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Review

Tumor Progression and Metastasis: Role of Translational Deregulation

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Abstract. *Translation deregulation is implicated in cellular transformation. Aberrant flux through signalling pathways that impinge on the translation process and perturbations in the relative levels of key regulatory translation initiation factors has been documented in a variety of human cancer types. Recently, studies have demonstrated that translation deregulation also contributes to the metastatic phenotype through selective effects on the translation of mRNAs whose products are involved in various steps of metastasis including migration, invasion, angiogenesis, homing, and activation of survival loops at distal sites. Herein, we present the latest findings implicating perturbed translational control in the metastatic process.*

Mutations that activate oncogenes or inactivate tumor suppressor genes predispose to tumourigenesis. Additional genetic changes are acquired during progression to the fully malignant phenotype, some of which impart to tumour cells novel properties, such as the ability to evade immune surveillance, invade, metastasize, and colonize distal sites. Although our knowledge of the metastatic process is still in its infancy, this process requires the expression of a set of gene products that will allow the tumour cell to stimulate angiogenesis (*e.g.* through increased expression of vascular endothelial growth factor, VEGF), break through the primary tumour site by degrading extracellular matrix (ECM), invade surrounding tissues (*e.g.* through increased expression of the matrix metalloproteases, MMPs), survive in the haematopoietic or lymphatic systems, and finally exit the circulation at distal sites to establish satellite growth in new microenvironments. A few examples of gene products involved in this process include the CD44 receptor, which has been implicated in chemotaxis and tumor cell migration, as well as in homing *via* binding to its ligands, osteopontin and hyaluronate (1, 2). Osteopontin binding mediates cell migration from the bloodstream to distal sites, whereas binding to hyaluronate mediates cell aggregation and growth in newly colonized sites. The different ligands of CD44 can account for the various effects observed on cell migration and implantation (1). The transcription factor cellular myelocytomatosis viral oncogene homolog (c-MYC) and the pro-survival protein, B-cell CLL/ lymphoma 2 (BCL-2) have been implicated in establishment of metastatic colonies by promoting cell survival and proliferation (3, 4) and inhibiting apoptosis of metastasizing cells (5), respectively. The ability of metastasizing cells to survive and grow at distal sites also depends on activation of autocrine stimulatory survival loops, as occurs upon stimulation of rat sarcoma (RAS) -extracellular signal regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) pathways (5, 6). Hence, a significant change in the primary tumour cell proteome must take place to enable a cell to acquire these additional features and overcome the multiple hurdles required for metastasis to distal sites.

Whereas the expression of many key gene products involved in the metastatic process appears to be a consequence of transcriptional activation (7), it is becoming clear that translation control also plays an important role in shaping the metastatic cell proteome. As such, a key regulatory step of protein synthesis is the recruitment of ribosomes to mRNA templates. This step not only commits the mRNA to protein synthesis but is also often thought to be rate-limiting for the process of translation. It is stimulated by eukaryotic initiation factor (eIF)4F, a heterotrimeric complex consisting of eIF4E, which binds the 5'-cap structure of mRNAs; eIF4A, an RNA-dependent ATPase thought to unwind local secondary structures; and eIF4G, a large scaffolding protein that recruits the 43S pre-initiation complex by interacting with ribosome-bound eIF3 (8).

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eIF4E is a convergence point of the PI3K/AKT/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signalling pathways (Figure 1). RAS activation ultimately leads to phosphorylation of mitogen-activated protein kinases MNK1 and MNK2, which in turn bind to eIF4G and phosphorylate eIF4E at Ser 209 (9). On the other hand, stimulation of the PI3K/AKT pathway leads to an mTOR-dependent phosphorylation of the negative regulators, eIF4E-binding proteins (4E-BP; there are three such proteins, of which the best characterized is 4E-BP1). Under normal cellular conditions, eIF4E is sequestered from the eIF4F complex *via* interaction with the 4E-BPs at a binding site similar to the one on eIF4G that interacts with eIF4E (8). Phosphorylation of the 4E-BPs causes their release from eIF4E, allowing eIF4E to bind to eIF4G and participate in the ribosome recruitment phase of translation initiation (10, 11).

The limited availability of eIF4E (0.3 copies/ribosome) is thought to render this step rate-limiting for translation initiation (12). In addition, there is a particular feature of this step that is generally not appreciated by individuals outside of the field of translation: mRNAs need to compete for access to the limiting supply of eIF4F and this sets up a situation where different mRNA transcripts are initiated at various rates. Features defining the competitive ability of an mRNA for the eIF4F complex include cap accessibility, 5' proximal secondary structure, and the presence of *trans*-acting proteins near the 5' end (13). Several mRNAs encoding proteins involved in survival, angiogenesis and growth are in fact poorly competitive transcripts and under conditions when eIF4E levels increase, their translation is preferentially stimulated (14).

A Role for Deregulated Translation Initiation in Transformation

Many lines of evidence support the idea that disruption of eIF4F activity has oncogenic consequences: i) Transformed cells exhibit higher rates of protein synthesis than normal cells (15); ii) ectopic overexpression of eIF4E in cell culture systems is oncogenic (16); iii) over-expression of eIF4E in the *Eμ-myc* mouse, a lymphoma model characterized by overexpression of the *c-Myc* oncogene in the B-cell lineage, accelerates tumourigenesis *in vivo* and modulates chemosensitivity – thus recapitulating key oncogenic functions of Akt and antagonizing the pro-apoptotic activity of c-Myc (17-19); iv) the tumour suppressor programmed cell death 4 (PDCD4) blocks eIF4F assembly by sequestering eIF4A from eIF4G to inhibit tumour growth (20); v) several studies targeting translation initiation as an anticancer approach have shown promising results *in vitro* and in pre-clinical models; these studies included the use of antisense oligonucleotides (ASOs) directed to eIF4E, small molecule inhibitors of eIF4E-eIF4G interaction and inhibitors of eIF4A function – several of which have shown efficacy in preclinical cancer models (21-25).

eIF4E was the first translation initiation factor found to be capable of inducing transformation in cultured fibroblasts and epithelial cells (16, 26). Subsequently, ectopic overexpression of eIF4G and some of the eIF3 subunits also revealed its ability to stimulate transformation (27, 28). Subsequent studies sought to explore eIF4F targeting activity as an approach for targeting tumour-specific vulnerability, betting on the dependency that tumour cells appear to have towards higher eIF4F activity (13). Overexpression or inhibition of 4E-BP1 phosphorylation has a negative impact on the transformation process. In breast cancer cells, overexpression of 4E-BP1 mutants (incapable of being phosphorylated by mTOR) was shown to inhibit cell proliferation and malignancy (29-31). In an ovarian cancer model, injection of 4E-BP-based peptides that block the interaction between eIF4E and eIF4G inhibited cap-dependent translation and decreased ascites and tumour growth with no observable toxic effect (32). The use of antisense RNA to target eIF4E in epithelial and fibroblast tumors led to the reduction of both tumour growth and metastatic burden, in concert with reductions in translation of eIF4E targets related to malignancy and metastasis: ornithine decarboxylase (ODC), VEGF, and fibroblast growth factor (FGF-2) (33-36).

One strategy that has advanced to clinical trials involved the use of second-generation ASOs (22). Targeting eIF4E with these ASOs led to just a 20% decrease in global translation, but a profound reduction in the levels of key-malignancy related gene products, including c-MYC, cyclin D1, VEGF, survivin, and BCL-2, indicating a selective translational effect on a specific subset of genes. Delivery of eIF4E ASOs to nude mice bearing tumours suppressed eIF4E levels in tumour tissues by ~50% and suppressed breast and prostate tumour growth due to inhibition of vascularisation and proliferation. These results seemed specific to tumour cells, with no adverse effects on normal tissues being noted, indicating that this approach may offer a manageable therapeutic index (22). The advantage of these second-generation ASOs is their ability to successfully reach tumour tissues in a dose-dependent manner and target eIF4E in tumour cells, as illustrated in recent Phase I human clinical trials (37). Promising, but not as advanced, are strategies aimed at targeting the eIF4A helicase component of eIF4F or the eIF4E:eIF4G interaction (13).

eIF4E and the Metastatic Process

Deregulated translation has also been implicated in the metastatic process. Firstly, perturbations of the mTOR signalling pathway have shown interesting results vis-à-vis effects on metastasis. Activation of mTOR in fibroblasts

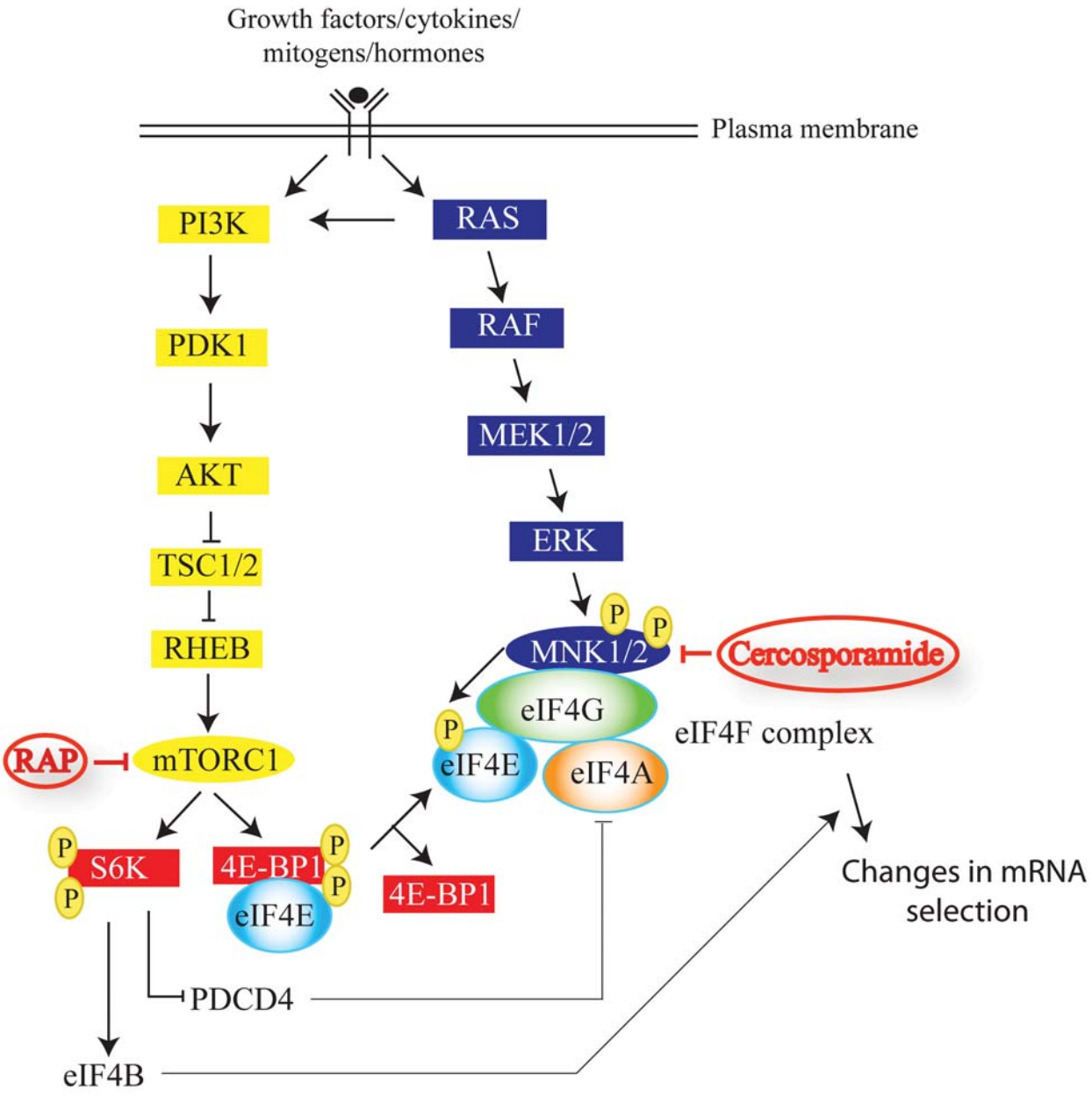

Figure 1. *Schematic representation of the PI3K/AKT/mTOR and RAS/MAPK pathways affecting eIF4F formation and activity. Both pathways are activated by extracellular stimuli, such as growth factors, mitogens, and hormones. Activated RAS ultimately leads to phosphorylation and activation of the mitogen-activated protein kinases, MNK1 and MNK2. MNK bound to eIF4G phosphorylates eIF4E in the eIF4F complex. PI3K activates AKT, which in turn ultimately leads to activation of the rapamycin-sensitive mTOR complex 1 (mTORC1). mTORC1 phosphorylation of 4E-BP1 releases eIF4E, allowing the latter to bind eIF4G and become assembled into the eIF4F complex. This leads to a selective increase in the translation of a subset of mRNAs. PDK1: phosphoinositide-dependent kinase 1; TSC1/2: tuberous sclerosis complex; Rheb: RAS homolog enriched in brain; RAP: rapamycin; S6K: S6 protein kinase; 4E-BP1: eIF4E-binding protein 1; P: phosphate; PDCD4: programmed cell death 4; RAF: rapidly accelerated fibrosarcoma; MEK1/2: map kinase 1 and 2; ERK: extracellular signal regulated kinase.*

leads to increased VEGF production that is reduced to normal levels by treatment with the specific mTOR inhibitor, rapamycin (38). In lymphatic endothelial cells, rapamycin inhibits VEGF expression, resulting in decreased invasion and migration (39). *In vivo*, immunosuppressive doses of rapamycin led to long-term anti-angiogenic effects and suppression of tumour growth and progression, a feature correlated with inhibition of VEGF secretion (40, 41). By

inhibiting secretion of VEGF, more precisely VEGF-C, rapamycin suppressed not only lymphangiogenesis, but also lymphatic metastasis (41). Therefore, it is important to assess the effects of more potent mTOR kinase inhibitors on the metastatic process.

eIF4E and 4E-BPs as biomarkers of cancer progression. eIF4E has been at the centre of several studies trying to elucidate its potential role in metastasis. Several studies have indicated that the eIF4E level can be used as a biomarker to distinguish between cancer having low *versus* high invasive and metastatic potential (42). In lung adenocarcinomas, overexpression of eIF4E was shown to be associated with poorer survival and higher incidence of haematogenous metastasis (43). In patients with triple negative breast cancer, high levels of eIF4E are associated with an unfavourable prognostic outcome (44). Additionally, in both node-positive and node-negative breast cancer, western blot analysis on patient tissues have shown that an elevated eIF4E level is an independent prognostic marker for disease recurrence and poorer outcome, independently of nodal status (45, 46). In angiosarcomas, components of the AKT/mTOR pathway can be used as a marker for disease due to the overexpression of p-AKT, p-4E-BP1, and eIF4E, as shown by immunohistological studies of tissues from patients (47). Although phosphorylated eIF4E levels are also elevated in human tumour tissues, there is no difference in p-eIF4E levels in tumours with or without lymph node metastases, and p-eIF4E expression has been reported to be higher in early-stage carcinomas compared to late-stage ones (48).

Not only are elevated levels of eIF4E associated with malignant progression, but phosphorylation of 4E-BP1 is also found to be highly related to disease progression in many settings, including prostate, breast and ovarian cancer, as well as rhabdomyosarcomas (49). The elevated level of 4E-BP1 phosphorylation is expected to result in increased eIF4E availability for the eIF4F complex (50). Immunohistochemical profiles of human epidermal growth factor receptor (HER)-2 positive and HER-2 negative breast cancer patients showed that the levels of p-4E-BP1 expression correlated with disease progression and recurrence, as well as lymph node metastasis, independently of Her-2 expression, making p-4E-BP1 a potential prognostic marker for disease outcome and grade in this setting (51). In esophageal cancer, p-4E-BP1 expression in patient tissues did not change with age, gender, or tumour grade, but phosphorylation of 4E-BP1 at Thr37/46 (8) was elevated in patients with early-stage carcinomas (52). These data indicate that eIF4E and its binding partner 4E-BP1 are potential markers for disease progression in different types of human cancer.

Translational deregulation by eIF4E in metastasis. The potential contribution of eIF4E to the metastatic phenotype was first observed in Ras-transformed cloned rat embryo fibroblast (CREF) cells (53, 54). In these CREF cells, overexpression of RAS resulted in increased levels of eIF4E phosphorylation, correlating with stimulation of protein synthesis rates and increased transformation and metastasis (54). Metastatic colonies were found to be selective for eIF4E-overexpressing cells compared to their non-metastatic counterparts. Alternatively, reducing eIF4E levels using antisense RNA in these cells reduced eIF4E expression by 60% and correlated with suppression of soft agar colonization and reduced pulmonary metastasis when cells were injected into the tail vein of nude mice (33). Interestingly, the pulmonary metastases that formed had escaped suppression by the antisense RNA and exhibited eIF4E levels comparable to those of vector control cells. These results are consistent with the notion that metastatic cells select for higher levels of eIF4E. The reduction of eIF4E upon suppression by antisense RNA also correlated with a decrease in polyamine transport, in the levels of ODC mRNA(36), and in metastasis-related proteins CD44v6 and MMP9, and surprisingly, with increased levels of metastasis-suppressor protein nucleoside diphosphate kinase A (NM23) (33). Interestingly, in metastatic lesions where eIF4E expression had escaped suppression by antisense RNA, the levels of ODC, CD44v6, MMP9, and NM23 proteins were restored to levels comparable to those of vector controls. The observation that eIF4E and NM23 expression levels were inversely related was also supported by another study in a highly metastatic human neuroblastoma cell line, where NM23 expression was reduced as eIF4E levels increased (55). eIF4E also exerts effects on the metastatic phenotype by regulating expression of VEGF and hence directly influencing angiogenesis, an event that predicts increased vascularity, dissemination and invasion (56, 57). Taken together, these data suggest that eIF4E suppression may contribute to the metastatic process by influencing expression of key mRNAs involved in this program (33).

These results were capitalized on for the development of a novel therapeutic approach by DeBenedetti and colleagues, who developed a suicide gene therapy strategy based on the discriminatory role that eIF4E plays in translation (58, 59). The strategy consisted of injecting metastatic breast cancer cells into the tail vein of mice and, upon formation of lung metastases, injecting mice with a plasmid encoding the herpes simplex virus (HSV) thymidine kinase (TK) gene under control of the FGF-2 5' untranslated region (UTR), an element that renders TK expression eIF4E dependent (60). Subsequent delivery of ganciclovir (which is converted into toxic dGTP analogue by HSV TK) to tumor-bearing mice led to a reduction in pulmonary burden by 90% in animals having received the FGF-2/TK chimeric expression plasmid, but not in animals with the TK expression vector lacking the FGF-2 5' UTR. Indeed, mice from this latter cohort exhibited

signs of systemic toxicity since HSV-TK was expressed in all tissues (58, 59). A similar suicide gene therapy approach based on adenovirus infection in a soft-tissue head and neck squamous cell carcinoma (SCC-7) metastasis model was developed by Li and colleagues (61). SCC-7 cells were infected with adenovirus-encoding HSV TK expression cassettes with TK mRNA translation under regulation of the FGF-2 5' UTR, and injected into mice. SCC-7 cells exhibiting elevated eIF4E expression had increased TK levels and were sensitive to ganciclovir. After treatment with ganciclovir, mice infected with FGF-2/TK adenovirus exhibited extended disease-free survival periods compared to control mice, illustrating that this suicide gene therapy can prolong survival through selective killing of eIF4E-overexpressing cancer cells (61).

More recent experiments on the effects of soy isoflavones on cancer and metastasis in MDA-MB-435 breast cancer cell lines injected into fat pads of nude mice led to a higher incidence in metastasis and this was associated with increased eIF4E levels (62). An independent study, also using MDA-MB-435 cells, showed that expression of an eIF4E mutant impaired for mRNA 5' cap binding, reduced VEGF expression and both cell growth and metastasis (63). Moreover, in a microarray study on six different human metastatic cell lines (64), eIF4E and three other proteins, desmoyokin, septin-9 and S100 calcium binding protein A11, were identified as being involved in protrusion of cell pseudopodia, an event dependent on actin and essential for epithelial to mesenchymal transitions (EMT) leading to cell migration and invasion. Knockdown of eIF4E in these metastatic cells induced mesenchymal to epithelial transition (MET) and inhibited metastasis. Another microarray study on late-stage colorectal cancer (65), analyzing the transition from invasive carcinoma to metastasis, indicated that expression of mRNAs with longer, more structured 5'UTRs are more likely altered at the translational than at the transcriptional level, in concert with elevated p-4E-BP1 levels, events that would be consistent with increased eIF4E availability and initiation rates. Taken together, these studies show a correlation between elevated eIF4E expression, increased translation of an mRNA subset whose products are involved in metastasis, and progression to the metastatic phenotype.

As mentioned earlier, eIF4E is phosphorylated at Ser 209 by MNK1 and MNK2, an event necessary for the oncogenic properties of eIF4E (66). The regulation of eIF4E phosphorylation by the MAPK pathway indicates that small molecule inhibitors of this pathway might impede the effect of eIF4E on tumour progression as well as on metastasis. Indeed, pharmacological inhibition of MNK1 and MNK2 by cercosporamide (Figure 1) blocks eIF4E phosphorylation and leads to the inhibition of lung metastasis in a B16 melanoma cell model (67). Cercosporamide was also shown to suppress growth of xenograft tumors from HCT116 colon carcinoma with no reported toxicity on the host (67). These data demonstrate the importance of eIF4E phosphorylation in the establishment of metastasis and suggest that the effects of cercosporamide are selective on tumour and metastatic cells, marking it as a potential avenue to explore for cancer therapy.

We have shown the contribution of eIF4E to metastatic progression in a breast cancer model. Using short hairpin (sh)RNAs targeting eIF4E in TM15 cells, a highly metastatic breast cancer cell line, we showed that suppression of eIF4E leads to a decrease in both the invasion and migration of these cells *in vitro* (68). Polysome profiling and quantitative real time (qRT)-polymerase chain reaction (PCR) experiments of metastasis-related mRNAs VEGF, MMP9 and cyclin D1, showed a decrease in the translation of these key transcripts when eIF4E was suppressed in TM15 cells. These results were recapitulated in the highly metastatic human breast cancer cell line MDA-MB-231, where eIF4E suppression led to a significant decrease in the ability of these cells to migrate and invade *in vitro*. As predicted, overexpression of eIF4E in the low metastatic cell line MDA-MB-468 increased the migration and invasion ability of these cells *in vitro* (68). *In vivo*, TM15 cells injected into the fat pads of nude mice exhibited a significant decrease in tumour growth and pulmonary metastasis when eIF4E was suppressed. To assess the direct contribution of eIF4E to the metastatic process, these cells were also injected into the tail vein of nude mice and the pulmonary metastasis burden assessed at different time points. eIF4E suppression led to a diminished ability of these cells to colonize the lungs (68). Taken together, these findings support the idea that eIF4E contributes to metastatic progression through the regulation of translation of key metastasis-related genes.

As indicated, MYC contributes to the metastatic phenotype by stimulating proliferation and cell survival. Moreover, MYC directly regulates several genes implicated in specific steps of the metastatic process. These include the regulation of transforming growth factor (TGF)β-mediated zinc finger SNAIL transcription factor activation, an event necessary for EMT, stimulating migration and invasion (69). MYC also regulates the expression of osteopontin, hence playing a role in cell-cell and cell-ECM interactions (70). A recent study by Wolfer *et al*. showed that MYC coordinately regulates 13 different multigene expression signatures of poor prognosis in cancer, identified by microarray profiling (71). They also showed that knockdown of MYC by small-interfering (si)RNAs in MDA-MB-231 cells inhibits migration, invasion and metastasis in experimental xenografts (71). These results indicate that MYC appears to be required for the invasion and metastasis of cancer cells in a manner that is different from its effects on cell proliferation and survival. However, it remains to be established which effectors of MYC are required for its role in metastasis (71, 72). Recently, we defined eIF4F as a key MYC client required for lymphoma initiation. Given the

above noted role of eIF4E in supporting metastasis, it will be interesting to assess whether the MYC-eIF4E relationship is also essential in driving the metastatic process (73, 74).

Conclusion

EIF4E is considered a central regulatory node for translational control whose activity is under the regulation of both the PI3K/AKT/mTOR and MAPK pathways. Signals that stimulate both pathways affect eIF4E availability and activity, leading to de-regulated expression of a subset of targets, some of which have been implicated in tumour progression and metastasis. Many of these signals cooperate to help establish a metastatic signature that characterizes this process. Several lines of experimentation have shown that eIF4E suppression selectively impairs proliferation and survival of tumour cells with minimal toxicity on normal cells (22, 74). It will be important to assess the value of targeting eIF4E and the eIF4F complex in various metastatic models in order to assess the potential therapeutic benefit, not only in curtailing tumour cell maintenance, but also in blocking the metastatic gene expression program.

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