

Phosphorylated 4E-BP1 Is Associated with Poor Survival in Melanoma

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Abstract Purpose: Both phosphatidylinositol 3-kinase/AKT and RAS/mitogen-activated protein kinase signal transduction pathways mediate 4E-BP1 phosphorylation, releasing 4E-BP1 from the mRNA cap and permitting translation initiation. Given the prevalence of PTEN and BRAF mutations in melanoma, we first examined translation initiation, as measured by phosphorylated 4E-BP1 (p-4E-BP1), in metastatic melanoma tissues and cell lines. We then tested the association between amounts of total and p-4E-BP1 and patient survival.

Experimental Design: Seven human metastatic melanoma cells lines and 72 metastatic melanoma patients with accessible metastatic tumor tissues and extended follow-up information were studied. Expression of 4E-BP1 transcript, total 4E-BP1 protein, and p-4E-BP1 was examined. The relationship between 4E-BP1 transcript and protein expression was assessed in a subset of patient tumors ($n = 41$). The association between total and p-4E-BP1 levels and survival was examined in the larger cohort of patients ($n = 72$).

Results: 4E-BP1 was hyperphosphorylated in 4 of 7 melanoma cell lines harboring both BRAF and PTEN mutations compared with untransformed melanocytes or RAS/RAF/PTEN wild-type melanoma cells. 4E-BP1 transcript correlated with 4E-BP1 total protein levels as measured by the semiquantitative reverse-phase protein array ($P = 0.012$). High levels of p-4E-BP1 were associated with worse overall and post-recurrence survival ($P = 0.02$ and 0.0003 , respectively).

Conclusion: Our data show that translation initiation is a common event in human metastatic melanoma and correlates with worse prognosis. Therefore, effective inhibition of the pathways responsible for 4E-BP1 phosphorylation should be considered to improve the treatment outcome of metastatic melanoma patients.

Over the last few years, our understanding of the molecular alterations underlying melanoma development and progression has rapidly advanced, with several independent signaling pathways having been implicated in melanoma pathogenesis,

including the RAS/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (1–3). These recent discoveries have translated into the development of new molecularly targeted therapies, such as specific BRAF, MEK, and mammalian target of rapamycin inhibitors. However, these therapeutic agents have shown only modest activity against melanoma in phase II clinical testing (4, 5). Alternative strategies based on a better understanding of the complex molecular pathogenesis of melanoma are needed before improvement in treatment outcomes for metastatic melanoma patients can be appreciated.

The PI3K/AKT pathway canonically regulates translation via activation of mammalian target of rapamycin kinase and subsequent phosphorylation of its substrates, 4E-BP1 and S6K, which leads to formation of the eIF4F translation initiation complex (6–8). Sustained activation of this translation machinery is essential for the transformation of human cells in culture and the maintenance of the malignant phenotype (9, 10). In this regard, 4E-BP1 phosphorylation has recently been shown to correlate with worse pathologic grade and prognosis in human breast cancer (11). The RAS/MAPK pathway also contributes to the formation of eIF4F and activation of translation through extracellular signal-regulated kinase-mediated phosphorylation of both S6K and 4E-BP1 and MNK-mediated phosphorylation of eIF4E. Thus, both RAS/MAPK and PI3K/AKT signaling pathways mediate phosphorylation of

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Translational Relevance

Our study reveals that the translation repressor 4E-BP1 is widely expressed and frequently phosphorylated in metastatic melanoma. We also show that higher levels of phosphorylated 4E-BP1 impart a worse prognosis in metastatic melanoma patients compared with those patients with relatively low levels of phosphorylated 4E-BP1. As phosphorylation disengages 4E-BP1 from the mRNA cap and results in disinhibited protein biosynthesis, our work suggests that unrestrained protein translation may be a hallmark of the most aggressive subset of metastatic melanomas. Therefore, strategies to hamper deregulated translation, such as 4E-BP1 dephosphorylation and/or up-regulation, should be studied as potential therapies in this cancer.

4E-BP1, thereby disengaging the protein from the mRNA cap and disinhibiting translation (12, 13).

In this study, we assessed eIF4F complex formation by measuring total and phosphorylated 4E-BP1 (p-4E-BP1) in both cell lines and in metastatic melanoma tumors. We first assessed the levels of 4E-BP1 phosphorylated at different sites (T37/46, T70, and S65) as well as total and phosphorylated levels of the translation initiation factors eIF4G and eIF4E in a panel of 7 human melanoma cell lines. We found that levels of p-4E-BP1 varied widely among the 7 melanoma cell lines and were higher in the BRAF and PTEN mutated cells. As p-4E-BP1 was differentially regulated among melanoma cell lines, we then employed a combination of assays to study the expression of total and p-4E-BP1 in the tumors of metastatic melanoma patients and studied the association of both protein expression and phosphorylation with survival.

Our data show that translation initiation, as measured by p-4E-BP1, is a common event in human metastatic melanoma and correlates with worse prognosis. Given the convergence of RAS/MAPK and PI3K/AKT signaling that mediates phosphorylation of 4E-BP1, dephosphorylation of 4E-BP1 may prove to be an effective strategy to improve treatment outcome in patients with metastatic melanoma.

Materials and Methods

Tissue culture and Western blot analysis. Seven human metastatic melanoma cell lines, including SkMel-31, SkMel-2, SkMel-11, SkMel-37, SkMel-39, SkMel-28, and SkMel-5 (kindly provided by Dr. Alan Houghton, Memorial Sloan-Kettering Cancer Center), and the PI3K mutant human breast adenocarcinoma cell line MCF-7 (American Type Culture Collection) were studied. Cells were maintained in either RPMI or a 1:1 mixture of DMEM/F-12 supplemented with 2 mmol/L glutamine, 50 units/mL penicillin, 50 units/mL streptomycin, and 10% fetal bovine serum and incubated at 37°C in 5% CO₂. HeMnLP cells were maintained in medium 254 (Life Technologies) supplemented with human melanocyte growth supplement-2 (Life Technologies). Cells were harvested and lysed in mRIPA buffer. Protein concentration was determined with the BCA method (Pierce). Samples were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane. Membranes were blocked with 5% milk and probed with 4E-BP1, p-4E-BP1 (T37/46), p-4E-BP1 (T70), p-4E-BP1 (S65), eIF4G, p-eIF4G (S1108), elongation initiation factor 4E (eIF4e), p-eIF4e (S209), PTEN (Cell Signaling Technology), and β -actin (Sigma-Aldrich)

antibodies. Proteins were detected using the enhanced chemiluminescence kit (Amersham Biosciences) and densitometry was done with MultiGuage Software (FujiFilm Life Sciences).

Patient characteristics. The study cohort consisted of 72 metastatic melanoma patients identified through the Interdisciplinary Melanoma Cooperative Group database at New York University School of Medicine (48 male, 24 female; median age, 60.0 years). Of the 77 specimens from 72 patients, 34 were lymph node metastases, 28 were skin metastases, and 15 were visceral metastases. Seventy-seven specimens from 72 patients were used for immunohistochemistry, and 41 specimens from 36 patients were used for transcript expression analysis and reverse-phase protein array (RPPA). Of the 77 specimens, 39 were analyzed by immunohistochemistry, microarray analysis, and RPPA for total and p-4E-BP1 based on corresponding paraffin tissue availability. Serum lactate dehydrogenase (LDH) was measured in 47 of the 72 patients. BRAF mutation status was determined by DNA sequencing in tissue specimens from 29 of the 72 patients. The median follow-up time for the cohort from the time of primary diagnosis to last follow-up date was 44.32 months. The study was approved by the New York University Institutional Review Board, and all patients signed informed consent before enrollment. Relevant clinicopathologic, demographic, and survival data were recorded for all patients.

Immunohistochemistry. Immunohistochemistry was done on 77 formalin-fixed, paraffin-embedded tissues using rabbit anti-human T70-p4E-BP1 and total 4E-BP1 (Cell Signaling Technologies). In brief, sections were deparaffinized in xylene (3 changes), rehydrated through graded alcohols (3 changes of 100% ethanol and 3 changes of 95% ethanol), and rinsed in distilled water. Heat-induced epitope retrieval was done in 10 mmol/L citrate buffer (pH 6.0) for 10 min (both antibodies) in a 1,200 W microwave oven at 90% power. Sections were allowed to cool for 30 min and then rinsed in distilled water. Antibody incubations and detection were carried out at 37°C on a NEXes instrument (Ventana Medical Systems) using Ventana reagent buffer and detection kits unless otherwise noted. Endogenous peroxidase activity was blocked with hydrogen peroxide. Antibodies against T70-p4E-BP1 and total 4E-BP1 were diluted 1:50 and 1:40, respectively, in PBS and incubated overnight at room temperature. Primary antibodies were detected with Ventana biotinylated goat anti-rabbit secondary followed by streptavidin-horseradish peroxidase conjugate. The complex was visualized with naphthol-AS-MX phosphatase and Fast Red complex. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with permanent medium. Appropriate positive and negative controls were included with the study sections. The levels of 4E-BP1 and p-4E-BP1 expression were based on the proportion of melanoma cells with positive cytoplasmic and/or nuclear staining and were scored on a continuous scale from 0% (undetectable) to 100% (homogeneous staining) by an attending pathologist (H.Y.) who was blinded to the clinical data. Several fields were scanned before an estimate of the percent tumor cells expressing either 4E-BP1 or p-4E-BP1 was recorded as a continuous variable from 0 to 100%. The intensity of the signal (0, 1+, 2+, or 3+) was also recorded.

RPPA. For detailed protocol, see ref. 14. One to two fixed, paraffin-embedded sections (5 μ m thick) were harvested from glass slides and transferred to a microcentrifuge tube in 40 to 50 μ L of 1% SDS lysis buffer. The lysate was then sonicated in a water bath for 20 min, boiled at 100°C for 20 min, and incubated in a heat block at 60°C for 20 min. The lysate was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a microcentrifuge tube and protein quantitation was carried out with the BCA assay (Pierce). β -Mercaptoethanol was then added to samples to 2.5% by volume. The samples were boiled again before printing of the lysate on nitrocellulose-coated glass slides with an automated robotic arrayer. Before exposure to primary antibody, array slides were blocked for endogenous peroxidase, avidin, and biotin protein activity. Primary antibody signal was amplified by the 3,3'-diaminobenzidine tetrachloride DAKO signal amplification system. Signal intensity was measured by scanning the slides with ImageQuant (Molecular Dynamics) and quantified using

the MicroVigene automated RPPA module (VigeneTech). Using MicroVigene software, the intensity of each spot was calculated and an intensity concentration curve was calculated with a slope and intercept. For comparison with mRNA results, differences in loading were assessed and corrected for by normalizing the expression intensities of 4E-BP1 and p-4E-BP1 to extracellular signal-regulated kinase 2.

Affymetrix gene expression. Data mining of Affymetrix U133 Plus 2.0 GeneChip array results for 4E-BP1 transcript levels was done for all available fresh tumor tissue specimens, which included 42 metastatic melanoma specimens from 37 patients (included were 3 patients who had 2 metastases and 1 patient who had 3 metastases). Tissue collection, whole RNA extraction, preparation of cRNA, and raw data analysis were done as described previously (15).

Statistical analysis. Descriptive statistics were calculated for baseline demographic and clinicopathologic characteristics. Cox proportional hazards model was used to examine the association between total 4E-BP1 and p-4E-BP1 transcript levels and protein expression with overall survival (time from initial diagnosis of melanoma to death) and post-recurrence survival (time from first recurrence to death). 4E-BP1 and p-4E-BP1 levels were analyzed as continuous variables and as positive/negative expression using 40% as a cutoff point as values occurred in a bimodal distribution with no values occurring between 40% and 80%. As the intensity of staining was uniformly strong (2-3+), the intensity of staining was not incorporated into the analysis. Serum LDH was analyzed as a dichotomous variable. High LDH was defined as greater than the upper limit of normal at New York University Medical Center (>618 units/L). Multivariate survival analysis of LDH, site of metastasis, and p-4E-BP1 as a continuous variable was done using the multivariable Cox proportional hazards model in a subset of patients (n = 47). Multivariate analysis of LDH, site of metastasis, and p-4E-BP1 as a dichotomous variable was done with the stratified log-rank test. Spearman correlation coefficients were used to examine the relationship between transcript levels and protein expression. To examine the agreement between positive and negative expression of the markers, κ statistic and its approximate SE were calculated. All P values were two-sided with statistical significance at the 0.05 α level. All analyses were done in statistical programming and software package R.⁷

Results

4E-BP1 is hyperphosphorylated in melanoma cell lines. Expression levels of the translation repressor protein 4E-BP1 were measured by Western blot in a panel of 9 cell lines including the PI3K mutant breast cancer cell line MCF-7, the melanocyte cell line HeMnLP, and 7 melanoma cell lines characterized for N-RAS, BRAF, and PTEN mutation status. 4E-BP1 was detected in each of the 9 cell lines (Fig. 1). 4E-BP1 was strongly expressed in 4 of the 7 melanoma cell lines (SkMel-39, SkMel-28, SkMel-5, and SkMel-11) and was also hyperphosphorylated in 4 of the 7 melanoma cell lines (SkMel-39, SkMel-28, SkMel-5, and SkMel-37). BRAF/PTEN mutants SkMel-11, SkMel-39, SkMel-28, and SkMel-5 were found to have 2- to 3-fold higher expression of 4E-BP1 compared with melanocytes or the wild-type N-RAS/BRAF SkMel-31. 4E-BP1 in SkMel-39, SkMel-28, and SkMel-5 was also strongly hyperphosphorylated at sites crucial for disengagement from the mRNA cap (T37/46, T70, and S65). These lines expressed 4- to 7-fold higher p-4E-BP1 T37/46 levels, 4- to 5-fold higher p-4E-BP1 (T70) levels, and 6- to 8-fold higher p-4E-BP1 S65 levels than melanocytes or SkMel-31. Although SkMel-37 did not overexpress 4E-BP1 compared with melanocytes, these cells expressed 6-fold higher

p-4E-BP1 (S65) levels compared with HeMnLP. Expression and phosphorylation of the translation initiation factors eIF4G and eIF4E were also measured. There was minimal variability in either total or phosphorylated eIF4G or eIF4E among all 9 cell lines. PTEN expression was also measured in the panel. The PTEN mutant lines SkMel-37, SkMel-39, SkMel-28, and SkMel-5 expressed less PTEN than melanocytes or SkMel-31.

p-4E-BP1 (T70) is associated with poor survival in human metastatic melanoma. Total and p-4E-BP1 (T70) expression was assessed in 77 specimens from 72 metastatic melanoma patients using immunohistochemistry. Ninety-seven percent of the specimens stained positively (>0%) for 4E-BP1 and 74% stained positively (>0%) for p-4E-BP1 (T70; Fig. 2A and B). Immunohistochemical staining for 4E-BP1 yielded values ranging from 0% to 100%, with a mean of 95%. Immunohistochemical staining for p-4E-BP1 (T70) yielded a range of values from 0% to 100% with a mean of 53%. Total and p-4E-BP1 staining was found in the nucleus and cytoplasm of melanoma cells, whereas tumor lymphocytes did not stain positively for either 4E-BP1 or p-4E-BP1. p-4E-BP1 was particularly enriched in the nucleus, whereas 4E-BP1 staining was more evenly distributed between the nucleus and the cytoplasm. Cells displaying mitotic figures strongly expressed p-4E-BP1 (T70) (Fig. 2C and D).

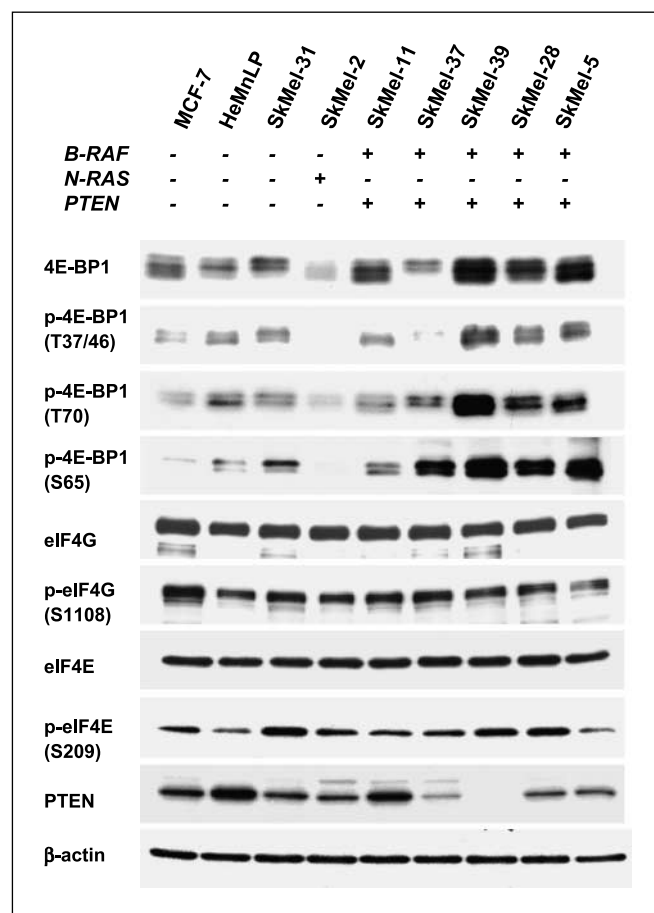
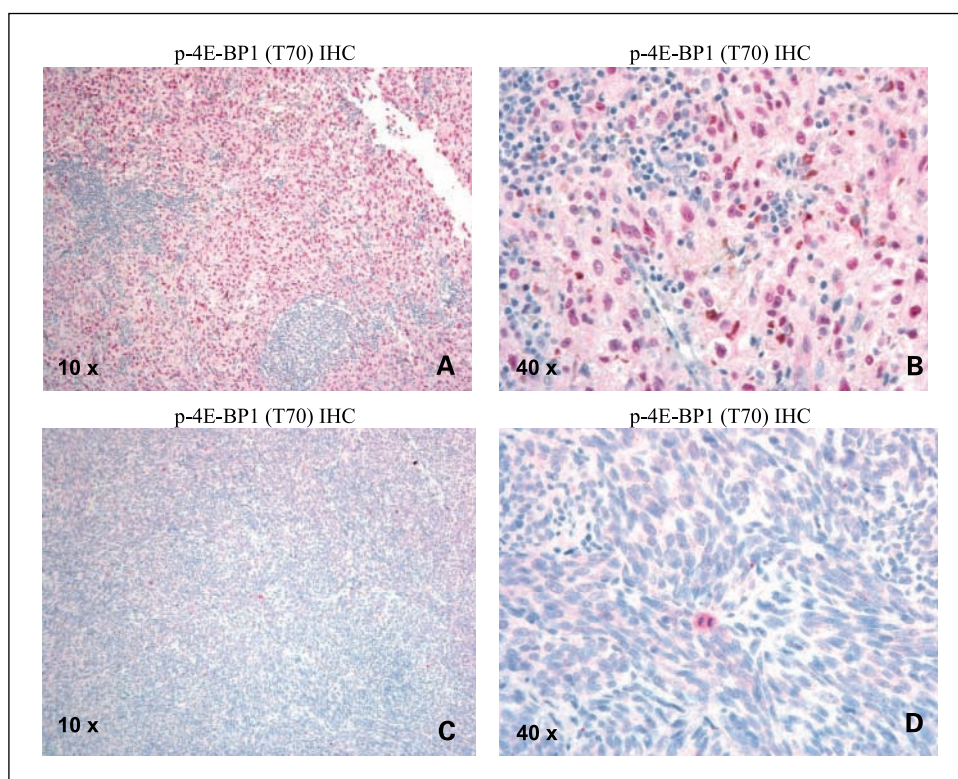


Fig. 1. 4E-BP1 is expressed and phosphorylated in melanoma cell lines. A, breast cancer cell line MCF-7, melanocytes, and 7 melanoma cell lines were grown in 10 cm dishes in their respective maintenance medium for 24 h before harvesting. Expression levels of total and phosphorylated forms of 4E-BP1, eIF4G, and eIF4E were measured by Western blot. PTEN expression was also measured by western blot. B, actin was used as a loading control.

⁷ R Development Core Team. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing; 2007. <http://www.R-project.org>.

Fig. 2. Immunohistochemical detection of p-4E-BP1 in metastatic melanoma patients. Metastatic melanoma specimen showing diffuse cytoplasmic and nuclear p-4E-BP1 staining of melanoma cells with sparing of tumor-infiltrating lymphocytes at $\times 10$ (A) and $\times 40$ (B) magnification. Metastatic melanoma specimen staining negative for p-4E-BP1, with expression of p-4E-BP1 in an actively dividing cell (red staining with naphthol-AS-MX phosphatase and Fast Red complex) at $\times 10$ (C) and $\times 40$ (D) magnification.



A statistically significant association between p-4E-BP1 (T70) expression and worse overall and post-recurrence survival was detected [hazard ratio (HR), 1.01 and 1.01; $P = 0.033$ and 0.002 , respectively], which suggests that every 1% increase in p-4E-BP1 staining increases the hazard by 1%. A review of the immunohistochemistry showed a dichotomous distribution of p-4E-BP1 staining, as 35 samples had $\leq 40\%$ positivity and 42 samples had $\geq 80\%$ positivity. Patients with $>40\%$ p-4E-BP1 staining had worse overall 5-year survival than those with $\leq 40\%$ p-4E-BP1 staining (HR, 2.12; $P = 0.02$, log-rank test). Patients with higher p-4E-BP1 staining had a 5-year survival of 44.3% [95% confidence interval (95% CI), 30.6-64.2%] compared with 62.8% (95% CI, 46.5-84.9%) for those with $\leq 40\%$ staining (Fig. 3A). In addition to poor overall survival in the high p-4E-BP1 cohort compared with the low p-4E-BP1 cohort, there was a significant difference in post-recurrence survival between these two groups (HR, 3.75; $P = 0.0003$, log-rank test; Fig. 3B). Two-year post-recurrence survival probabilities were 79.5% (95% CI, 64.9-97.4%) for subjects with low immunohistochemistry p-4E-BP1 (T70) values and 38.0% (95% CI, 24.7-58.5%) for subjects with high immunohistochemistry p-4E-BP1 (T70) values.

As metastatic melanoma patients with visceral metastases have a worse prognosis compared with those with only lymph node or skin metastases, the distribution of metastatic sites in the high and low immunohistochemistry p-4E-BP1 categories was examined. The proportion of visceral metastases was slightly higher among those with low immunohistochemistry p-4E-BP1 compared with those with high immunohistochemistry p-4E-BP1 [25.7% (9 of 35) versus 14.3% (6 of 42), respectively], but the difference was not significant statistically ($P = 0.25$, Fisher's exact test). The time from primary diagnosis to resection of metastasis was also assessed in the high and low immunohistochemistry p-4E-BP1 cohorts. The median time

to metastasis (in months) for the low and high groups was 38.4 months (95% CI, 15.3-75.1) and 18.1 months (95% CI, 12.9-36.5), respectively. The difference was not, however, statistically significant ($P = 0.24$, log-rank test).

As 4 patients had multiple specimens (3 subjects had 2 specimens each and 1 subject had 3 specimens), frailty models with several commonly used frailty distributions (γ , Gaussian, and t distribution of various degrees of freedom) were further employed to examine the effect of accounting for clustering on the association between overall and post-recurrence survivals and immunohistochemistry p-4E-BP1. For overall survival, the P values remained significant and ranged from 0.023 to 0.038 using frailty models. For post-recurrence survival, the P values remained significant and ranged from 0.002 to 0.004.

p-4E-BP1 (T70) is associated with worse survival in melanoma patients independent of known prognostic factors. LDH serum values were measured at the time of first metastasis in a subset of our patient cohort ($n = 47$). In this subset, we found that high LDH was associated with worse survival. The association between high LDH and overall survival was not statistically significant (HR, 1.55; $P = 0.24$). However, significantly greater hazard of post-recurrence survival was observed for patients with high LDH levels (HR, 2.26; $P = 0.03$, log-rank test). Multivariate survival analysis in a subset of patients ($n = 47$) showed that p-4E-BP1 (T70) was associated with worse survival in melanoma patients independent of established prognostic indicators, specifically site of metastasis and serum LDH. The association between immunohistochemistry p-4E-BP1 and overall and post-recurrence survival remained significant (HR, 1.01 and 1.03; $P = 0.03$ and $P < 0.001$, respectively) after adjusting for LDH level and site of metastasis using multivariable Cox proportional hazard model. When p-4E-BP1 (T70) was treated as a dichotomous variable, after adjusting for LDH level and site of

metastasis using stratified log-rank test, patients with higher p-4E-BP1 staining had significantly worse overall and post-recurrence survival compared with those with low p-4E-BP1 (HR, 2.29 and 5.26; $P = 0.04$ and $P < 0.001$, respectively). There was no statistically significant association between level of p-4E-BP1 and site of metastasis or between p-4E-BP1 and LDH levels.

4E-BP1 and p-4E-BP1 (T37/46 and T70) can be detected in paraffin-embedded tumor specimens with RPPA. In an effort to quantify levels of total and p-4E-BP1 in paraffin-embedded tissue samples and to correlate total protein amounts with transcript levels, we employed the semiquantitative RPPA technology. Tumor lysates were derived from 41 formalin-fixed, paraffin-embedded metastatic melanoma sections. One to two sections were harvested per specimen. These lysates were printed onto

nitrocellulose-coated glass slides and RPPA was conducted with antibodies to 4E-BP1 and p-4E-BP1 (T37/46). To determine whether signals detected with RPPA correlate with levels detected by Western blotting, a subset of the samples with sufficient protein available for both techniques was analyzed (Fig. 4A and B). Western blotting analysis of total 4E-BP1 showed minimal protein degradation, indicating that the technique for protein isolation from the paraffin-embedded samples maintained the integrity of the protein (Supplementary Fig. S1). A comparison of the densitometry results of Western blotting with RPPA results showed a high degree of correlation for both total ($r^2 = 0.81$) and p-4E-BP1 ($r^2 = 0.93$) levels for these samples (Fig. 4C). In addition to both 4E-BP1 and p-4E-BP1 (T37/46), p-4E-BP1 (T70), AKT, and extracellular signal-regulated kinase 2 were also detected in these samples. Thus, 5 protein targets were detected in 41 human tumors using $<1 \mu\text{g}$ tumor protein/sample.

4E-BP1 transcript correlates with 4E-BP1 RPPA expression. Given the prevalence of total 4E-BP1 in melanoma cell lines, we profiled 42 metastatic melanoma specimens from 37 patients for 4E-BP1 mRNA using Affymetrix GeneChip microarray. Normalized 4E-BP1 microarray values ranged from 8.47 to 11.87, with a mean of 10.36. To determine whether higher transcript levels were associated with increased total 4E-BP1 protein expression, we employed Spearman's method to examine the correlation between semiquantitative RPPA results and microarray values. There was a positive correlation between 4E-BP1 transcript levels and 4E-BP1 RPPA expression ($r_s = 0.39$; $P = 0.012$). A weak correlation between 4E-BP1 transcript levels and 4E-BP1 immunohistochemistry expression was also found ($r_s = 0.27$), but this association was not statistically significant ($P = 0.12$).

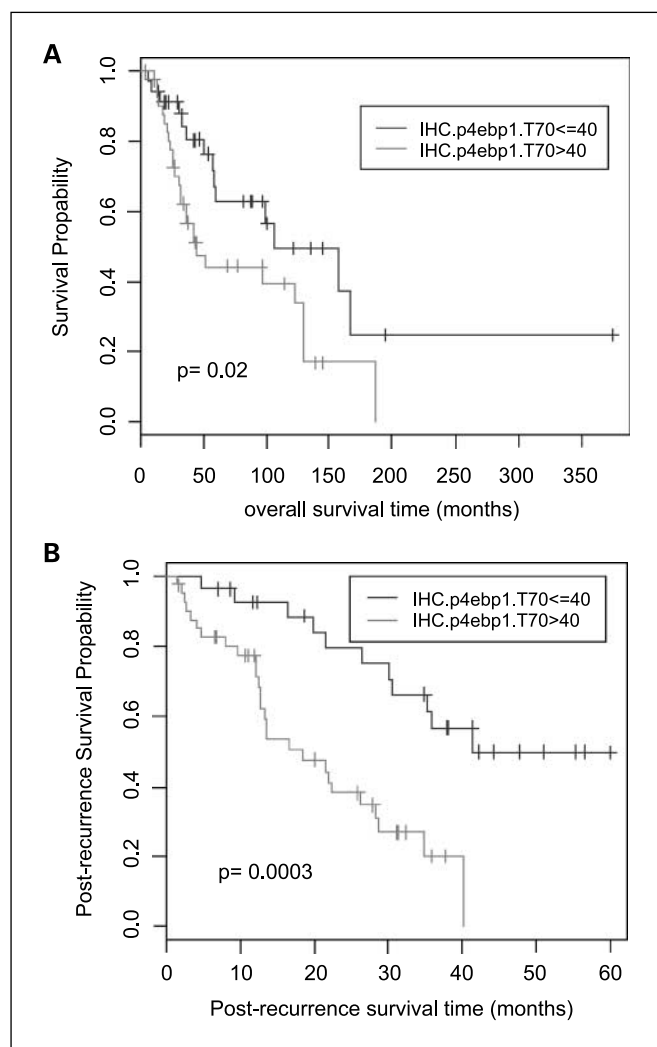


Fig. 3. High p-4E-BP1 levels are associated with poor overall and post-recurrence survival. *A*, Kaplan-Meier curves for patients with low/high IHC.p4ebp1.T70 values (based on data for 77 specimens without missing values). Five-year overall survival probabilities for the two groups are 62.8% (95% CI, 46.5-84.9%) and 44.3% (95% CI, 30.6-64.0%), respectively. The difference in the two groups is significant at the 0.05 significance level (HR, 2.12; $P = 0.02$, log-rank test). *B*, Kaplan-Meier post-recurrence survival curves for patients with low/high IHC.p4ebp1.T70 values (based on data for 77 specimens without missing values). There was a significant difference in post-recurrence survival between these two groups (HR, 3.75; $P = 0.0003$, log-rank test). Two-year post-recurrence survival probabilities are 79.5% (95% CI, 64.9-97.4%) for subjects with low IHC.p4ebp1.T70 values and 38.0% (95% CI, 24.7-58.5%) for subjects with high IHC.p4ebp1.T70 values.

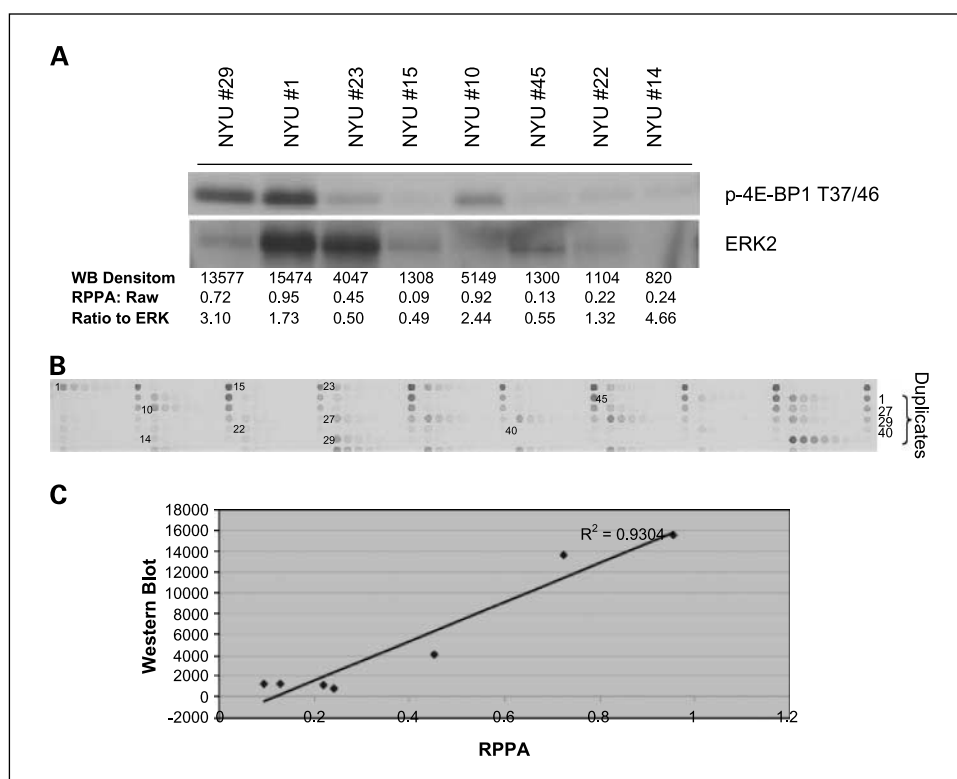
Discussion

Our study reveals several important findings. We found that 4E-BP1 is strongly expressed and hyperphosphorylated in melanoma cell lines with BRAF and PTEN mutations, illustrating the convergence of hyperactivated MAPK and PI3K signaling on 4E-BP1. We also showed that accumulation of total 4E-BP1 is associated with increased transcript expression. Moreover, we showed that p-4E-BP1 in melanoma metastases is associated with poor survival. The association between p-4E-BP1 and overall survival is largely due to the increased post-recurrence hazard associated with higher levels of p-4E-BP1.

The association between p-4E-BP1 in melanoma and worse survival is in concordance with data in breast (11) and ovarian (16) cancers that showed a significant correlation between p-4E-BP1 and both poor prognosis and high pathologic grade. Our observation of 4E-BP1 localized to both the nucleus and the cytoplasm corroborates previous reports of nuclear and cytoplasmic expression in breast cancer cells and mouse embryo fibroblasts (11, 17). The specificity of nuclear immunostaining was shown using a blocking experiment with 4E-BP1 antibody preadsorbed with purified recombinant 4E-BP1 protein. Both nuclear and cytoplasmic reactivity were abolished in the melanoma cells, thereby confirming the specificity of the 4E-BP1 antibody (data not shown).

In this study, we also employed a novel high-throughput protein array technology, which allowed detection of protein targets in as little as 10 ng tumor protein extracted from paraffin-embedded melanoma sections. This proved to be a useful approach for quantitative analysis of hyperactivated

Fig. 4. RPPA detection of p-4E-BP1 in paraffin-embedded metastatic melanoma sections. **A**, lysates derived from 5 μ m thick paraffin-embedded metastatic melanoma sections were subjected to immunoblotting for p-4E-BP1 (T37/46) and signals were quantitated by densitometry. For comparison with microarray data, all total and p-4E-BP1 RPPA data were normalized to total extracellular signal-regulated kinase protein for each sample. **B**, serial dilutions of lysates from 49 paraffin-embedded metastatic melanoma specimens were arrayed onto glass slides and incubated with primary p-4E-BP1 antibody. Brown signal amplification was achieved by horseradish peroxidase-mediated cleavage of 3,3'-diaminobenzidine tetrachloride. **C**, p-4E-BP1 RPPA signal correlates strongly with p-4E-BP1 Western signal ($R^2 = 0.93$).



signaling networks in melanoma. 4E-BP1 transcript levels were found to correlate with 4E-BP1 expression as measured by RPPA, suggesting 4E-BP1 accumulation occurs secondary to an increase in message expression. The fact that the weak correlation of 4E-BP1 transcript with immunohistochemistry protein expression did not reach statistical significance may be explained by the heterogeneity of different sections of tumor used to harvest mRNA and protein.

Our study reveals that p-4E-BP1 is significantly associated with worse survival independent of LDH and metastatic site. A limitation of this multivariate analysis is that the majority of metastases were derived from surgical resection of accessible tissues such as skin and lymph node. This predominance of readily accessible metastases in our cohort may therefore limit the validity of independent association with outcome.

Future studies are needed to determine whether PTEN and BRAF mutations are enriched in the tumors of patients that harbor high p-4E-BP1. Preliminary analyses of tissue from 29 patients from our cohort did not reveal a statistically significant association between p-4E-BP1 levels and BRAF mutation (DNS). However, sequencing for BRAF and PTEN mutations in a larger number of metastatic melanoma specimens is currently under way.

Although p-4E-BP1 was associated with poor overall and post-recurrence survival, a statistically significant association between total 4E-BP1 protein expression and overall or post-recurrence survival was not observed. The lack of correlation with poor survival might be a reflection of the translation suppressor function of 4E-BP1. Given that unphosphorylated 4E-BP1 binds to the mRNA cap and prevents translation of oncogenic proteins, one would expect that some survival advantage is imparted to patients whose tumor cells robustly express 4E-BP1. In fact, inducing 4E-BP1 expression to overwhelm the

ability of upstream signaling pathways to phosphorylate and inactivate 4E-BP1 might prove an effective anticancer strategy.

Given the prevalence of p-4E-BP1 in the most aggressive subset of metastatic melanomas, strategies to up-regulate and/or dephosphorylate 4E-BP1 may be powerful therapeutic options in this disease. Combined suppression of the upstream signaling pathways that activate 4E-BP1 (PI3K/AKT and RAS/MAPK) may also effectively restrain deregulated translation. Recently, combined mammalian target of rapamycin and MEK inhibition has been reported to effectively inhibit 4E-BP1 phosphorylation, polysome formation, and cell growth in non-small cell lung cancer (18). Targeting both mammalian target of rapamycin and MEK may therefore be a useful strategy to dephosphorylate 4E-BP1 and inhibit translation. Alternatively, directly modifying the translation machinery may be effective. Small molecules, such as phenethyl isothiocyanate, which induce 4E-BP1 expression and prevent 4E-BP1 phosphorylation, are in development (19). Also, small-molecule inhibitors of the eIF4e and inhibitors of the eIF4e-eIF4G interaction are being developed to prevent eIF4F complex assembly and inhibit translation in cancer cells (20).

In sum, the eIF4F complex receives signaling input from many mitogenic signal transduction pathways. Phosphorylation of 4E-BP1 is emerging as a common theme in the cancer cell's strategy to evade physiologic restraints on protein biosynthesis. Further study of the signaling pathways that regulate 4E-BP1 expression and phosphorylation in melanoma may reveal additional therapeutic strategies to attenuate unrestrained protein biosynthesis in this malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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