretumoral mice, spleen enlargement was due to increased proliferation and extramedullary hematopoiesis (Figures 2A and 2B), the latter being less developed in Eμ-Myc/eIF6$^{+/-}$ than in wt. The reduction in extramedullary hematopoiesis of Eμ-Myc/eIF6$^{+/-}$ mice was confirmed by lowered expression of hematopoietic GATA-1 transcription factor in the spleen of heterozygous mice than in wt mice (Figure 2C; Figure S2).

In summary, splenomegaly of Eμ-Myc/eIF6$^{+/-}$ was delayed for 30 days compared with wt mice, due to reduced extramedullary hematopoiesis, indicating a delay in the onset of lymphomas. In contrast, the survival of Eμ-Myc/eIF6$^{+/-}$ mice compared with Eμ-Myc/eIF6$^{+/+}$ mice was more than doubled. These two facts suggested that eIF6 haploinsufficiency limits both tumor onset and growth, which was further investigated.

Reduced Expression of eIF6 Protects from p53 Loss without Affecting Apoptosis

p53 deletion accelerates Eμ-Myc-driven lymphomagenesis by suppressing Myc-induced apoptosis. Since a small nucleolar pool of eIF6 is necessary for biogenesis of the ribosomal 60S subunit (Gandin et al., 2008; Sanvito et al., 1999), and haploinsufficiency of ribosomal proteins causes "ribosomal stress" and p53 induction (Zhang and Lu, 2009), we investigated whether p53 is required for the antitumorigenic effect of eIF6 haploinsufficiency in the Eμ-Myc model. In general, phenotypes due to ribosomal stress are reverted by p53 deletion (Zhang and Lu, 2009). No induction of p53 was seen in eIF6$^{+/-}$ mice (Figures S3A and S3B). The protective effect of eIF6 heterozygosity was observed also in the p53$^{+/-}$/Eμ-Myc mice (Figure 3A). To investigate whether eIF6 acted in the antiapoptotic pathway in pre-B cells, we purified them from several pretumoral 4-week-old mice and analyzed apoptosis. Early activation of Myc led to a strong increase in apoptosis, which was not statistically affected by eIF6 haploinsufficiency (Figure 3B). The spleens of 4-week-old p53$^{+/-}$/eIF6$^{+/-}$ mice showed reduced proliferation compared with that of p53$^{+/-}$/eIF6$^{+/+}$ mice (Figures S3C–S3E).

eIF6 Reduction Impairs Cell Cycle Progression of Pre-B Cells and Both Myc- and LPS/Interleukin 4-Induced Global Translation

To investigate the potential mechanism by which eIF6 reduction delays lymphomagenesis, we analyzed proliferation and differentiation of pre-B lymphocytes from control and Eμ-Myc mice. Pre-B cells from Eμ-Myc mice had a higher percentage of S-phase cells as compared with nontransgenics (Figure 4A). eIF6 heterozygosity caused a statistically significant reduction in the

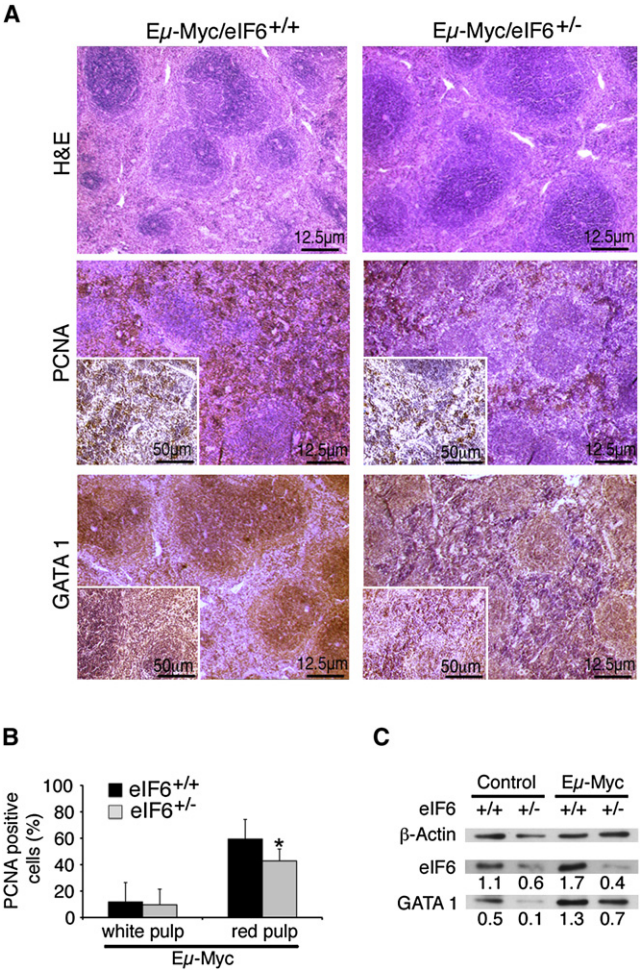

Figure 2. Eμ-Myc/eIF6$^{+/-}$ Mice Show Reduced Splenomegaly at Early Stages of Disease

(A) Representative staining of spleens of 4-week-old Eμ-Myc mice. Spleen morphology was analyzed with H&E staining, while proliferating cells were detected by anti-PCNA antibody. Changes in proliferation rate involve primarily the spleen red pulp (as shown in insets). Hemopoiesis in spleens was analyzed with an anti GATA1 antibody. Scale bar is indicated.

(B) Percentage of PCNA-positive cells in the spleens of 4-week-old mice.

(C) Western blot analysis for eIF6 and GATA1 in spleen extracts. Densitometric analysis is shown.

See also Figure S2.

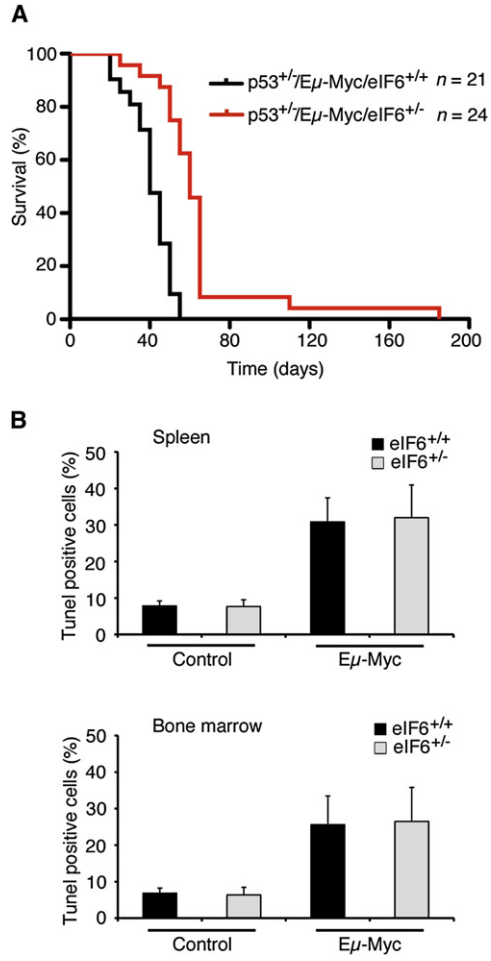

Figure 3. Eμ-Myc/eIF6$^{+/-}$ Mice Are Protected Also by p53 Depletion, without Affecting Apoptosis

(A) Kaplan-Meier curves of p53$^{+/-}$/Eμ-Myc/eIF6$^{+/-}$ mice (n = 24) compared with p53$^{+/-}$/Eμ-Myc/eIF6$^{+/+}$ ones (n = 21). ***p < 0.001.

(B) TUNEL assay on purified pre-B lymphocytes from spleens and bone marrow (n = 3 per genotype).

See also Figure S3.

percentage of S-phase cells and an increase in Go/G1 cells (Figure 4A). In Eμ-Myc mice, B cell development is characterized by a reduction of mature B cells and increase of pre-B cells (Langdon et al., 1986). We did not see a significant change in the percentages of mature and immature B cells in Eμ-Myc/eIF6$^{+/+}$ mice, compared to Eμ-Myc/eIF6$^{+/-}$ mice (Figure 4B). Taken together, data suggest that eIF6 impacts on cell cycle control downstream multiple oncogenic events such as Myc and p53 (see above).

In mouse fibroblasts, eIF6 heterozygosity impairs growth factor and PMA-stimulated translation (Gandin et al., 2008). Myc oncogenesis was shown to increase the translational rate (Barna et al., 2008; Iritani and Eisenman, 1999). Thus, we evaluated how eIF6 haploinsufficiency impacts translation of purified pre-B cells, using methionine incorporation assay. Myc overexpression caused an increase in methionine incorporation. eIF6 haploinsufficiency reduced both Myc-induced and LPS/IL4-induced translation (Figure 4C). These data demonstrate that in conditions of mitogenic signaling eIF6 is rate-limiting for translation in pre-B cells.

The PKCβII-eIF6 Axis Is Active in Lymphomas

To gain an insight on how the translation activity of eIF6 modulates the tumorigenesis process, we exploited its activation properties. RACK1, a ribosomal scaffold protein and intracellular PKC receptor, interacts with eIF6 and modulates its capability to keep ribosomal subunits dissociated (Ceci et al., 2003). PKCβII is the most effective PKC isoform binding RACK1 (Stebbins and Mochly-Rosen, 2001). Ser235 of eIF6 is phosphorylated in vivo upon pharmacological stimulation of PKC and is found phosphorylated in cycling cells (Dephoure et al., 2008). Consequently, we examined whether lymphomas and pretumoral B cells expressed PKCβII and whether this expression could be

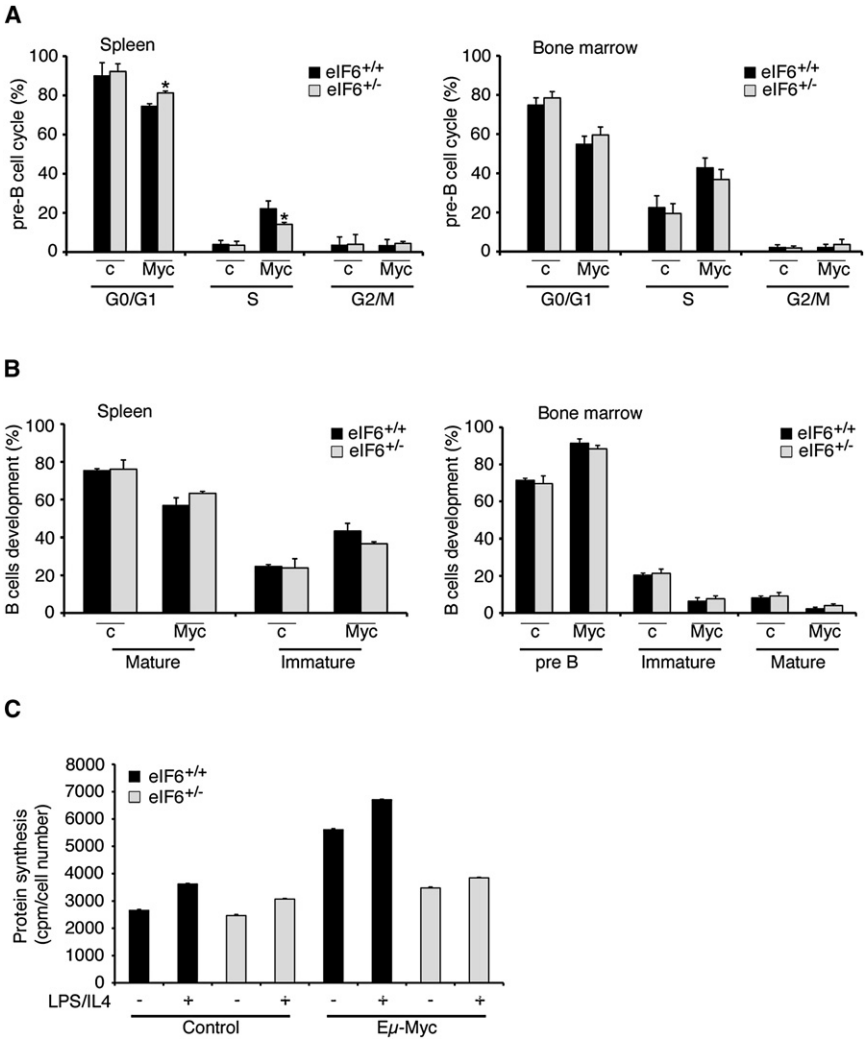

Figure 4. eIF6 Reduction Affects Myc Oncogenesis by Reducing Pre-B Cell Proliferation and Methionine Incorporation

(A) FACS analysis on primary pre-B lymphocytes isolated from spleens and bone marrow of 4-week-old control mice (c) and Eμ-Myc mice (Myc), previously injected i.p. with 1 mg BrdU (n = 3 per genotype). *$p < 0.05$ and **$p < 0.01$.

(B) Development of B cells. B cells of 4-week-old mice isolated from spleen and bone marrow of control (c) mice and Eμ-Myc ones (Myc). Total bone marrow lymphocytes were stained with FITC-anti-mouse B220 and PE-anti-mouse IgM, while total spleen cells were stained with FITC-anti-mouse IgD and PE-anti-mouse IgM; nuclei were counterstained with PI marker and all samples were analyzed by FACS. Data are expressed in percentages (n = 3).

(C) Representative results of ^{35}S-methionine labeling experiment in purified pre-B lymphocytes either unstimulated or upon stimulation with LPS/IL4.

correlated to the phosphorylation of eIF6. PKCβII was expressed in pretumoral B cells already in 4-week-old mice (Figure S4A) and later in all lymphomas (Figure 5A). We immunoprecipitated mTOR and PKCβII from lymphomas and assayed their capability to phosphorylate eIF6. eIF6 was not phosphorylated by mTOR, while 4E-BP1 was (Figure 5B). In the same condition, PKCβII immunoprecipitated from lymphomas phosphorylated eIF6, as well as its control substrate MARCKS1 (Figure 5C). PKCβII activity was already evident in pretumoral samples of 4-week-old mice (Figures S4B–S4D). Next, we assayed by 2D-gel analysis whether endogenous eIF6 was phosphorylated in lymphomas; eIF6 from lymphomas showed 3 spots compatible with phosphorylation (Figure 5D). In summary, data show that PKCβII is abundant in lymphomas and can phosphorylate eIF6 in vitro and in vivo. Since Ser235 is the only consensus site for PKCβII phosphorylation, we addressed its role in translation, tumorigenesis, and tumor growth.

Mutated eIF6 Abrogates Tumorigenesis In Vivo

We analyzed the relevance of Ser235 phosphorylation in transformation and tumor growth in wt and eIF6 heterozygous cells. We transformed wt and eIF6$^{+/-}$ primary fibroblasts using DNp53 + H-ras^{V12}, eIF6$^{+/-}$ fibroblasts transformed at lower efficiency than do wt cells (Figure 6A; Figure S5A). Similar to what we saw in Myc-driven lymphomagenesis, eIF6$^{+/-}$ transformed cells expressed lower levels of eIF6 than do wt cells (Figure 6B). We then proceeded to conduct reconstitution experiments. Transformed primary fibroblasts were reconstituted as follows: (1) eIF6$^{+/-}$ cells with either eIF6 wt, eIF6^{S235A} or control GFP, and (2) wt fibroblasts with either eIF6^{S235A} or control GFP. Analysis of the levels of eIF6 in each population indicated that each construct was mildly overexpressed (Figure 6C). Reconstitution of eIF6$^{+/-}$ fibroblasts with wt eIF6 increased their anchorage-independent colony formation, whereas the reconstitution with eIF6^{S235A} decreased it (Figure 6D). Similarly, wt eIF6 fibroblasts expressing eIF6^{S235A} showed a reduction of the transformation rate (Figure 6D). Similar results were obtained in primary fibroblasts transformed with Myc + H-ras^{V12} (Figure S5B). Taken together, these data imply that eIF6 levels affect transformation in vitro and that mutation of Ser235 reduces, but not abolishes, the rate of transformation. We evaluated global translation of MEFs transformed by Myc + H-ras^{V12} and found that the translational rate of cells expressing eIF6^{S235A} reduced considerably during transformation (Figure 6E).

Next, we injected equal numbers of transformed MEFs in nude mice. We found that transformed eIF6 wt fibroblasts grew faster than do their eIF6$^{+/-}$ counterpart (Figure 7A). Furthermore, the expression of eIF6^{S235A} in both transformed wt eIF6 (Figure 7B) and eIF6$^{+/-}$ cells (Figure 7C) reduced the growth of tumors. In transformed eIF6$^{+/-}$ cells, the re-expression of wt eIF6 accelerated tumor growth (Figure 7C). We analyzed morphology, proliferation, apoptosis, and angiogenesis in tumor xenografts. Tumors expressing wild-type levels of eIF6 had more BrdU

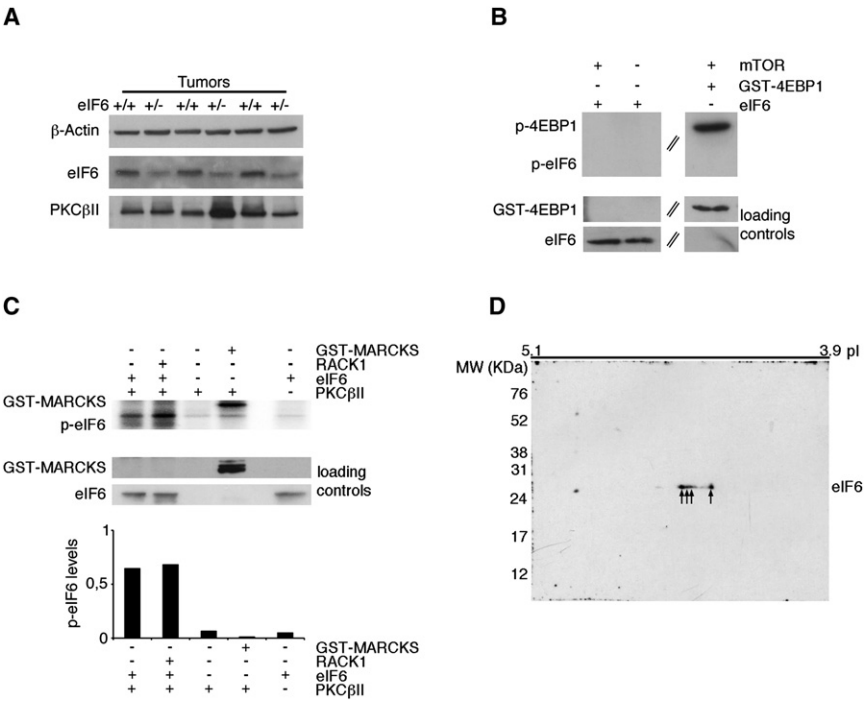

Figure 5. eIF6 Is Phosphorylated in Myc-Driven Lymphomas Expressing PKCβII

(A) Lymphomas express PKCβII. Representative western blot of proteins recovered from tumors of Eμ-Myc mice presenting comparable weight.

(B and C) Kinase assay on tumor samples at indicated conditions. eIF6 is directly phosphorylated by PKCβII (C), but not by mTOR (B). Corresponding densitometric analyses were normalized to background and appropriate controls indicated.

(D) Representative 2D gel electrophoresis of a lymphoma sample: black arrows indicate eIF6. See also Figure S4.

labeling, a marker of S-phase entry, than eIF6$^{+/-}$ tumors (Figures 7D and 7E). Compared with wt, eIF6$^{+/-}$ tumors also presented reduced cellular density (Figure 7E; Figure S6). We did not detect differences in the apoptotic rate between wt and eIF6$^{+/-}$ tumors, as determined by TUNEL and caspase 3 stainings, and both tumor types presented sporadic clusters of apoptotic cells in necrotic or infiltrated areas (Figure S6). In addition, eIF6$^{+/-}$ tumors show fewer CD31-positive cells. In conclusion, Ser235 of eIF6 is not essential for normal growth, but it is important for transformation and for growth of tumor cells, in vivo.

DISCUSSION

We have presented evidence that eIF6 activation is rate-limiting in tumorigenesis and tumor growth in vivo. We showed that eIF6$^{+/-}$ mice are resistant to lymphomagenesis also in conditions of p53 haploinsufficiency, suggesting that the protective effect is independent from ribosomal stress. We found that PKCβII, but not mTORC1, phosphorylates eIF6, suggesting that eIF6 activity is not under mTOR control. Since eIF6 haploinsufficiency has limited or no negative side effects, we hypothesize that eIF6 would be an attractive therapeutic target for treating tumors, in particular, those insensitive to mTOR inhibition.

Upstream Regulation of eIF6

Understanding the transcriptional network regulating eIF6 expression is important to define which oncogenes could control eIF6 during the process of tumorigenesis. It has been proposed that Myc acts as a master regulator of translational control, by affecting multiple targets that are rate limiting in translation (Ruggero, 2009; White, 2008). Myc regulates the expression of genes of the eIF4F complex, which are necessary for cap-dependent translation and for efficient translation of structured mRNAs like cyclin D (Ruggero, 2009). These data suggest that Myc evolved as a powerful transcriptional regulator affecting the translation of specific classes of mRNAs.

Our data show that eIF6 is not directly regulated by the oncogene Myc. In the past, we showed that eIF6 expression is regulated by transcription factor complex GA-binding protein (GABP) (Donadini et al., 2006). GABP regulates the expression of nuclear genes involved in mitochondrial respiration (Ristevski et al., 2004). In addition, among GABP targets there are also ribosomal proteins (Perry, 2005). Thus, GABP may regulate the transcription of genes of the ribosomal and the mitochondrial machineries upon increased needs of metabolic rates. So far, a role for GABP in tumorigenesis has not been described. Considering that GABP may have an essential role in mediating the proliferative response, it will be of interest to define if some oncogenes directly act on GABP expression.

Finally, the strong gene dosage effect of eIF6 may suggest that in some conditions, tumor cells may have an advantage to amplify eIF6 gene. In this sense, amplification of the 20q11.2 has been reported in myelodispastic syndrome and acute myeloid leukemya (Mackinnon and Campbell, 2007), but specific studies on eIF6 gene are lacking.

Downstream Targets of eIF6: Are They Specific or Not?

The specific effect of eIF6 haploinsufficiency in restricting tumorigenesis without obvious negative side effects is remarkable. In heterozygous mice, the only phenotype we could observe was a nonpathogenic reduction in liver mass and in adipose tissue (Gandin et al., 2008). This observation raises the question on whether eIF6 regulates the translation of specific mRNAs. Studies on eIF6$^{+/-}$ cells expressing Myc or with inactivated p53 showed a delay in G1/S phase progression suggesting that specific mRNAs involved in cell cycle progression can be affected at the level of translation. Thus, at first sight proliferating cells seem more sensitive to eIF6 depletion. Expression data support this hypothesis; eIF6 is enriched in the stem cell and proliferating compartment of colonic epithelium (Sanvito et al., 2000) and is generally highly expressed in embryonic and epithelial tissues (Biffo et al., 1997). Furthermore, we found that

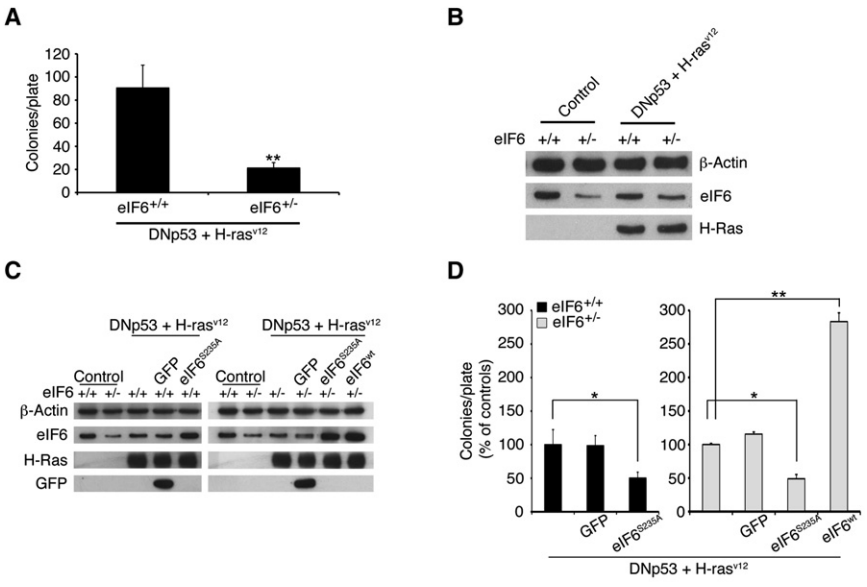

Figure 6. eIF6 Haploinsufficiency and Mutation of eIF6^{S235A} Reduce Transformation and Protein Synthesis

(A) Transformation rate of wt and eIF6$^{+/-}$ fibroblasts with indicated retroviruses (n = 6).

(B) eIF6 levels were determined by western blotting in wt and heterozygous eIF6 transformed cells. Anti H-Ras antibody was used as a control for retroviral infection.

(C) Western blot analysis of eIF6 levels in eIF6wt and eIF6^{S235A} transduced cells. Anti H-Ras and anti-GFP antibody were used as a control for retroviral and lentiviral infection, respectively.

(D) Transformation assay on eIF6$^{+/+}$ and eIF6$^{+/-}$ primary fibroblasts transformed with DNp53 plus H-ras^{V12}. Both genotypes were then infected with lentivirus carrying GFP as an internal control and mutant eIF6^{S235A}, while eIF6$^{+/-}$ MEFs received also a lentivirus bearing full-length eIF6 (eIF6^{wt}) to rescue normal eIF6 protein levels. Single retroviral infections are indicated as a control (100%). (n = 6 per genotype).

(E) ^{35}S-methionine incorporation on MEFs cells sequentially infected with lentiviral vector expressing either GFP, as control, or mutated eIF6 ^{S235A}, followed by retrovirus bearing Myc + H-ras^{V12} oncogenes. Methionine incorporation was measured before retroviral infection with Myc + H-ras^{V12} (left), 3 days after retroviral infection (middle), and 10 days after retroviral infection when cells showed signs of transformation (right). Results are expressed in percentage of control. See also Figure S5.

translation upon growth factor and insulin stimulation is impaired in conditions of eIF6 haploinsufficiency (Gandin et al., 2008), suggesting that mRNAs involved in the mitogenic response and glucose metabolism are more susceptible to changes in eIF6 levels.

The molecular mechanism by which eIF6 regulates translation is to prevent improper 80S complex formation. eIF6 binds, in a regulated fashion, 60S subunits, preventing their association with 40S subunits that are not loaded with mRNA. This mechanism allows the presence of a pool of free ribosomes in the

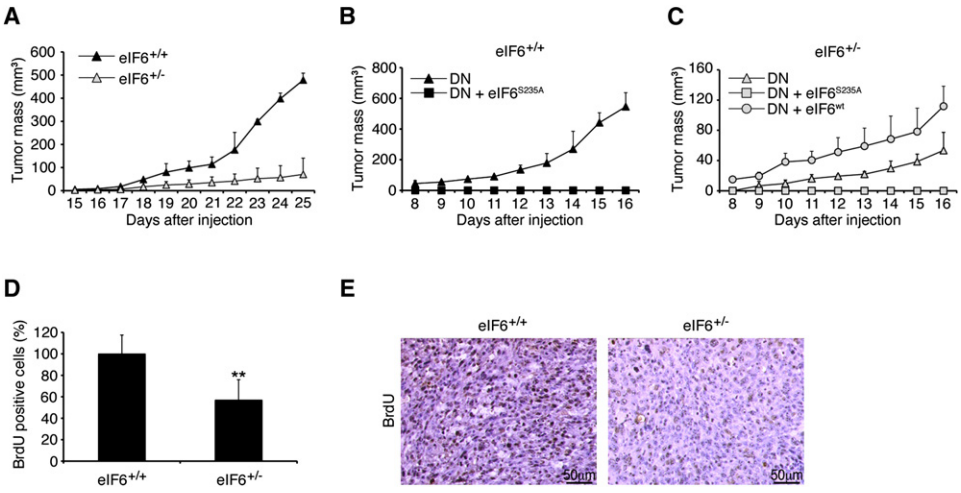

Figure 7. eIF6 Reduction and Mutation of eIF6^{S235A} Reduce Tumor Growth in Xenograft

(A–C) Subcutaneous injection of transformed eIF6$^{+/+}$ and eIF6$^{+/-}$ MEFs with DNp53 plus H-ras^{V12} (DN) reconstituted with either wt eIF6 or eIF6^{S235A} (n = 3 for A, n = 6 for B and C).

(D and E) Proliferation rate was measured by BrdU incorporation. Quantification was performed setting at 100% the values of control mice; **p < 0.01 (D). Corresponding representative immunohistochemical staining (E). Scale bar is indicated.

See also Figure S6.

cytoplasm (Ceci et al., 2003; Valenzuela et al., 1982). Since eIF6 binds 60S subunits, it is not expected to actively select for mRNAs for translation. In principle, there are two possible mechanisms by which eIF6 could affect the translation of specific mRNAs. One, indirectly, by reducing available 60S, eIF6 haploinsufficiency generates a strong competition between mRNA-loaded 48S subunits, which may lead to a competitive disadvantage for some mRNAs. In this case, it will be difficult to identify specific mRNAs affected, but rather it is expected the narrowing of the translational landscape, as recently suggested for eIF4G depletion (Park et al., 2011). In this context, it would be interesting to study whether inhibition of eIF6 acts synergistically to 4E-BP dephosphorylation, which affects the translation of eIF4F-dependent mRNAs. A second possibility is that eIF6 regulates the translation of a specific class of mRNAs. Myc-hyperactivation increases cap-dependent translation and reduces translation of IRES-containing mRNAs, whereas deficiency of large ribosomal protein rpL24 reverts IRES translation, at mitosis, and reduces lymphomagenesis (Barna et al., 2008). The mechanism by which eIF6 acts is, however, very different from rpL24 because eIF6 haploinsufficiency limits not only Myc-induced translation, but also growth factor-induced translation. Indeed, eIF6 haploinsufficiency also restricts proliferation in conditions of p53 depletion, thus resembling a general translation node regulating cell cycle progression. In addition, we have not observed in eIF6 heterozygous cells changes in the translation efficiency of HCV and Myc IRES reporters. Further work to identify specific mRNA targets is required.

eIF6 Broadens Cancer Treatment as Target of the Translational Machinery

A growing interest has risen regarding translational control as a determinant of cancer tumorigenesis and as a pharmacologically targetable process (Silvera et al., 2010; Sonenberg and Hinnebusch, 2009). There are more than 20 initiation factors (Jackson et al., 2010), but only eIF4F complex formation has reached sufficient genetic and pharmacological evidence to be an important target in cancer (Hsieh et al., 2010; Ruggero et al., 2004; Wendel et al., 2004). eIF4F targeting can be obtained through mTOR inhibition using rapalogs (Robert and Pelletier, 2009) or ATP site mTOR inhibitors (Feldman et al., 2009; Hsieh et al., 2010; Janes et al., 2010). More recently, blockers of eIF4E/eIF4G interaction (Moerke et al., 2007) or eIF4A have also been proposed (Cencic et al., 2010). Due to the fact that eIF4F inhibition acts exclusively at the level of 48S formation, and that resistance to mTORC1 inhibition may be reverted by activation of the Ras pathway (Di Nicolantonio et al., 2010), it may be of value to target other steps of initiation that are under growth factor activation and are rate limiting. For this, eIF6 is particularly attractive because its activity is independent from mTORC1 phosphorylation, but specific to conditions of growth factor activation.

Phosphorylation of Ser 235 is important for protumorigenic activity of eIF6 (Figure 8). The phosphorylation of eIF6 is stimulated by agonists of phorbol esters, hence of PKC and of the Ras cascade (Ceci et al., 2003). Tumor-derived PKCβII phosphorylates Ser235 of eIF6, in vitro. A clear mechanistic role of eIF6 phosphorylation in vivo is not available, whereas, in vitro,

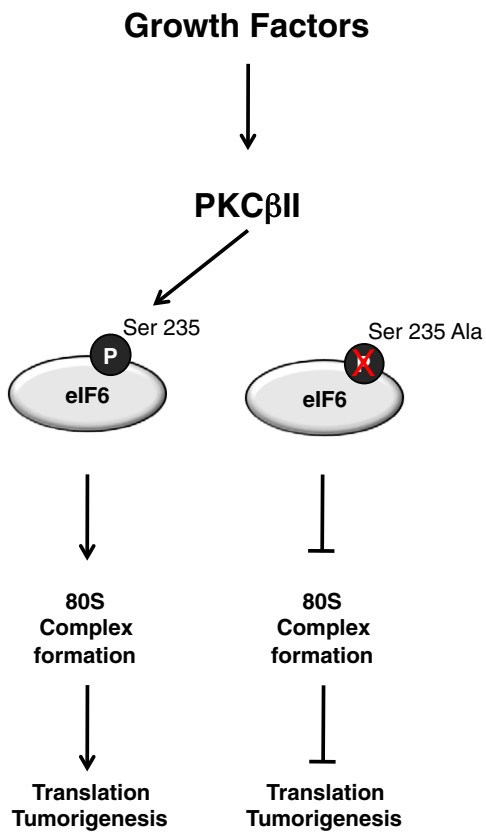

Figure 8. Model of eIF6 Activation

Growth factors activate PKC. In our model, eIF6 is phosphorylated by PKCβII on Ser235, the only consensus site for PKCβII phosphorylation; eIF6 activity is modulated by PKC, resulting in increased translation and tumorigenesis.

phosphorylation of Ser235 causes release of eIF6 from 60S (Ceci et al., 2003), which presumably is a necessary step for 60S activation. PKCβII is the preferred partner of RACK1, a PKC receptor shown to be part of ribosomal particles (Sengupta et al., 2004). PKCβII is upregulated in human lymphomas and its upregulation is linked to poor outcome in B cell malignancies (Chaiwatanatorn et al., 2009; Schaffel et al., 2007). PKCβ inhibition reduces translation without affecting mTORC1 targets (Grosso et al., 2008). Taken together these observations suggest a role for the PKC axis in the regulation of translation, in conditions of elevated growth factor signaling. Enzastaurin is a PKCβ inhibitor with a broad range of inhibition in tumor cell lines and is currently being evaluated in clinical trials against lymphomas (Leonard et al., 2008). It will be interesting to define if part of the action of enzastaurin is through inhibition of eIF6 activity.

The binding activity of eIF6 to ribosomes is well defined and amenable to the development of high-throughput screenings for agonists/antagonists. However, our knowledge of the eIF6 molecular mechanism is still too limited to predict the effects of agonists or antagonists of eIF6 binding to the 60S. Theoretically, antagonists of eIF6 binding to 60S should be effective blockers of eIF6 function. All these hypotheses must be now experimentally addressed.

EXPERIMENTAL PROCEDURES

Mice

All experiments were approved by the Ethical Committee of San Raffaele and comply with E.C. regulations (IACUC authorization SK397). eIF6$^{+/-}$ mice were generated as previously described (Gandin et al., 2008), and backcrossed to C57BL6/N strain for a minimum of eight generations. eIF6$^{+/-}$, p53$^{-/-}$, and Eμ-Myc transgenic mice were intercrossed to obtain the genotypic combinations, as described in the manuscript. Mice were monitored daily for the tumor development until they died spontaneously or were sacrificed if showing evident signs of distress. Kaplan-Meier curve was used to examine the survival rate of all considered animals. Cervical, axillary, and inguinal lymph nodes and spleens of these mice were recovered, weighted, and used for further analysis. Eight-week-old CD1 athymic nude mice were used for detecting tumor growth after a subcutaneous (s.c.) injection of transformed MEFs cells, as indicated in this paper. Mice with tumors larger than 600 mm^3 were sacrificed. Genotyping of the offspring mice was detected by PCR using AmpliTaq Gold (Roche) according to the manufacturer's protocol. The PCR primers for genotyping of eIF6 and Eμ-Myc transgenic mice were previously reported (Gandin et al., 2008; Gorrini et al., 2007); p53 genotyping was performed using the specific primers: 5′-ACAGCGTGGTGGTACCTTAT-3′ (wt allele), 5′-TATACTCAGAG CCGGCCT-3′ (common primer), and 5′-CTATCAGGACATAGCGTTGG-3′ (mutant allele). PCR products were resolved on 2% agarose gels.

Antibodies and Reagents

The following antibodies were used: rabbit polyclonal antibodies against eIF6 (Biffo et al., 1997), c-Myc, Cleaved Caspase 3, RACK1 (Cell Signaling), PKCβII, H-Ras (Santa Cruz), PCNA, GATA1 (Abcam); mouse monoclonal antibodies against BrdU (Sigma), p53 (Cell Signaling), β-Actin (Sigma) and eIF6. LPS, IL4, 4-hydroxy-tamoxifen (OHT) and all powders and reagents were from Sigma.

Primary Cell Culture

Primary MEFs were isolated from 13.5 d.p.c. embryos as previously described (Gandin et al., 2008). Primary B lymphocytes were isolated from spleen and bone marrow of 4-week-old mice by labeling with CD45R (B220) microbeads and using an autoMACS separator (Milteny Biotec), according to manufacturer's instruction and (Gorrini et al., 2007). All the analyses were performed at least three times on different genetic backgrounds.

Cell cycle rate was analyzed on freshly isolated B lymphocytes from 4-week-old mice (eIF6$^{+/+}$/Eμ-Myc and eIF6$^{+/-}$/Eμ-Myc mice). These mice received 1 mg of BrdU (Sigma) by intraperitoneal (i.p.) injection 2 hr before being sacrificed. Primary B lymphocytes were recovered from spleen and bone marrow, fixed in 95% ethanol, and labeled with FITC-conjugated anti-BrdU, using the BD Biosciences BrdU Flow Kit, following manufacturer's instructions. B lymphocytes development was examined by labeling specific surface markers with FITC-anti-mouse IgD, FITC-anti-mouse B220, and PE-anti-mouse IgM (BD Pharmingen). Nuclei were counterstained with propidium iodide (PI) marker. For both analyses, 10,000 events per tube were acquired by BD FACS CANTO II flow cytometer and then analyzed using the FCS Express software (BD). The apoptotic rate was also measured on the same cells. B lymphocytes were fixed in paraformaldehyde, permeabilized, and stained using In Situ Cell Death Detection Kit, AP (Roche), following the manufacturer's instructions. All the results were expressed as percentage and each experiment was done in triplicate.

Translation Analysis

Cells were pulsed with ^{35}S-labeled methionine (Perkin-Elmer) for 30 min, in the tested conditions. Cells were lysed in 50 μl of RIPA buffer without SDS (10 mM Tris-HCl [pH 7.5], 1% Na-deoxycholate, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail). Aliquots of 10 μl of extracts were TCA-precipitated on glass microfiber filters (Whatman) and counted. Obtained values were normalized on viable cell number. Each sample was performed in triplicate.

Histological Staining, Immunohistochemistry, and In Situ TUNEL Assay

Immunohistochemical and histological analysis were performed on paraffin-embedded sections obtained from spleens of eIF6$^{+/+}$/Eμ-Myc, eIF6$^{+/-}$/Eμ-Myc, and p53$^{+/-}$ mice and from tumors derived from nude mice. Hematoxylin and eosin staining (H&E) was performed on all tissues for morphological analysis, while immunohistochemistry for the considered targets was done using the Vectastain Elite ABC kit (Vector), according to the manufacturer's instructions. Apoptotic cells were identified on tumor sections using a commercially available In Situ Cell Death Detection Kit, AP (Roche), according to manufacturer's protocol.

Transformation Analysis

Primary fibroblasts were infected at early passage with a retrovirus carrying both Myc and DNp53 + oncogenic H-ras^{V12} as previously described (Gandin et al., 2008). After 2 days these cells were also infected with lentiviral vectors. The lentiviral vectors used in this study express full-length wt eIF6, mutant eIF6^{S235A} and GFP as internal control. Packaging plasmid VSV-G, PMDLg/pRRE, pREV, and transfer vector pCCL-PPT-hPGK-pre used in this paper was previously described and full-length wt eIF6, mutant eIF6^{S235A}, and GFP cloning was performed as previously described (Gandin et al., 2008). Foci were counted 2–3 weeks after infections and transformed cells were recovered for subcutaneous injection in nude mice (500,000 cells/mouse).

Kinase Assay

Proteins from Eu-Myc tumoral lymph nodes were extracted in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 1× Protease Inhibitors (Sigma), 1 mM NaF, 10 mM β-glycerophosphate), clarified by centrifugation and protein concentration was quantified by the BiCinchonic Acid (BCA) protein assay (EuroClone). For PKC assays, lysates were subjected to preclearing with Protein G (GE Healthcare) for 2 hr at 4°C, and precleared protein extracts were incubated with antibody against PKCβII (Santa Cruz Biotechnology) overnight at 4°C using constant rotation. Immunoprecipitation was performed with protein G for 2 hr. Beads were washed three times and resuspended in lysis buffer. The kinase assay was performed by adding 3 μg of eIF6 recombinant protein, 3 μg of recombinant RACK1 (Ceci et al., 2003), or 5 μg of GST-MARCKS (Myristoylated Alanine-Rich C-Kinase Substrate (Soh and Weinstein, 2003) in the PKCβII specific buffer (100 mM $MgCl_2$, 10 mM $CaCl_2$, lipid mixture) either in the presence or absence of immunoprecipitated PKCβII. mTOR kinase assay was instead performed by adding either 3 μg of eIF6 or 5 μg of GST- 4E-BP1 recombinant protein to immunoprecipitated mTOR in a specific buffer (10 mM HEPES, 50 mM β-glycerophosphate, 150 mM NaCl). The negative control was an immunoprecipitate obtained with irrelevant antibodies.

Four μCi of γ32-ATP (Perkin Elmer) were added to each sample. The reaction was run at 30°C for 1 hr and terminated by adding one volume of sample buffer. The samples were boiled 5 min, separated by SDS-PAGE and stained with Coomassie Brilliant blue R-250. Autoradiography was performed on dried gels. Recombinant eIF6 was prepared in *E. coli*. N-terminal histidine tagged human eIF6 was coexpressed with a mixture of molecular chaperones (de Marco et al., 2007). Protein production was induced at 16°C overnight and the recombinant protein was first recovered by metal affinity chromatography. Monomeric active eIF6 was separated by inactive, dimeric and aggregated eIF6 by gel filtration. GST-4E-BP1 was a kind gift of Dr. C. Proud (University of Southampton, UK).

Two-Dimensional Gel Electrophoresis

Tumor samples were examined in two-dimensional gel electrophoresis. Samples were lysed in RIPA buffer and proteins were precipitated with 10% Trichloroacetic acid. Pellets were resuspended in two-dimensional buffer (7 M Urea, 2 M Thiourea, 50 mM DTT and 4% CHAPS) and 100 μg of proteins were isoelectrofocused. The first dimension was performed on ReadyStrip IPG Strips (pH 3.9–5.1; Biorad) For the reduction/alkylation step, the strips were incubated with re-equlibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) plus DTT and re-equilibration buffer plus iodoacetamide, respectively.

Then, the strips were subjected to SDS/PAGE for the second dimension. Proteins were transferred on PVDF membrane and subsequently incubated with eIF6 monoclonal antibodies. The signal was detected with an anti-mouse secondary antibody and ECL substrate kit (GE Healthcare). Each experimental sample was run at least twice, and at least three different biological replicates were analyzed.

Statistical Analysis

Each experiment was repeated a minimum of three times, as biological replicates; means and standard deviations between different experiments were calculated. Statistical *P*-values obtained by Student t test were indicated: three asterisks *** for p values less than 0.001, two asterisks ** for p values less than 0.01 and one asterisk * for p values less than 0.05. Kaplan-Meier curves were validated by the log-rank test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at doi:10.1016/j.ccr.2011.04.018.

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