



Beyond molecular tumor heterogeneity: protein synthesis takes control

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Abstract

One of the daunting challenges facing modern medicine lies in the understanding and treatment of tumor heterogeneity. Most tumors show intra-tumor heterogeneity at both genomic and proteomic levels, with marked impacts on the responses of therapeutic targets. Therapeutic target-related gene expression pathways are affected by hypoxia and cellular stress. However, the finding that targets such as eukaryotic initiation factor (eIF) 4E (and its phosphorylated form, p-eIF4E) are generally homogeneously expressed throughout tumors, regardless of the presence of hypoxia or other cellular stress conditions, opens the exciting possibility that malignancies could be treated with therapies that combine targeting of eIF4E phosphorylation with immune checkpoint inhibitors or chemotherapy.

Introduction

Owing to increased incidence, cancer is now the second most common cause of death in developed countries and the leading cause of death in individuals above 40 years of age [1]. The number of cancer-related deaths is expected to grow due to increases in life expectancy and lifestyle risk factors. Although current treatments have improved patient survival, the results remain dismal for advanced disease. For example, the 5-year survival rate is only 2% for stage IV lung cancer and 25% for breast cancer patients with metastatic disease [2]. In contrast, the 5-year survival of patients with in situ breast cancer exceeds 90%. Indeed, despite the development of a new arsenal of molecular targeted

therapies over the last decade, patient survival with advanced cancer has improved by only 15% [1, 2]. One of the main reasons for these disappointing outcomes is the pervasive heterogeneous expression of drug targets within human tumors.

Cancer can be viewed as a group of heterogeneous diseases that arise from a small number of initiation events, but phenotypically diverge during progression due to environmental context (site of origin), the acquisition of different mutations required for survival, and individual patient responses to the tumor. Heterogeneity is observed at the genetic, proteomic, morphological, and environmental levels.

Tumor cell adaptability often leads to the use of redundant signaling pathways in response to stress, such as hypoxia and reduced nutrient availability. Within a tumor bed, variations in the “strength” of these stressor events and the corresponding responses can result in a significant degree of heterogeneity in gene expression, with some cells needing to respond more acutely than others. In this review, we underscore the nature of eukaryotic initiation factor (eIF) 4E in malignant tumors as a critical effector of cell signaling networks. We summarize the findings that the phosphorylated forms of eIF4E and 4E-BP1, termed p-eIF4E and p-4E-BP1, show a predominantly homogenous expression pattern within tumor beds, a feature that we predict to be actionable and to hold significant consequences for cancer therapy.

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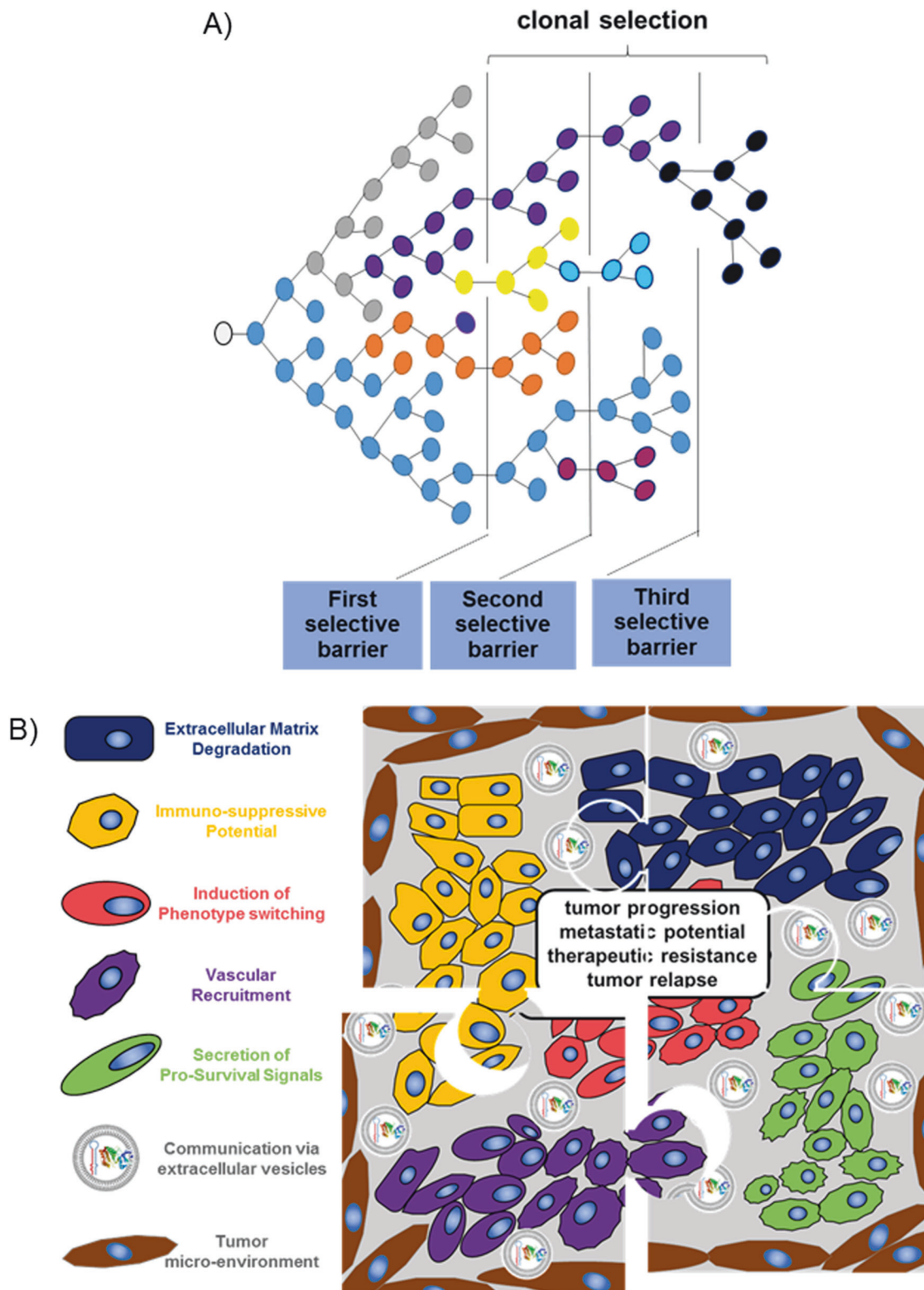


Fig. 1 a Diagram representing clonal selection according to a Darwinian model. The best-adapted clones due to genetic or epigenetic advantages or with better interplay with neighboring cells will survive and proliferate, becoming the dominant clone until a new “selective barrier” appears. The tumor clonal composition varies over time, although, microscopically, these changes can be subtle or not evident. **b** Clonal cooperation and feature complementation. Puzzle diagram

illustrating the contribution of individual cell clones with different tumor-promoting features to the formation of a tumor. The main feature of each clonal population within a tumor is shown as legend on the left side. The cooperation between different clones results in different functional consequences for the tumor, which are summarized in the middle of the figure

Clonal evolution during cancer progression

A complex molecular scenario is responsible for tumor initiation and tumor progression. It is well established that, in a single tumor, cell clones with novel genetic alterations arise constantly and are selected according to a Darwinian model [3]. Concomitantly, there is also cross talk between the tumor clones and the microenvironment that affect the ability of tumors to survive and proliferate. In fact, the cooperation between clones and the microenvironment is similar to that of a tumor consortium (Fig. 1) [4]. The new genetic alterations are driven by genetic instability, one of the hallmarks of tumor cells [5]. Only a small proportion of the total mutational burden is related to the process of clonal evolution because most are passenger mutations with no biological relevance [5]. In addition, treatments can alter clonal heterogeneity by selecting for more resistant cells or perturbing the microenvironmental conditions [6].

Epigenetic differences between clones are critical to tumor heterogeneity. Many of them are associated with an aberrant DNA methylation pattern, histone modification, or microRNA transcriptome and can be related to microenvironment factors [7]. Thus, both genome and epigenome diversity enables malignant tumor clones to acquire all of the capabilities to survive, proliferate, and invade neighboring or distant tissues [8–12].

The microenvironment appears to be important for the selection of the best-adapted clones. Contributors include neighboring cells (e.g., fibroblasts and immune cells), growth factors, cytokines, hypoxia, and nutrient availability [13–16]. Hence, variations among tumor microenvironments may be responsible for some of the phenotypic heterogeneity observed within tumor beds. Consequently, the microenvironment may also have an impact on the selection of specific clones with different driver/maintenance mutations in topologically segregated areas of a tumor, which together affect the evolutionary trajectory of the disease (Fig. 1a).

Clonal accumulation and response to anticancer agents

The proteomic complexity of tumors must be fully understood to develop more effective therapeutic strategies. Pathologists have long recognized that not all cells within a given tumor express the same amounts of a large number of proteins. For example, the expression of cyclin D1 in mantle cell lymphoma is not homogeneous even when all of the tumor cells carry the signature CCND1-IGH translocation [17]. Likewise, hormone receptor expression in breast carcinoma is often non-homogenous and irregular within a given tumor bed. The protein intra-tumor heterogeneity is

mirrored in some cases at the transcriptional level and has been well documented by single-cell RNA-sequencing in glioblastomas [18]. Moreover, the latter study noted variations in the expression level and differences in cell signaling receptor and cell proliferation markers. In addition, differences in post-translational modifications of a given protein among cancer cells in a single tumor have been documented [19, 20].

The consequences of this pervasive lack of uniformity between cancer cells are grim for patients. It is a major cause of treatment failure in many patients, particularly in those treated with molecular targeted therapies [21]. If a fraction of cancer cells in the tumor do not express a particular drug target or have evolved to no longer be dependent on its presence/activity, then it stands to reason that these cells will fail to be eliminated by targeted therapies. A case in point is HER2+ (human epidermal growth factor receptor 2) breast cancers, with the classification requiring that only 30% of the cells have to stain positive for HER2 by immunocytochemistry. Clearly, treatment with anti-HER2 therapies cannot be expected to be curative in such a context. Similarly, therapies based on rapamycin fail because of the uneven and heterogeneous expression of p-mTOR [22–24].

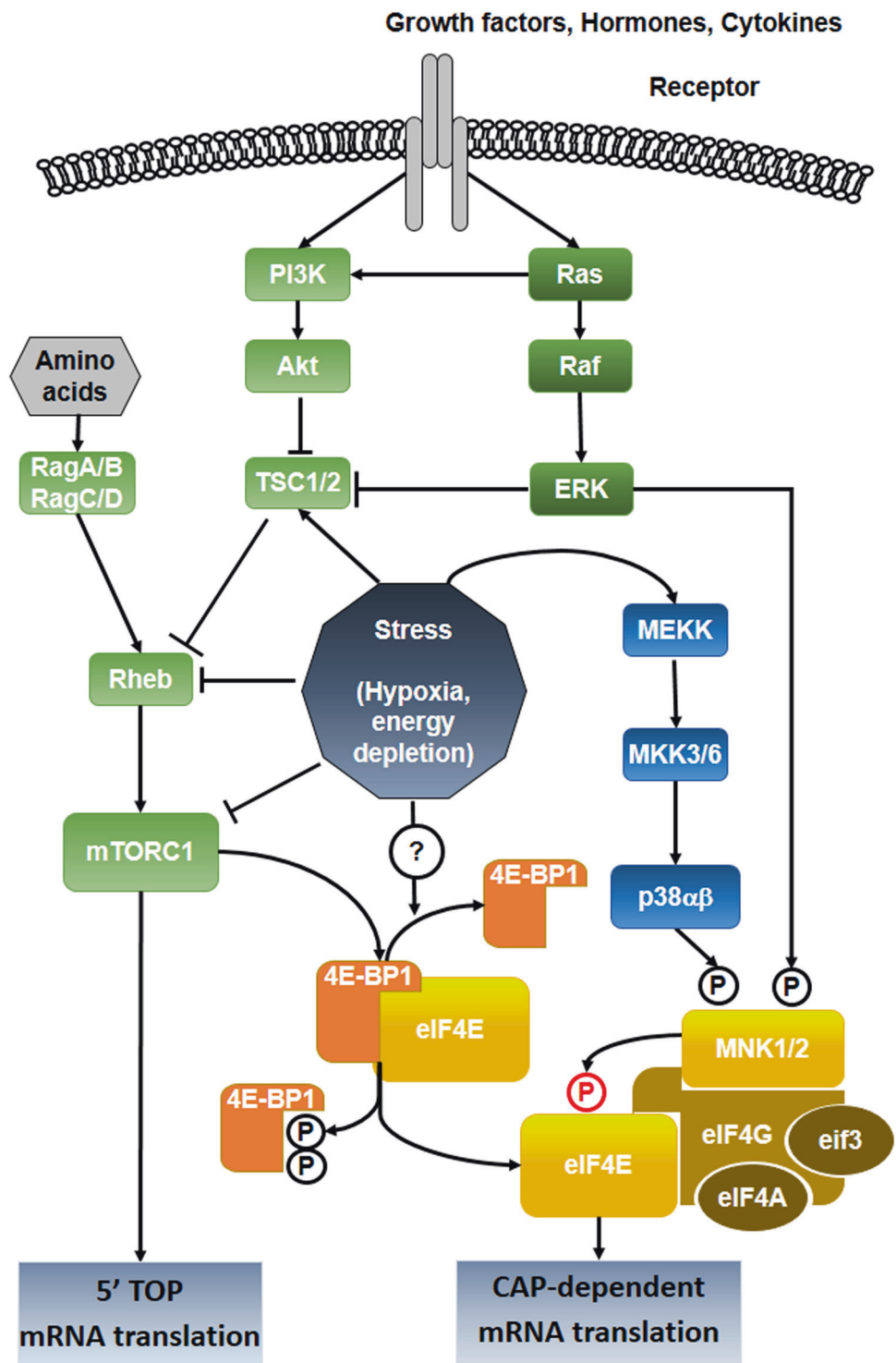
It is noteworthy that this same target expression issue may also affect the outcomes of immunotherapeutic approaches. Most patients with B cell malignancies who are treated with chimeric antigen receptor (CAR) T cells targeting CD19 will initially respond to therapy, but about 30% will relapse. The relapse appears to be because the tumor cells express a novel CD19 isoform arising from alternative splicing and lacking the exon encoding the antigenic epitope [25]. One way around this problem is to combine multiple therapies after tumor mutational profiling [26–28], but appropriate clinical trials are required to ensure that the resulting combination is not antagonistic.

Identifying the status of the protein synthesis machinery and the key regulators of translational control

Choosing targets whose expression levels do not vary significantly among cancer cells and whose expression cannot be extinguished (i.e., essential targets) is a logical step to deal with the issue of heterogeneity. Such potential targets include components of the translation apparatus, an essential biochemical process, with recent experience showing that an optimal therapeutic index can be obtained when this process is targeted in cancer cells [20, 21, 26, 27]

Two of the most important regulatory signal transduction pathways that modulate cellular translation rates are the RAS-RAF-ERK1/2 and PI3K-AKT/mTOR [29–31]

Fig. 2 Schematic representing the signaling cascade regulating translation initiation. The key event is the dissociation of eIF4E from 4E-BP1 by different signaling pathways under normal growth conditions and in response to stress



pathways. These pathways are crucial to the development of targeted therapies because many of their components are changed in the vast majority of human cancers (e.g., HER2, PI3K, RAS, and RAF) [32]. What is generally underappreciated is that components of the translation regulatory machinery (namely, 4E-BP1, eIF4E, and eIF4A) involved in the ribosome recruitment phase of translation initiation fall under the control of these pathways (Fig. 2) [33, 34].

The eIF4F complex, consisting of eIF4E (the cap-binding protein), eIF4A (a DEAD-box RNA helicase), and eIF4G (a large scaffolding protein), regulates ribosome recruitment to mRNA templates [35]. This step in translation initiation is thought to be rate-limiting for protein synthesis. The assembly of eIF4F is regulated by mTOR via phosphorylation of 4E-BPs (of which there are three, with 4E-BP1 being the best studied), as well as of PDCD4 [36–39].

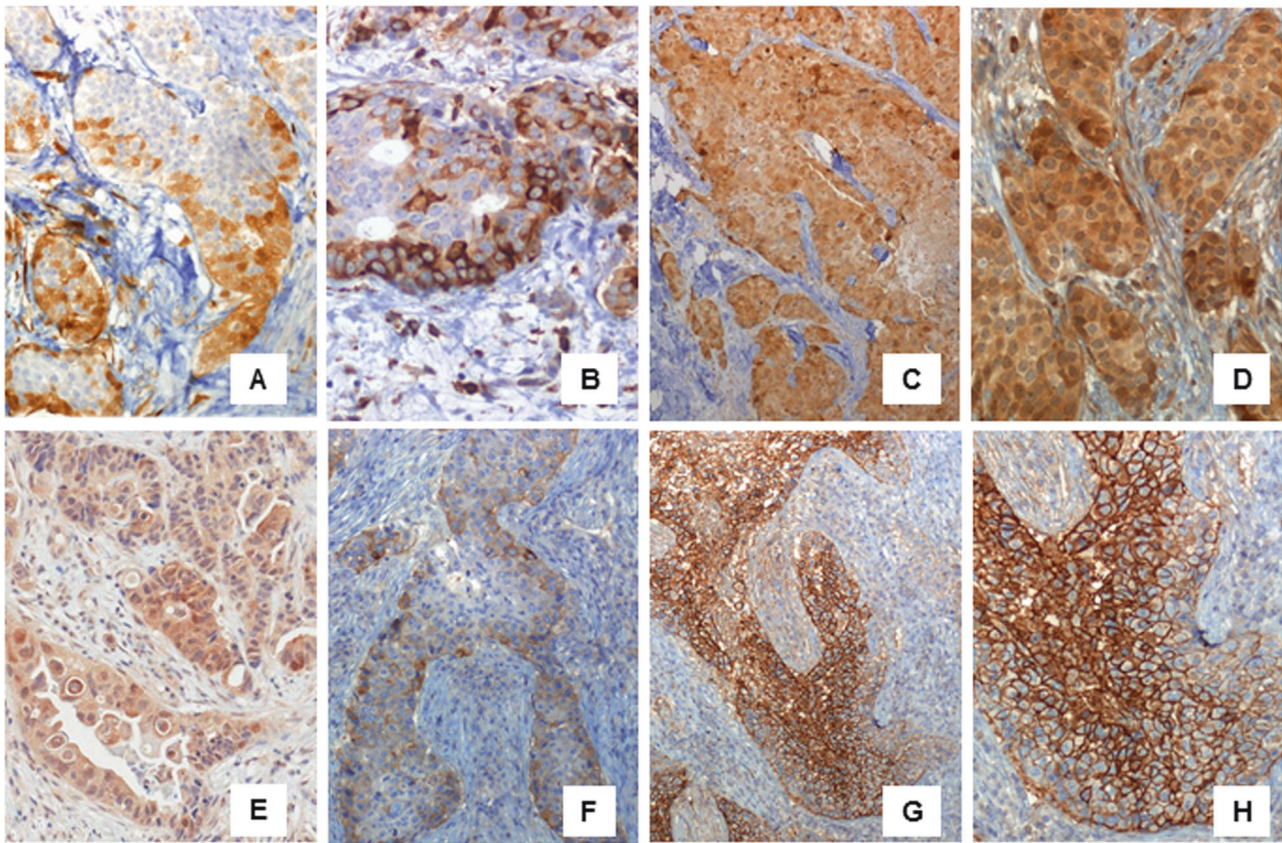


Fig. 3 a–h Invasive ductal carcinoma **a–g** ($\times 200$), **h** ($\times 400$). Immunohistochemistry for **a** p-ERK1/2, **b** p-S6, **c** p-4E-BP1, **d** p-eIF4E, **e** p-AKT, and **f** p-mTOR. **g**, **h** GLUT-1 Immunohistochemistry was performed as described previously [58] using the following primary antibodies: p-eIF4E (Abcam, Ab76256), p-4E-BP1 (T37/46) (Cell

Signaling, #2855), pS6 (S235/236) (Cell Signaling, #2211), p-ERK1/2 (T202/Y204) (Cell Signaling, #9101), p-mTOR (Ser2448) (Cell Signaling #2971); p-Akt (Ser473) (Cell Signaling #3787), GLUT-1 (Abcam, Ab652)

Binding of 4E-BP1 to eIF4E prevents eIF4F complex formation [40]. mTOR activation (as occurs in a broad range of human cancers) causes direct phosphorylation of 4E-BP1 and its dissociation from eIF4E to consequently stimulate eIF4F formation [41–43]. eIF4F discriminates between different mRNAs and therefore an increase in eIF4F levels or activity causes a selective change in the translome. Although the features responsible for mRNA discrimination by eIF4F are not completely understood, cap accessibility and 5' leader secondary structure are important contributors [44–46]. PDCD4 forms an inhibitory complex with eIF4A and phosphorylation of the former by S6K1/2 leads to its degradation and allows eIF4A to enter the eIF4F complex [38, 39]. In addition, eIF4E can be directly phosphorylated upon activation of the RAS-RAF-ERK1/2-MNK pathway or through p38 and this is also associated with a selective increase in translation, the mechanistic basis of which remains to be elucidated [47–51].

4E-BP1 harbors seven phosphorylation sites and, although mTOR is the most prominent kinase targeting these sites, other kinases, such as CDK1, ATM, PI3K-AKT, ERK1/2, and PIM1, also phosphorylate 4E-BP1 [52, 53].

Therefore, 4E-BP1 phosphorylation can be the consequence of many different oncogenic events that modulate disparate signaling pathways or that occur via several mechanisms, including amplification or mutation of growth factor receptors or mutations in critical oncogenes (e.g., PTEN, ATM, p53, PI3K, or RAS). Our current understanding of perturbed translation initiation in cancer cells is that the eIF4E/4E-BP1 ratio is critical to sustain the oncogenic features of a transformed cell. Ultimately, this essential node may act as a “bottleneck” or “funnel factor” to sustain transformation, regardless of the upstream oncogenic alterations [54] (Fig. 2).

Expression of signaling factors in human tumors

In the past decade, by analyzing more than 2500 human tumor samples [55–60], we have assessed the expression of membrane receptors such as EGFR and HER2, components of the RAS/RAF/ERK and PI3K/AKT/mTOR pathways, and their effectors such as p70S6K, 4E-BP1, eIF4E, and p-

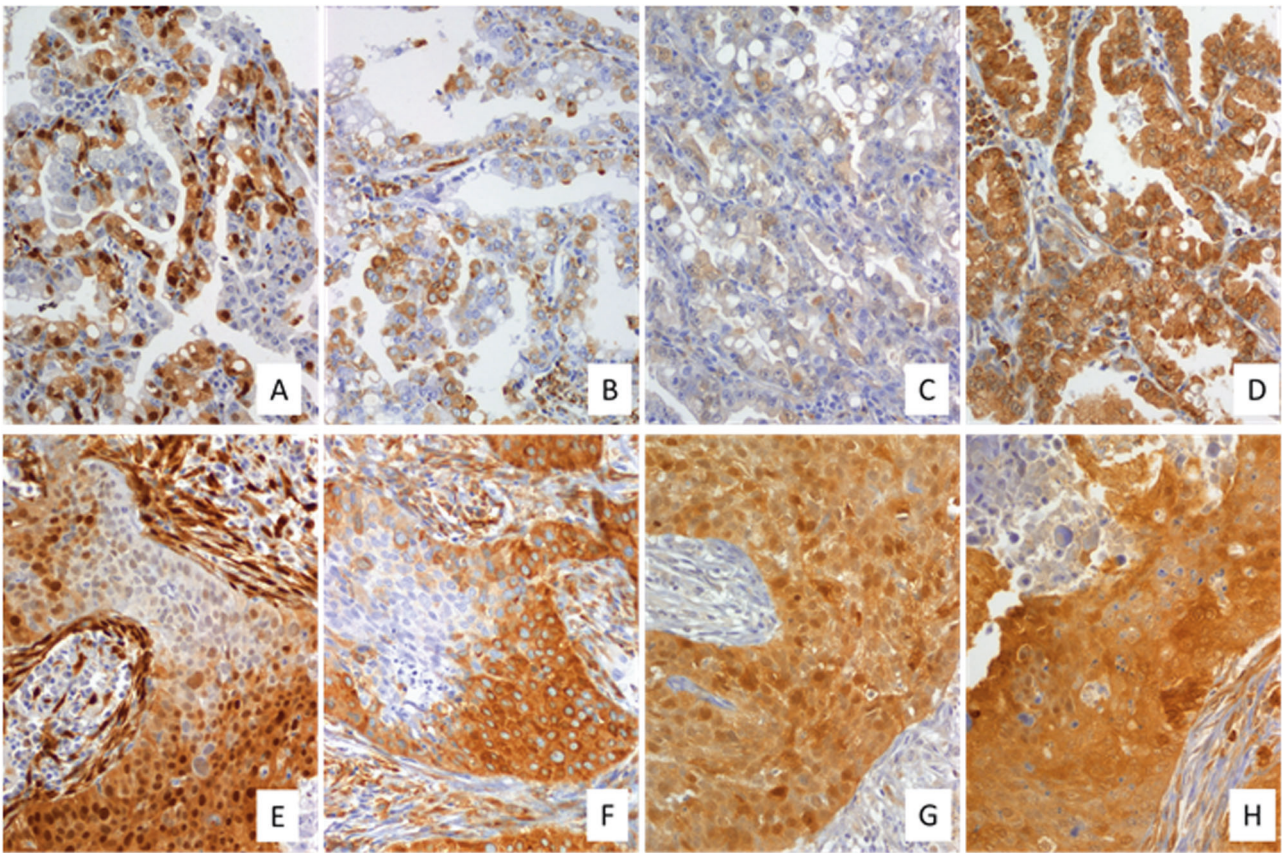


Fig. 4 **a–d** Low-grade lung adenocarcinoma ($\times 400$). **e–h** High-grade lung squamous carcinoma ($\times 200$). Immunohistochemistry for p-ERK1/2 (**a**, **e**), p-S6 (**b**, **f**), p-4E-BP1 (**c**, **g**), and p-eIF4E (**d**, **h**). Immunohistochemistry was performed as described previously [58]

eIF4E. We have found that increased amounts of total or p-eIF4E, as well as p-4E-BP1, are associated with malignant progression and adverse prognosis in several tumors, including breast, lung, ovary, endometrium, glioma, and prostate cancers, regardless of the upstream oncogenic alterations (Figs. 3 and 4) [22]. Other groups have confirmed the prognostic importance of these factors in additional tumor types (Table 1), including colon cancer [61, 62], nasopharyngeal carcinoma [63], hepatocellular carcinoma [64], astrocytomas [65], lung cancer [66, 67], and melanoma [68]. Importantly, eIF4E is a central regulator of metastatic progression [69–71] (Fig. 4).

Thus, the eIF4E/4E-BP1 node appears to act as a restriction point for essential oncogenic features such as self-sufficiency in growth signals and should serve as a highly relevant molecular marker of malignant potential. Interestingly, the expression of eIF4E and 4E-BP1 and their phosphorylated forms is apparent even in the presence of upstream receptor or kinase overexpression (e.g., AKT, mTOR, or ERK), suggesting that other mechanisms are involved in their regulation. The expression of p-AKT or p-mTOR is highly heterogeneous within a tumor, whereas the expression of 4E-BP1 and eIF4E is more homogeneous (Fig. 3) [22]. This may be due to the activation status of the

global growth signaling and proliferative network in being able to maintain a certain flux threshold rather than the necessity of maintaining activity of a specific player. Even in tumors showing constitutive expression of EGFR and HER2, the global gene expression program is not necessarily permanently fixed or homogeneous in all cells. Interestingly, the geographic context of the tumor cell may impinge on the expression levels of these pathways [72]. For example, some markers are more highly expressed at the invasive front or around necrotic areas, suggesting that ischemia or other microenvironmental factors impinge on their expression or activity (Figs. 3 and 5).

Perspectives in tumor heterogeneity beyond genetics

The microenvironment has a key role in selecting the best-adapted cancer clone and can alter communication networks between different cancer cell types. The aberrant information flow in cancer cells leads to alterations in gene regulatory networks that support the cancer hallmarks [73] and can be influenced by features such as cytokines, exosomes, hypoxia, starvation, and oxidative stress (Fig. 5). In this

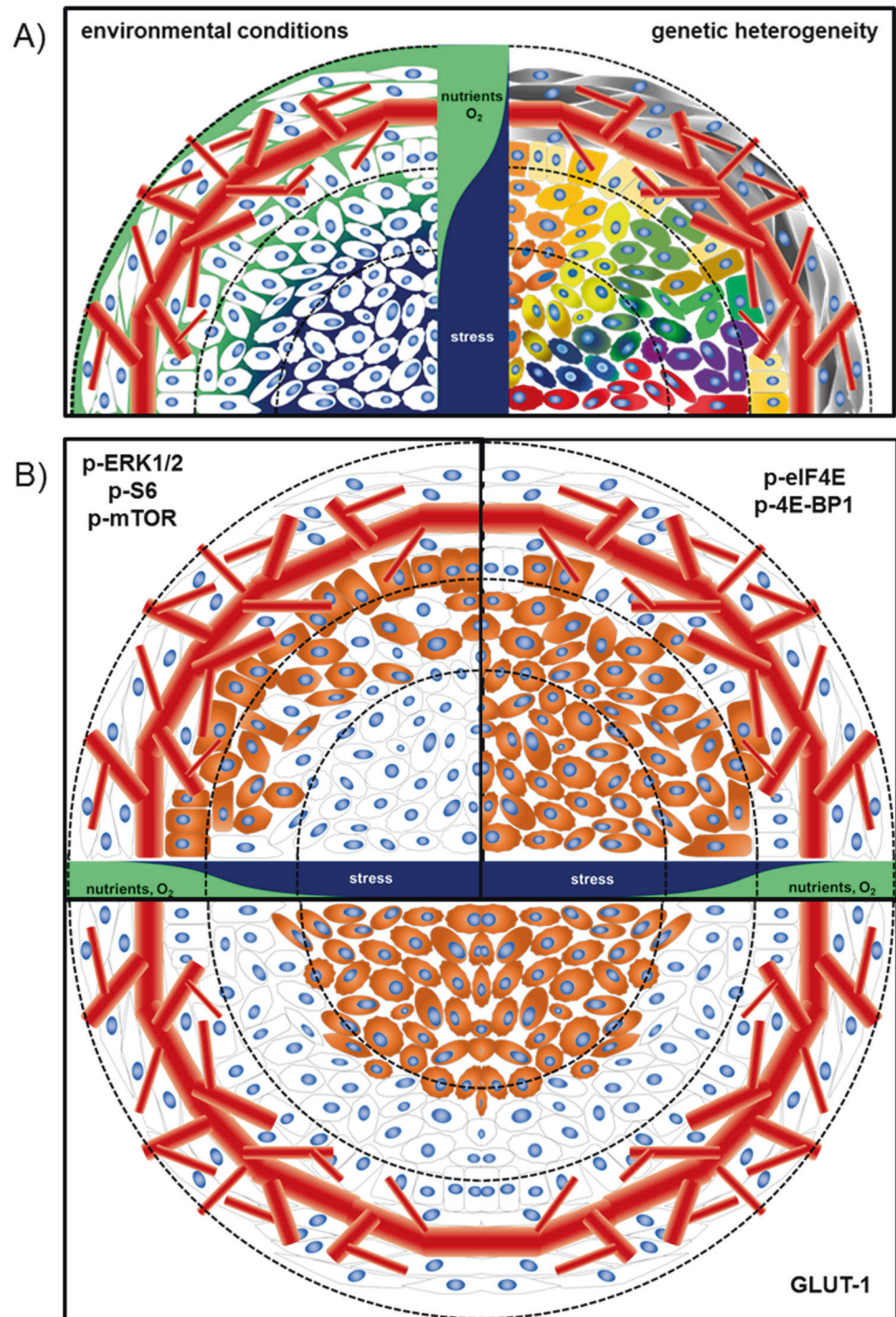
Table 1 Prognostic significance of total and phosphorylated eIF4E and 4E-BP1 in malignant tumors

Primary tumor	Clinical significance	References
Bladder	p-4E-BP1 correlates with prognosis in patients with muscle-invasive bladder cancer	Nishikawa et al. [96]
Breast	Increased expression of eIF4E in invasive ductal carcinoma correlate with presence of lymph node metastasis 4E-BP1 is an independent prognostic factor and is associated a poor response to endocrine therapy eIF4E predicts survival after anthracycline chemotherapy in breast cancer patients eIF4E expression is related to breast cancer survival and it is modulated by 4E-BP1 p-4E-BP1 correlates with grade and prognosis in breast cancer High eIF4E is an independent predictor of recurrence in breast cancer.	Hu et al. [97] Karlsson et al. [98] Heikkinen et al. [99] Coleman et al. [100] Rojo et al. [60] Li et al. [101]
Cervix	Overexpression of p-4E-BP1 predicts recurrence and reduced survival in cervical carcinoma	Benavente et al. [55]
CNS	p-eIF4E is an independent prognostic factor in astrocytoma p-4E-BP1 expression increase with tumor grade and predicts survival in astrocytomas	Martínez-Saez et al. [58] Korkolopoulou et al. [102]
Colon	High 4E-BP1 expression is associated with poor prognosis High expression of eIF4E is associated with advanced stage and poor prognosis	Chen et al. [103] Chao et al. [61]
Endometrium	p-4E-BP1 is associated with high-grade endometrial carcinomas and worse prognosis p-4E-BP1 is associated with stage and high-grade tumors	Castellvi et al. [57] Darb-Esfahani et al. [104]
Esophagus	p-4E-BP1 expression after chemoradiotherapy is a predictor for recurrence and worse survival in esophageal carcinoma p-4E-BP1 is associated with poor prognosis in early stage esophageal carcinoma	Chao et al. [105] Yeh et al. [106]
Head and neck	eIF4E expression is associated with tumor stage, lymph node metastasis and grade of differentiation p-eIF4E and p-MNK1 are independent prognostic factors in nasopharyngeal carcinoma	Han et al. [107] Zheng et al. [63]
Kidney	p-4E-BP1 is associated with poor prognosis in Xp11.2 translocated renal cell carcinoma p-4E-BP1 and eIF4E are independent prognostic factors in clear cell renal cell carcinoma p-4E-BP1 is a prognostic predictor in patients with metastatic renal cell carcinoma	Qu et al. [108] Campbell et al. [109] Nishikawa et al. [110]
Liver	eIF4E overexpression is an independent indicator for overall survival in hepatocarcinoma p-4E-BP1 is overexpressed in cholangiocarcinomas with poor differentiation and lymph node metastasis, and is an independent prognostic factor	Jiang et al. [64] Fang et al. [111]
Lung	p-4E-BP1 expression is associated with poor prognosis in small-cell lung cancer p-4E-BP1 Thr70 predicts poor prognosis in non-small-cell lung cancer p-4E-BP1 and eIF4E are prognostic factors in stage I lung adenocarcinoma High eIF4E expression correlates with poor prognosis in lung adenocarcinomas	Roh et al. [66] Lee et al. [112] Seki et al. [113] Wang et al. [114]
Melanoma	eIF4E is associated with melanoma thickness and overall survival p-4E-BP1 is associated with poor survival in melanoma	Khosravi et al. [68] O'Reilly et al. [115]
Ovary	p-4E-BP1 is a prognostic factor in ovarian cancer	Castellvi et al. [56]
Stomach	p-eIF4E is overexpressed in tumors with lymph node metastasis p-4E-BP1 is a prognostic factor in gastric cancer patients and correlates with advanced stage	Tapia et al. [116] Jiao et al. [117]

respect, modulating the translational program is thought to ensure the expression of factors, which confer resistance to cellular stress [74] (Fig. 3). In most malignant cells, the cap-dependent pathway is highly upregulated and interference with this translational program has been shown to be an attractive venue for novel therapeutics that ultimately prevent the adaptation of tumor cells to stress conditions. The main therapeutic approaches targeting the 5' cap-dependent translational machinery (summarized in ref. [33]) are directed against, the expression of eIF4E [75, 76], the interaction between eIF4E-4G [77, 78], the binding of eIF4F complex to the 5'-cap structure [79, 80], the eIF4A

helicase activity [81–84], the phosphorylation status of eIF2 α [85, 86], and the kinase activity of MNK1/2 [87–91]. Among the different strategies that prevent 5' cap-dependent translation under stress conditions, it is believed that inhibition of MNKs may be a powerful way to increase the efficacy of other anti-tumor agents, as phosphorylation of its downstream target eIF4E has been shown to confer resistance to cellular stress, genomic damage, lack of nutrients, and oxidative stress (Fig. 5) [92, 93]. In fact, several companies are developing inhibitors of MNK1/2 activity [94], and at least one of them (eFT508) is already being studied in a clinical phase II trial. Impressive data

Fig. 5 a Schematic drawing of a tumor and the tumor microenvironment. Left: Cells within a tumor exposed to different microenvironmental cues. Nutrient and O₂ supply decreases from the periphery to the center of the tumor while stress conditions are elevated. Right: Genetic alterations of cells within a tumor are depicted by different colors. Importantly, genetic alterations are not strictly limited to the different environmental conditions. **b** Scheme depicting the staining pattern of different proteins involved in the signaling cascade regulating translation initiation (top), as shown in Figs. 3 and 4. GLUT1 serves as a marker for hypoxic conditions within the tumor (bottom)



have been obtained from preclinical models of diffuse large B cell lymphoma, non-small-cell lung carcinoma, and breast adenocarcinoma. Moreover, the inhibitor eFT508 enhances the efficiency of anti-PDL1 checkpoint blockade inhibitors [95]. Similarly, blockage of 4E-BP1 phosphorylation by inhibition of upstream signaling activity (e.g., mTOR) will decrease eIF4F levels and dampen cap-dependent translation and tumor cell growth [40].

In summary, tumor heterogeneity must first and foremost be considered by a treating oncologist after a cancer diagnosis and be a key factor in the determination of a therapeutic target following mutation profiling. We know that intra-tumor heterogeneity is dynamic, occurs at multiple levels, and follows a Darwinian model. Still unresolved is the number of biopsies or sections required from the primary specimen to determine the extent of molecular target

heterogeneity. Moreover, relapses and metastases need to be analyzed to understand how they differ from the primary tumor. Given the complexities of these issues, collaboration among oncologists, radiologists, pathologists, bioinformaticians, and molecular biologists is required to offer the best care to patients. Finally, it is clearly of paramount importance to explore intervention strategies that target critical factors involved in regulating translation, such as eIF4E. With the rigorous evaluation of combinations of small-molecule eIF4E or MNK1/2 inhibitors with other therapeutics (e.g., cytotoxics, targeted therapies, immunotherapy), the issue of proteomic heterogeneity can start to be therapeutically addressed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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