

4E-Binding Protein 1, A Cell Signaling Hallmark in Breast Cancer that Correlates with Pathologic Grade and Prognosis

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Abstract Purpose: Cell signaling pathways include a complex myriad of interconnected factors from the membrane to the nucleus, such as erbB family receptors and the phosphoinositide-3-kinase/Akt/mTOR and Ras-Raf-ERK cascades, which drive proliferative signals, promote survival, and regulate protein synthesis.

Experimental Design: To find pivotal factors in these pathways, which provide prognostic information in malignancies, we studied 103 human breast tumors with an immunohistochemical profile, including total and phosphorylated (p) proteins: human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor, extracellular signal-regulated kinase 1/2, Akt, 4E-binding protein 1 (4EBP1), eukaryotic initiation factor 4E, phosphorylated ribosomal protein S6 kinase 1, phosphorylated ribosomal protein S6, and Ki67. Western blot and reverse lysate protein arrays were also done in a subset of tumors.

Results: Significantly, activation of the phosphoinositide-3-kinase/Akt/mTOR cascade was detected in a high proportion of tumors (41.9%). Tumors with HER2 overexpression showed higher p-Akt as compared with negative tumors ($P < 0.001$). Levels of p-Akt correlated with the downstream molecules, p-4EBP1 ($P = 0.001$) and p-p70S6K ($P = 0.05$). Although 81.5% of tumors expressed p-4EBP1, in 16.3% of these tumors, concomitant activation of the upstream factors was not detected. Interestingly, p-4EBP1 was mainly expressed in poorly differentiated tumors ($P < 0.001$) and correlated with tumor size ($P < 0.001$), presence of lymph node metastasis ($P = 0.002$), and locoregional recurrences ($P = 0.002$). Coexpression of p-4EBP1 and p-eIF4G correlated with a high tumor proliferation rate ($P = 0.012$).

Conclusion: In this study, p-4EBP1 was the main factor in signaling pathways that associate with prognosis and grade of malignancy in breast tumors. Moreover, p-4EBP1 was detected in both HER2-positive and HER2-negative tumors. This factor seems to be a channeling point at which different upstream oncogenic alterations converge and transmit their proliferative signal, modulating protein translation.

Human tumors are characterized by their great heterogeneity and histopathologic variability. Currently, >250 malignant tumors and thousands of subtypes and histologic variants have been described. Nevertheless, the classic pathologic criteria, such as tumor size, grade of malignancy, and metastatic dissemination, are generally the most relevant prognostic factors in cancer. In addition to the variability of histopathologic subtypes, molecular study of tumors is even more complex. In all malignant tumors, at least six genetic alterations

should affect the main mechanisms of cellular transformation, including growth factor and cell signaling pathways, the cell cycle, apoptosis, and mechanisms implicated in cellular invasiveness, and angiogenesis (1–3). Overall, >350 genes associated with cancer have been identified, representing >1% of the human genome (4).

Studies using cDNA expression arrays have detected hundreds of genetic alterations whose true clinical and pathologic significance remains to be described and validated. It is relevant that except in lymphoma, leukemia, small round cell tumors in children, and a large group of sarcomas, which are known to have translocations, the genetic alterations thought to be involved in malignant transformation show considerable redundancy in most carcinomas (4). In breast cancer, one of the main genetic alterations is the amplification of the oncogene human epidermal growth factor receptor 2 (HER2)/neu and the overexpression of its product. Nevertheless, this gene is amplified in only 25% of breast tumors (5–7), a fact suggesting that other factors in these pathways must be activated to maintain the oncogenic signal.

Epidermal growth factor receptor (EGFR) and HER2/neu, as well as the downstream activation factors extracellular

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Table 1. Characteristics of the breast tumors

	N (%)
Infiltrating carcinomas	103
Histologic type	
Ductal	97 (94.2)
Lobular	6 (5.8)
Histologic grade	
1	3 (2.9)
2	56 (54.4)
3	44 (42.7)
pT	
1	33 (32.0)
2	64 (62.2)
3	6 (5.8)
pN	
0	50 (48.5)
1	37 (35.9)
2	6 (5.8)
3	10 (9.8)
Hormone receptors	
ER/PR+	75 (72.8)
HER2/neu	
Overexpression	23 (22.3)
Amplification	21 (20.4)
Ki67 (MIB1)	
>20%	29 (28.2)
≤20%	74 (71.8)

signal-regulated kinase 1/2 (ERK1/2), Akt, initiation factor 4E-binding protein 1 (4EBP1), phosphorylated ribosomal protein S6 kinase 1 (p70S6K1), and ribosomal protein S6 (S6) are among the most important and representative elements of the signaling pathway (8, 9). Activation of membrane growth factor receptors drives cell proliferation signals through at least two major biochemical cell pathways, ERK1/2 and Akt. Akt activates many downstream factors, and one of the most important is mTOR which, in turn, activates 4EBP1 and p70S6K1. 4EBP1 plays a critical role in controlling ribosome protein synthesis, and hence, in cell survival and proliferation (10, 11) through the phosphorylation of eukaryotic initiation factor 4E (eIF4E). Phosphorylation of 4EBP1 (p-4EBP1) results in the release of eIF4E and activation of cell protein synthesis. Recent experimental studies have shown that 4EBP1 has a central role in cell transformation, and conversely, when it is inactivated, a reversion of the transformed phenotype is observed (12). In the cell signaling routes, several factors can be activated or inactivated to promote oncogenic proliferation, and more than one gene or epigenetic alteration can be present, including HER2/neu amplification or various mutations in EGFR, PTEN,

phosphoinositide-3-kinase, or Ras, illustrating the redundancy of oncogenic events in tumor cells. This redundancy and the oncogenic alterations in other cellular pathways could explain the low reported response rates to specific biological therapies in cancer (13, 14).

The central aim of the present study is to identify a simplified molecular signature in cell signaling in breast tumors on the basis that a channeling downstream factor or "wild card" might orchestrate the transduction of oncogenic signals to the ribosomes or nucleus through a kind of "bottleneck", regardless of the individual upstream oncogenic alterations. To investigate this hypothesis, activation of the membrane receptors, EGFR and HER2/neu, two major biochemical pathways, Akt and ERK1/2, and the downstream factors, 4EBP1, p70S6K1, and S6, were studied by immunohistochemistry using phosphorylation-specific antibodies. Expression of these factors was measured in a breast malignant tumor series and correlated with pathologic grade, patient survival, and tumor recurrence to determine which, if any, of the factors fulfilled the concept of a channeling factor or an oncogenic wild card. This study showed that 4EBP1 is activated in a high percentage of breast tumors, is associated with higher malignant grade, tumor size, and local recurrence regardless of HER2/neu status, and could be proposed as a hallmark in cell signaling.

Materials and Methods

Tumor cell lines and Western blot assays. For the current study, we selected the following human cell lines containing different levels of EGFR and HER2/neu from the American Type Culture Collection (Manassas, VA): vulvar squamous carcinoma (A431; ref. 15), colon carcinoma (DiFi; ref. 16), and breast carcinoma (SK-BR-3, MDA-MB-468, T-47D, and BT-474; refs. 17, 18). Cancer cell lines were maintained as monolayers at 37°C and 5% CO₂/air in DMEM/Ham's F-12 supplemented with 10% fetal bovine serum and glutamine (2 mmol/L); in the case of BT-474, insulin at 0.01 µg/mL was added.

Western blot assays were done as previously reported (19, 20) with minor modifications. In brief, tumor cells were grown in 100 mm dishes to subconfluence and exposed to rapamycin analogue mTOR inhibitor for 2 h at doses superior to the IC₅₀ for these cell lines (10 and 100 nmol/L; ref. 21). After removal of the medium, cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer [50 mmol/L HEPES (pH 7.0), 10% glycerol, 1% Triton X-100, 5 mmol/L EDTA, 1 mmol/L MgCl₂, 25 mmol/L NaF, 50 µg/mL leupeptin, 50 µg/mL aprotinin, 0.5 mmol/L orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride]. After removal of cell debris by centrifugation, protein concentration was determined by the Lowry assay (DC Protein Assay; Bio-Rad, Hercules, CA). Lysate samples containing equal amounts of protein were then added to SDS-PAGE loading buffer with 5%

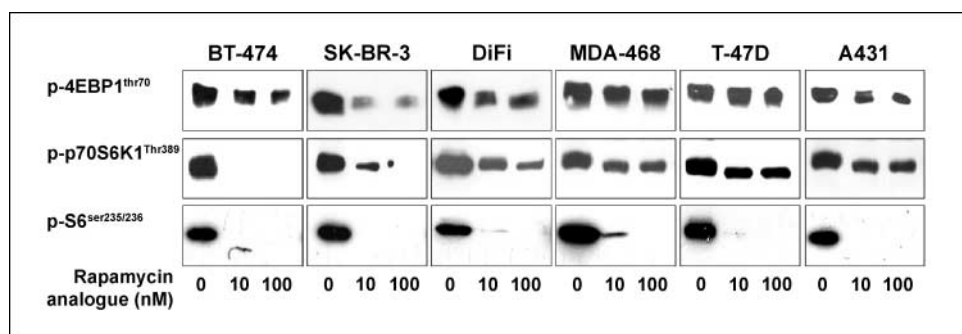
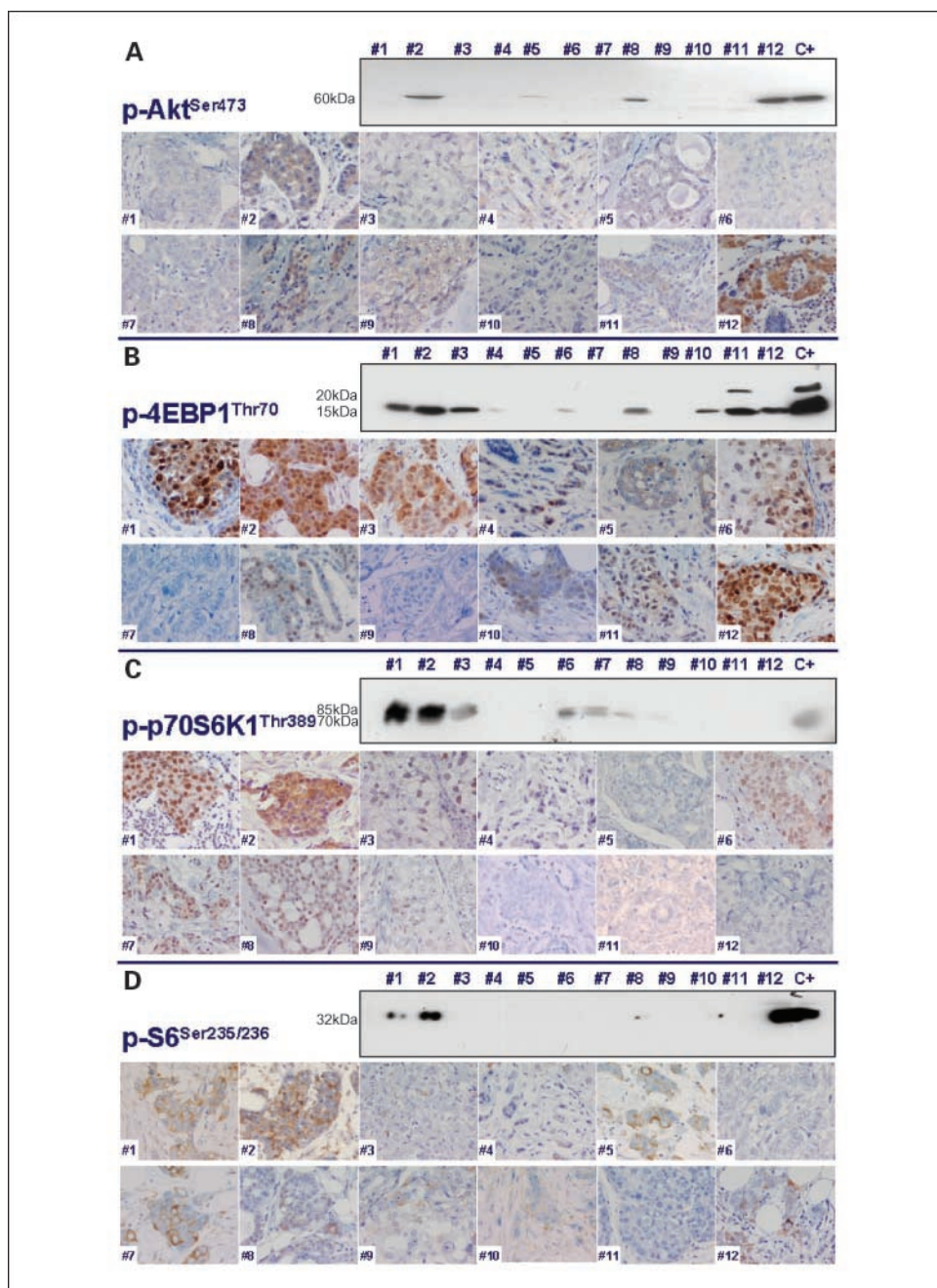


Fig. 1. Dose-dependent effects of mTOR inhibition on phosphorylated initiation factor 4EBP1, p70S6K1, and S6 in a panel of cell lines including breast cancer cells (BT-474, SK-BR-3, MDA-468, and T-47D), colon cancer cells (DiFi), and squamous cancer cells (A431). Expression was detected by Western blot.

Fig. 2. Protein levels in fresh-frozen tissue specimens by Western blot and in formalin-fixed tissue from the same specimens by immunohistochemistry. The BT-474 cell line was used as a positive control for Western blot. **A**, detection of p-Akt at Ser⁴⁷³ with the same antibody in 12 different breast tumors. Tumors 2, 5, 8, and 12 showed the highest p-Akt levels in frozen and paraffin-embedded samples. A cytoplasmic and nuclear staining pattern was observed in tumor cells. Faint staining was present in stromal and lymphoid cells. **B**, assays for p-4EBP1 at Thr⁷⁰ detected bands at 15 and 20 kDa in Western blot and strong levels were observed for tumors 1, 2, 3, 8, 10, 11, and 12. Tumor cells showed cytoplasmic and nuclear staining. Stromal and nontumoral breast epithelia expressed p-4EBP1 in a nuclear fashion. **C**, expression of phosphorylated p70S6K1 showed double bands at 70 and 85 kDa by Western blot. Tumors 1, 2, 3, 6, 7, and 8 had detectable levels of protein in gel and high expression by immunohistochemistry. **D**, phosphorylated S6 was detected in tumors 1, 2, 8, and 11 by Western blot at 32 kDa. Expression of pS6 by immunohistochemistry was predominantly observed at the infiltrating edge of tumors with a heterogeneous distribution inside this area.



β -mercaptoethanol and heated for 5 min at 100°C. Electrophoretic transfer to nitrocellulose membranes was followed by immunoblotting with the primary antibodies, as previously described. Finally, membranes were hybridized with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and detected by chemiluminescence with the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

Patients. A total of 103 malignant infiltrating breast tumors (97 ductal and 6 lobular carcinomas) were randomly obtained from the Pathology Department of Vall d'Hebron University Hospital (Barcelona, Spain) during the period from 1998 to 2002. Patient ages ranged from 33 to 75 years, with a mean age of 53. Tumor size, histologic grade, and the number of nodes showing metastasis were determined (Table 1). Histologic grades 1, 2, and 3 were defined according to the criteria of Scarff-Bloom-Richardson, modified by

Elston (22). The tumor-node-metastasis stage was defined according to WHO criteria. Hormonal status and the proliferation marker Ki67 were determined by immunohistochemistry. All the patients studied were included in clinical protocols and follow-up was obtained in all cases. The Institutional Review Board Committee on Human Research of the Vall d'Hebron University Hospital approved the collection of tissues and the procedures conducted on these tissues.

Reagents. The following primary antibodies were assayed, as described previously (13, 19, 23): mouse monoclonal anti-EGFR clone K1494 (Dako, Carpinteria, CA), mouse monoclonal anti-p-EGFR clone 74 (Chemicon, Temecula, CA), rabbit anti-HER2 Herceptest (Dako), rabbit polyclonal anti-p-HER2 Tyr¹²⁴⁸, rabbit polyclonal anti-ERK1/2, rabbit polyclonal anti-Thr²⁰²/Tyr²⁰⁴ p-ERK1/2, rabbit polyclonal anti-Akt, rabbit polyclonal anti-Ser⁴⁷³ p-Akt, rabbit polyclonal anti-4EBP1, rabbit polyclonal anti-Thr⁷⁰ p-4EBP1, rabbit polyclonal anti-Thr³⁸⁹ p-p70S6K1, and rabbit polyclonal anti-Ser^{240/244} p-S6, from Cell

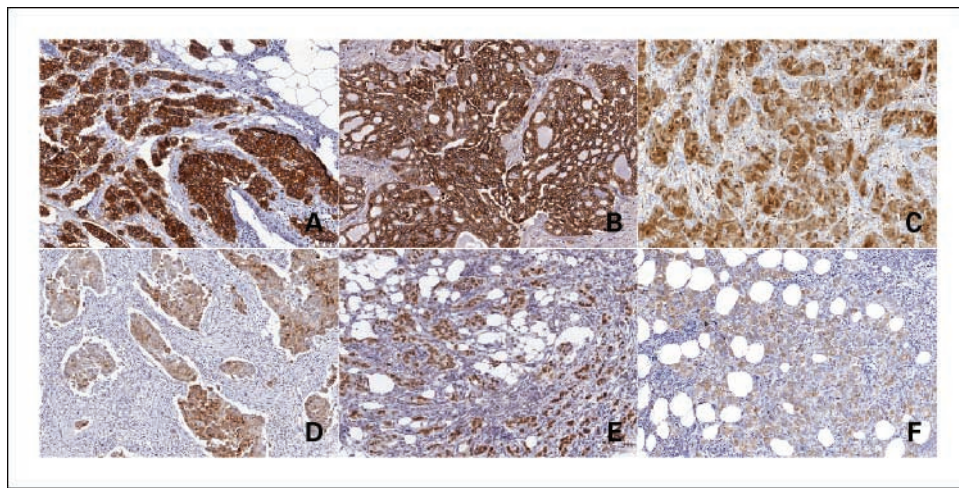


Fig. 3. Expression of biomarkers in various breast tumors. *A*, strong, diffuse HER2/neu membrane overexpression in an infiltrating ductal breast tumor. *B*, EGFR expression in low-grade carcinoma with membranous and cytoplasmic staining patterns. Some stromal cells express moderate levels of EGFR. *C*, nuclear and cytoplasmic staining for p-ERK1/2 in a breast carcinoma. Only nuclear expression due to activated translocation of protein was considered p-ERK1/2 expression. Proteins were detected in stromal elements. *D*, p-Akt was present in the cytoplasm and nuclei of tumor cells, with a heterogeneous distribution within the tumor. *E*, p-p70S6K1 was observed in tumor cells with strong nuclear and weak cytoplasmic expression. *F*, cytoplasmic expression of p-S6 protein. Staining is focal in distribution.

Signaling Technology (Beverly, MA) as well as estrogen (clone M7047; Dako) and progesterone (clone MU328UCE; Biogenex, San Ramon, CA) receptors and the proliferation marker Ki67 (clone M7240; Dako). Heat-induced antigen retrieval in a water bath was done using 0.01 mol/L of citrate buffer at pH 6, except for p-EGFR, which required pH 10 buffer, and EGFR, which used proteinase K. The appropriate EnVision detection system (Dako) was used for each antibody. p-EGFR and p-HER2 were detected with the Catalytic System of Amplification from Dako, following the manufacturer's guidelines.

Immunohistochemistry evaluation. To score a tumor cell as positive, membrane staining was required for EGFR, membrane or cytoplasmic staining for p-EGFR, strong and complete membrane staining for HER2/neu, membrane staining for p-HER2/neu, nuclear staining for ERK1/2, p-ERK1/2, estrogen receptor, progesterone receptor, and Ki67, and cytoplasmic and nuclear staining for p-Akt. Samples were assessed in a blinded fashion by two investigators (F. Rojo and L. Najera). For the quantitative analysis, a Histo score (Hscore) based on the percentage of stained cells and intensity of staining was calculated evaluating 10 high-power representative fields ($\times 400$) on complete tumor sections using optical microscopy (24). The intensity score was defined as follows: 0, no appreciable staining in cells; 1, weak intensity cells, comparable to stromal cells; 2, intermediate intensity of staining; and 3, strong intensity of staining. The score was based on the fraction of positive cells (0-100%) and the Hscore was calculated by multiplying the intensity score and the fraction score, producing a total range of 0 to 300. A tumor was considered positive when at least 1% of stained cells were visualized. This scoring was used for the statistical analysis.

Fluorescent in situ hybridization for HER2/neu. Fluorescent *in situ* hybridization was done according to the PathVysion (Vysis Inc., Downers Grove, IL) guidelines, described in the package insert as approved by the U.S. Food and Drug Administration. In brief, the PathVysion guidelines involve the rehydration of a paraffin-embedded 5- μ m-thick section. The section was air-dried, pretreated, and digested with proteinase K before being hybridized with fluorescent-labeled probes for the *HER2* gene, and α -satellite DNA for chromosome 17. Nuclei were routinely counterstained with an intercalating fluorescent counterstain, 4',6-diamidino-2-phenylindole. For each tumor, 60 tumor cell nuclei from invasive areas were identified using a Nikon Eclipse E400 fluorescence microscope with a Rhodamine and FITC double filter, and scored for both HER2 and chromosome 17 centromere numbers. *HER2* gene amplification was defined as a HER2-to-chromosome 17 ratio of ≥ 2.0 , as required by the guidelines.

Western blot assays. From the same patients, a subset of frozen breast tumor samples was analyzed by Western blot, as described previously (25, 26). Several controls and extraction procedures were used to ensure that a degradation artifact was not generated (26).

Approximately 0.1 g of tumor tissue, which had been fresh-frozen and stored at -80°C , was minced on dry ice, and suspended in buffer containing protease inhibitors (1% aprotinin, 2 mmol/L of phenylmethylsulfonyl fluoride, and 10 $\mu\text{g}/\text{mL}$ of leupeptin) and a phosphatase inhibitor (2 mmol/L of sodium orthovanadate). After homogenization and fractionation, equal amounts of protein from each sample (20 μg) were resolved under denaturing and reducing conditions by SDS-PAGE in 8% gels. Each gel also contained 3 μg of protein from extracts of 17-3-1 and 4-aminophenylmercuric acetate-treated BT-474 cells (26) to mark the migration of the bands and to provide a standard for the entire study. Electrophoretic transfer to nitrocellulose membranes was followed by immunoblotting with the same antibodies as those used for immunohistochemistry. Finally, membranes were incubated as described for the cell line assays.

Reverse lysate protein arrays. The previously described tissue processing and lysate preparation (27-29) were done with minor variations. Tissue lysates were loaded onto 96-well plates in serial dilutions (neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128). Dilution series of samples and a reference standard were printed in duplicate onto nitrocellulose-coated glass slides (Schleicher & Schuell Bioscience, Keene, NH) using a ring-and-pin robotic arrayer (GeneTAC G3 Library Management System; Genomic Solutions, Ann Arbor, MI). The reference standard was prepared from EGF-stimulated MDA-468 and MDA-435 cells. Microarrays were stained as previously described (29) using a biotinyl-linked catalyzed signal amplification system (Dako). Stained arrays were scanned on an HP scanner. Mean pixel intensities

Table 2. Expression levels of markers in 103 breast tumors

	Total levels, N (%)	Hscore [cutoff, 25], N (%)
EGFR	35 (33.8)	
p-EGFR	18 (17.4)	18 (17.4)
HER2	23 (22.3)	
p-HER2	11 (10.7)	11 (10.7)
p-ERK1/2	90 (87.3)	48 (46.6)
p-Akt	61 (59.2)	48 (46.6)
p-4EBP1	90 (87.3)	61 (59.2)
pp70S6K1	80 (77.7)	39 (37.8)
p-S6	80 (77.7)	34 (32.9)

NOTE: Total expression was described for all markers. The Hscore cutoff of 25 was considered overexpression, based on median expression in nontumoral breast epithelium.

Table 3. Statistical significance for biomarkers related to tumor size, histologic grade, lymph node status, and recurrence

	EGFR	HER2	p-ERK1/2	p-Akt	p-4EBP1	p-eIF4G	p-p70S6K1	p-S6
Tumor size	0.292	0.078	0.623	0.621	<0.001*	0.057	0.493	0.898
Histologic grade	0.051	0.057	0.098	0.502	<0.001*	0.039*	0.69	0.649
Lymph node metastasis	0.844	0.026*	0.119	0.015*	0.02*	0.096	0.016*	0.69
Locoregional recurrence	0.191	0.035*	0.236	0.01*	0.002*	0.114	0.347	0.402

NOTE: Statistical significance (Mann-Whitney *U* test).

*Statistically significant.

were calculated with background correction using MicroVigene software (v. 2.006, VigeneTech, Inc., North Billerica, MA). For each sample, the slope of the regression line best fitting the linear range of the dilution curve was used to determine the relative protein expression (30). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase as reference standard.

Statistical analysis. Statistical analyses were carried out using the SPSS Data Analysis Program, version 10.0 (SPSS, Inc., Chicago, IL). Spearman's nonparametric correlation test was used to calculate the statistical significance of continuous variables, and the Mann-Whitney *U* test was used to compare group means. All statistical tests were conducted at a two-sided significance level of 0.05.

Results

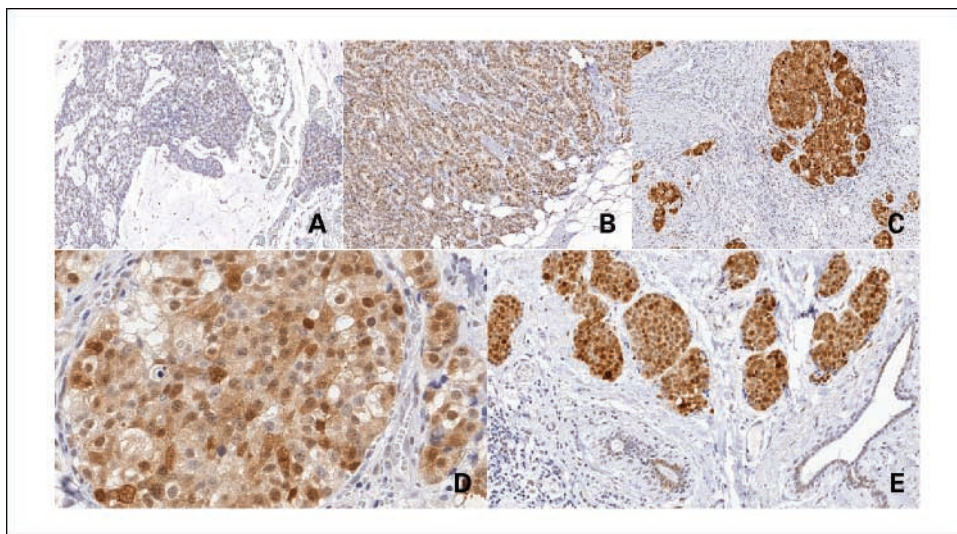
Validation of reagents. To validate antibody specificity, expression of the proteins studied was determined on a panel of cell lines treated with specific erbB receptors and mTOR inhibitors. To show the specificity of p-p70S6K1, p-4EBP1, and p-S6 antibodies, cell lines were treated with a rapamycin-analogue mTOR inhibitor and protein levels were detected by Western blot (Fig. 1). Complete inhibition of p-S6 protein was observed at both doses of mTOR inhibitor in all cell lines. In contrast, p-4EBP1 and p-p70S6K1 levels showed a dose-dependent reduction, as reported by other groups (21). The specificity of the antibodies against membrane receptors, total and phosphorylated ERK1/2, and p-Akt was previously shown (19, 24).

To validate the antibodies in paraffin-embedded tissues, protein levels were compared by Western blot in fresh-frozen tissue samples and in formalin-fixed tissue from the same specimens by immunohistochemistry. Strong significant correlations between the Hscore and relative expression levels by Western blot were shown for EGFR, p-EGFR, HER2/neu, p-HER2/neu, p-ERK1/2, p-Akt, 4EBP1, p-4EBP1, and p-p70S6K1 (Spearman's correlation test, $P < 0.05$), but not for p-S6 (Fig. 2). This absence of a correlation might be explained by the low and heterogeneous p-S6 expression in tumors.

Finally, to test the Hscore as a reproducible and reliable quantification method for immunohistochemistry, a reverse tissue lysate array approach was done on frozen tissue samples, and results were compared with the Hscore from the same paraffin-embedded specimens. Statistically significant correlations between the Hscore and reverse lysate array results were found for EGFR, p-EGFR, HER2/neu, p-HER2/neu, p-ERK1/2, and p-Akt (Spearman's correlation test, $P < 0.05$; Fig. 3).

Protein expression profile in breast tumors. HER2/neu overexpression was detected in 22.3% of breast tumors and gene amplification was confirmed by fluorescent *in situ* hybridization in 20.4% of cases. EGFR was observed in 33.8% of tumors. Among the signaling factors studied, p-ERK1/2 was detected to some degree in 87.3%, p-Akt in 59.2%, p-4EBP1 in 87.3%, p-p70S6K1 in 77.7%, and p-S6 in 77.7%. Overexpression for these signaling factors was defined by a cutoff of 25 on the Hscore, considering this value as the median of expression in

Fig. 4. Expression of p-4EBP1 in various breast tumors. *A*, low-grade carcinoma with mucinous differentiation shows faint nuclear staining for p-4EBP1 in scattered tumor cells. *B*, phosphorylated 4EBP1 expression in grade 2 infiltrating ductal carcinoma. Intermediate cytoplasmic staining was observed in some tumor cells. Nuclear staining was diffusely present in tumor and stromal cells. *C*, strong expression in grade 3 infiltrating ductal carcinoma, with clearly defined cytoplasmic staining in tumor cells. The presence of stained nuclei was also shown. *D*, high magnification of an infiltrating breast carcinoma with heterogeneous cytoplasmic and nuclear staining in tumor cells. Different intensities were observed in the same tumor area. Weak nuclear staining was present in stromal and lymphoid cells. *E*, representative microscopic field with strongly stained tumor cells and preserved normal duct with a faint nuclear pattern of expression.



adjacent normal breast epithelium. Overexpression was detected in <40% of cases for all these markers with the exception of p-4EBP1, which was observed in 59.2% of breast tumors (Table 2; Fig. 4).

Correlations among the signaling factors. Tumors with HER2/neu overexpression showed higher expression levels of p-Akt (Mann-Whitney *U* test, $P < 0.001$), and a significant association was found between EGFR expression and p-ERK1/2 (Spearman's correlation test, $P < 0.002$). Similarly, there was a correlation between p-Akt expression and both p-4EBP1 and p-p70S6K1 (Spearman's correlation test, $P = 0.001$ and $P = 0.05$, respectively). Additionally, p-4EBP1 expression levels significantly correlated with p-p70S6K1 and p-eIF4G (Spearman's correlation test, $P = 0.002$ and $P = 0.010$, respectively) but not with p-S6 (Spearman's correlation test, $P = 0.421$). Nevertheless, an important subset of these breast tumors (16.39% of cases) had high p-4EBP1 levels, but nonrelevant upstream marker expression, including erbB receptor overexpression and signaling factors, suggesting that 4EBP1 can be phosphorylated by various cellular pathways. Exploring individual factors, HER2 and EGFR were undetectable in 65.57%, p-ERK1/2 in 45.9% and p-Akt in 44.26% of tumors with p-4EBP1 overexpression. This finding was supported by a significant correlation between proliferation marker Ki67 and p-4EBP1 (Spearman's correlation test, $P = 0.012$). Expression of the total forms was compared with the phosphorylated levels of each protein and significant correlations were observed for EGFR and HER2 (Spearman's correlation test, $P < 0.001$). In contrast,

no correlations were observed for total and phosphorylated protein levels of ERK, Akt, and 4EBP1.

Correlations between expression profile and clinical and pathologic variables. Expression levels were correlated with tumor size, histologic grade, presence of lymph node metastasis, and evidence of local recurrence 5 years after diagnosis. Among the markers examined, p-4EBP1 showed the strongest correlations with tumor size (Mann-Whitney *U* test, $P < 0.001$), histologic grade ($P < 0.001$), and presence of positive lymph nodes ($P = 0.02$; Table 3). Moreover, patients with locoregional disease at 5 years from diagnosis showed higher p-4EBP1 as compared with patients with no recurrence (Mann-Whitney *U* test, $P = 0.002$; Fig. 5).

Discussion

The findings obtained from this study on cell signaling pathways in a series of breast tumors, including growth factor receptors and the ERK1/2 and Akt cascades, suggest that p-4EBP1 is a main factor of these biochemical pathways associated with histologic grade, lymph node metastases, and locoregional recurrences. p-4EBP1 was detected in breast tumors showing HER2/neu overexpression or EGFR expression as well as in a relevant number of tumors without these oncogenic alterations. Phosphorylation of 4EBP1 can be the consequence of several oncogenic events along these biochemical routes, including amplification or mutation of growth factor receptors, loss of function, or mutations in PTEN, phosphoinositide-3-kinase mutations, Ras mutations, and other collateral mechanisms of oncogenic cellular activation (7, 31). These might include phospholipase D activation (32), which could activate the mTOR cascade, the ATM-p53 pathway (33), and possibly other, as yet unknown, kinases or phosphatases. In this scenario of multiple genetic alterations that can trigger the oncogenic pathways, we propose that the phosphorylated form of 4EBP1 might act as a bottleneck or wild card factor through which many upstream oncogenic signals converge, regulating protein synthesis machinery, cell cycle progression, and cell proliferation (Fig. 6). Hence, analysis of p-4EBP1 could provide reliable molecular information on the oncogenic potential mediated by this pathway. Importantly, the results obtained by immunohistochemistry were validated by Western blotting and reverse lysate protein arrays using the same antibodies, underlining the fact that this factor can be studied in paraffin sections.

The results obtained in breast cancer are comparable to those observed in ovarian tumors, in which p-4EBP1 expression was also associated with high histologic grade and poor survival (34). Interestingly, we did not find similar significant correlations with p70S6K1, which was also activated by mTOR. The differences between p-4EBP1 and pp70SK1 expression observed in breast tumors may be an indication that p-4EBP1 could be phosphorylated by other pathways (35) or that mTOR phosphorylates 4EBP1 more actively in breast cancer. It is well known that mTOR-dependent phosphorylation of p70S6K1 is not linearly associated with 4EBP1 phosphorylation (36, 37), and this feature could explain the lack of prognostic value of p-p70S6K1 observed in our study. In our series, p-p70SK1 was detected by immunohistochemistry in the infiltrating margins of the tumors and not throughout the tumors, as was p-4EBP1. Other reports (38) have shown a

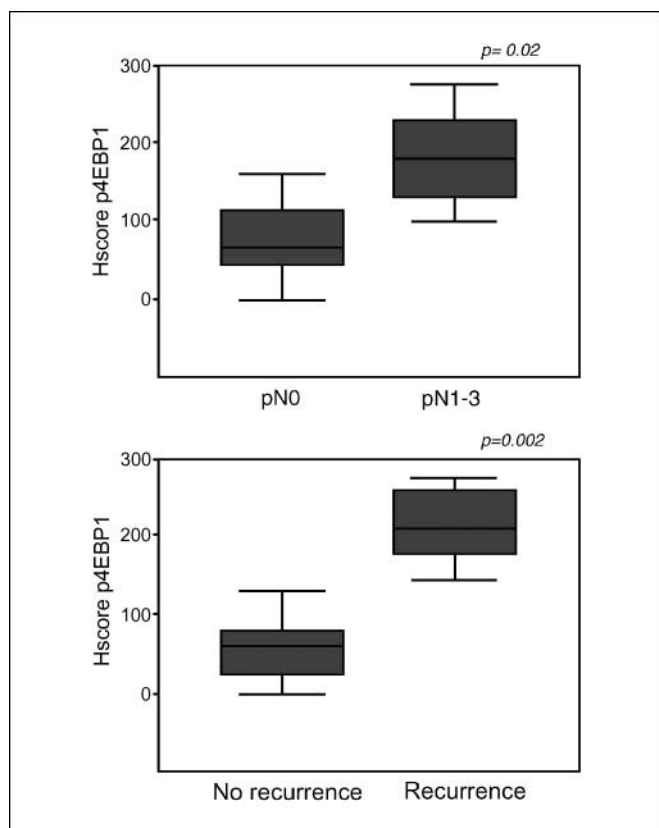
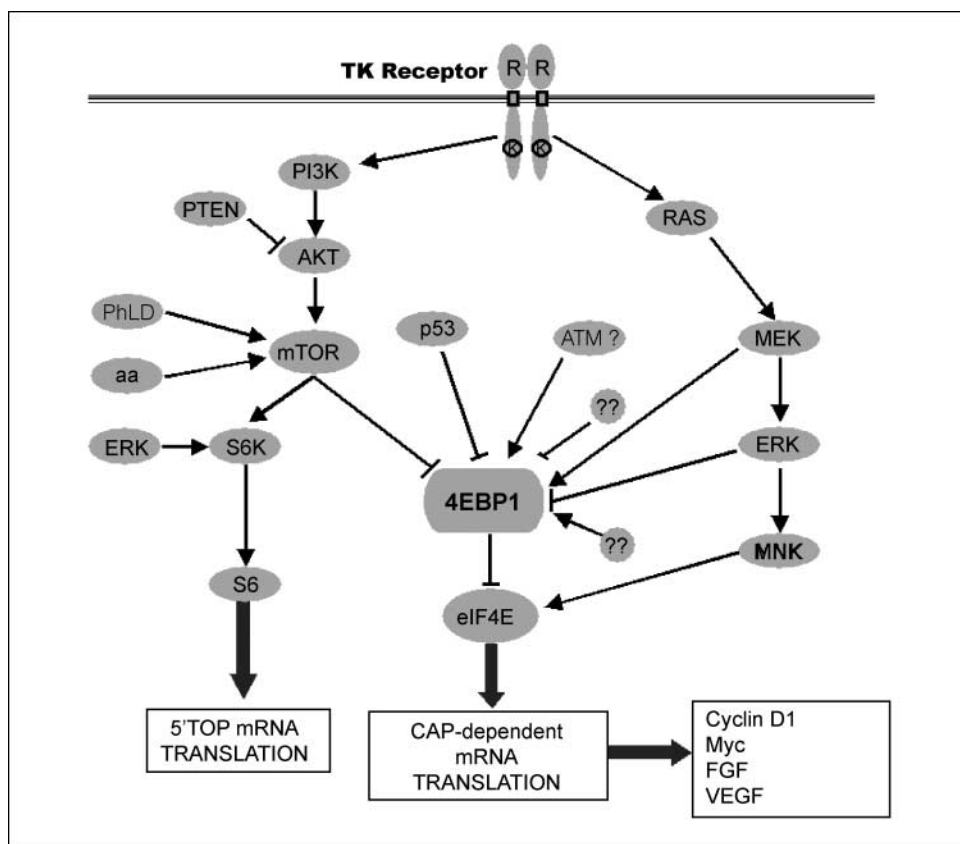


Fig. 5. Box graphs comparing p-4EBP1 expression for the presence of lymph node involvement (Mann-Whitney *U* test, $P = 0.02$) and locoregional recurrence (Mann-Whitney *U* test, $P = 0.002$).

Fig. 6. Schematic proposal of signaling pathways converging at 4EBP1.



prognostic role for p70S6K1 overexpression and amplification only in a subgroup of node-negative premenopausal breast cancer patients with an increased risk of locoregional disease, but this population was not fully represented in our study. In any case, further clinical studies and *in vitro* analyses are needed to clarify the dissociation between p70S6K and p-4EBP1 in breast tumors.

The results of this study support previous data (6), indicating that 4EBP1 expression can predict poor prognosis in breast tumors, particularly those with HER2/neu overexpression or amplification. In the present study, we found a significant correlation between high p-4EBP1 expression and poor prognosis, not only in tumors showing HER2/neu overexpression or amplification, but also in a group of high-grade breast tumors in which HER2/neu amplification/overexpression was not detected. These findings suggest that p-4EBP1 overexpression in breast cancer indicates poor prognosis regardless of whether there is concomitant HER2/neu overexpression or amplification. In fact, in our series, p-4EBP1 was overexpressed in 80% of breast tumors with negative HER2/neu expression, and >75% of tumors without EGFR expression, and even 16% of tumors were negative for all the upstream marker studies. Activation of the Akt pathway and 4EBP1 may be associated with the response to chemotherapy and radiotherapy because of the activation of cell survival signals (39), and might be a predictive factor of clinical response to specific oncogenic inhibitors. In fact, phospho-4EBP1 was detected in the tumors of patients treated with EGFR inhibitors that showed down-regulation of EGFR protein but no clinical response, indicating that p-4EBP1 was activated by other pathways (13).

We found that total 4EBP1 expression was increased in high-grade tumors as compared with low-grade tumors, and that nearly all the 4EBP1 proteins were phosphorylated. Conversely, in low-grade tumors, there was a clear difference between total 4EBP1 and the phosphorylated protein. 4EBP1 phosphorylation was also regulated at the transcriptional level, whereas 4EBP1 has been reported to be inhibited by p38-MAPK (40) and up-regulated by the forkhead transcriptional factor FOXO (41) as well as the Ras-Raf-ERK signaling pathway (42). Functional 4EBP1 status, i.e., phosphorylated 4EBP1, is essential for eIF4E release. 4EBP1 has eight described phosphorylation sites susceptible to phosphorylation by kinases such as mTOR, phosphoinositide-3-kinase, and ERK1/2. Moreover, mTOR can be activated by phospholipase D, Akt, and other kinases, such as cdc2-cyclin B, whereas 4EBP1 is dephosphorylated by a phosphatase controlled by p53 (10, 43). Thus, indirectly, if p53 is not functional, the activity of the phosphatase decreases and this favors 4EBP1 hyperphosphorylation. Moreover, p53 can inhibit mTOR through the activation of MAPK, the tuberous sclerosis TSC1/TSC2 complex and LKB-1 kinase (43), can activate the transcription of the *PTEN* gene and induce transcriptional repression of the eIF4E (44). Other kinases that can phosphorylate 4EBP1 are cdc2 (cdk1; ref. 10) and pim-2 (45). In fact, during mitosis, cdc2 could phosphorylate 4EBP1 on Thr⁷⁰, the same residue that is recognized by the antibody used in the present study.

Various studies have underlined the role of eIF4E in several malignancies (46). Overexpression of eIF4E has been associated with high-grade tumors, and detection of eIF4E expression in

the margins of head and neck carcinoma surgical specimens has been related with local recurrence (46). eIF4F and complexes with other members of the family are essential for ribosomal synthesis (47). Moreover, eIF4E can block the exit of cytochrome *c* from the mitochondria, blocking apoptosis (48). The interrelationship between 4EBP1 and eIF4E is essential for eIF4E control of protein synthesis and translation of key proteins in tumor transformation, such as cyclin D1, Myc, fibroblast growth factor, and vascular endothelial growth factor (49). In fact, *in vitro* studies have shown that eIF4 is crucial for maintaining malignant breast cell lines (12, 49). Thus, it is clear that an increase in free eIF4E can be critical for the control of apoptosis, protein synthesis, and indirectly, cell proliferation (50). Nevertheless, no significant correlation was found between total eIF4E or p-eIF4G and locoregional recurrence or grade of malignancy in our breast tumor series. Future studies are needed to validate these results with reliable anti-p-eIF4E antibodies in paraffin sections.

References

- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
- Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 1997;386:761–3.
- Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–31.
- Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. *Nat Rev Cancer* 2004;4:177–83.
- Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000;406:747–52.
- Zhou X, Tan M, Stone Hawthorne V, et al. Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. *Clin Cancer Res* 2004;10:6779–88.
- Panigrahi AR, Pinder SE, Chan SY, Paish EC, Robertson JF, Ellis IO. The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. *J Pathol* 2004;204:93–100.
- Carrera AC. TOR signaling in mammals. *J Cell Sci* 2004;117:4615–6.
- Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer* 2004;4:335–48.
- Heesom KJ, Gampel A, Mellor H, Denton RM. Cell cycle-dependent phosphorylation of the translational repressor eIF-4E binding protein-1 (4E-BP1). *Curr Biol* 2001;11:1374–9.
- Topisirovic I, Ruiz-Gutierrez M, Borden KL. Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities. *Cancer Res* 2004;64:8639–42.
- Avdulov S, Li S, Michalek V, et al. Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells. *Cancer Cell* 2004;5:553–63.
- Baselga J, Albanell J, Ruiz A, et al. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J Clin Oncol* 2005;23:5323–33.
- Jackson JG, St Clair P, Sliwkowski MX, Brattain MG. Blockade of epidermal growth factor- or heregulin-dependent ErbB2 activation with the anti-ErbB2 monoclonal antibody 2C4 has divergent downstream signaling and growth effects. *Cancer Res* 2004;64:2601–9.
- Arteaga CL, Winnier AR, Poirier MC, et al. p185^{erbB-2} signal enhances cisplatin induced cytotoxicity in human breast carcinoma cells: association between an oncogenic receptor tyrosine kinase and drug-induced DNA repair. *Cancer Res* 1994;54:3758–65.
- Wu X, Fan Z, Masui H, Rosen N, Mendelsohn J. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* 1995;95:1897–905.
- Bos M, Mendelsohn J, Kim YM, Albanell J, Fry DW, Baselga J. PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clin Cancer Res* 1997;3:2099–106.
- Wosikowski K, Schuurhuis D, Johnson K, et al. Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* 1997;89:1505–15.
- Albanell J, Codony-Servat J, Rojo F, et al. Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor α expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. *Cancer Res* 2001;61:6500–10.
- Guinea B, Ligos JM, Lain de Lera T, et al. Nucleocytoplasmic shuttling of STK16 (PKL12), a Golgi-resident serine/threonine kinase involved in VEGF expression regulation. *Exp Cell Res* 2006;312:135–44.
- Boulay A, Zumstein-Mecker S, Stephan C, et al. Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. *Cancer Res* 2004;64:252–61.
- Robbins P, Pinder S, de Klerk N, et al. Histological grading of breast carcinomas: a study of interobserver agreement. *Hum Pathol* 1995;26:873–9.
- Matar P, Rojo F, Cassia R, et al. Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. *Clin Cancer Res* 2004;10:6487–501.
- O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006;66:1500–8.
- Saez R, Molina MA, Ramsey EE, et al. p95^{HER-2} predicts worse outcome in patients with HER-2-positive breast cancer. *Clin Cancer Res* 2006;12:424–31.
- Molina MA, Saez R, Ramsey EE, et al. NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. *Clin Cancer Res* 2002;8:347–53.
- Gulmann C, Espina V, Petricoin E III, et al. Proteomic analysis of apoptotic pathways reveals prognostic factors in follicular lymphoma. *Clin Cancer Res* 2005;11:5847–55.
- Grubb RL, Calvert VS, Wulkuhle JD, et al. Signal pathway profiling of prostate cancer using reverse phase protein arrays. *Proteomics* 2003;3:2142–6.
- Wulkuhle JD, Aquino JA, Calvert VS, et al. Signal pathway profiling of ovarian cancer from human tissue specimens using reverse-phase protein microarrays. *Proteomics* 2003;3:2085–90.
- Herrmann PC, Gillespie JW, Charboneau L, et al. Mitochondrial proteome: altered cytochrome *c* oxidase subunit levels in prostate cancer. *Proteomics* 2003;3:1801–10.
- Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 2004;6:117–27.
- Chen Y, Rodrik V, Foster DA. Alternative phospholipase D/mTOR survival signal in human breast cancer cells. *Oncogene* 2005;24:672–9.
- Constantinou C, Clemens MJ. Regulation of the phosphorylation and integrity of protein synthesis initiation factor eIF4G1 and the translational repressor 4E-BP1 by p53. *Oncogene* 2005;24:4839–50.
- Castellvi J, Garcia A, Rojo F, et al. Phosphorylated 4E binding protein 1: a hallmark of cell signaling that correlates with survival in ovarian cancer. *Cancer* 2006;107:1801–11.
- Sarbasov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005;307:1098–101.
- Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem* 1998;273:14484–94.
- Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J. Regulation of the p70 S6 kinase by phosphorylation *in vivo*. Analysis using site-specific anti-phosphopeptide antibodies. *J Biol Chem* 1998;273:16621–9.
- van der Hage JA, van den Broek LJ, Legrand C, et al. Overexpression of P70 S6 kinase protein is associated with increased risk of locoregional recurrence in node-negative premenopausal early breast cancer patients. *Br J Cancer* 2004;90:1543–50.
- Wendel HG, De Stanchina E, Fridman JS, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* 2004;428:332–7.
- Rolli-Derkinderen M, Machavoine F, Baraban JM,

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- Grolleau A, Beretta L, Dy M. ERK and p38 inhibit the expression of 4E-BP1 repressor of translation through induction of Egr 1. *J Biol Chem* 2003;278:18859–67.
41. Puig O, Marr MT, Ruhf ML, Tjian R. Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* 2003;17:2006–20.
42. Sarbassov dos D, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005;17:596–603.
43. Feng Z, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A* 2005;102:8204–9.
44. Stambolic V, MacPherson D, Sas D, et al. Regulation of PTEN transcription by p53. *Mol Cell* 2001;8:317–25.
45. Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA, Thompson CB. The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. *Genes Dev* 2003;17:1841–54.
46. De Benedetti A, Graff JR. eIF-4E expression and its role in malignancies and metastases. *Oncogene* 2004;23:3189–99.
47. Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* 2005;433:477–80.
48. Li S, Takasu T, Perlman DM, et al. Translation factor eIF4E rescues cells from Myc dependent apoptosis by inhibiting cytochrome *c* release. *J Biol Chem* 2003;278:3015–22.
49. Ruggero D, Pandolfi PP. Does the ribosome translate cancer? *Nat Rev Cancer* 2003;3:179–92.
50. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004;18:1926–45.