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TARGETING THE eIF4F TRANSLATION INITIATION COMPLEX: A CRITICAL NEXUS FOR CANCER DEVELOPMENT

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Abstract

Elevated protein synthesis is an important feature of many cancer cells and often arises as a consequence of increased signaling flux channeled to eukaryotic initiation factor (eIF) 4F, the key regulator of the mRNA-ribosome recruitment phase of translation initiation. In many cellular and pre-clinical models of cancer, eIF4F deregulation results in changes in translational efficiency of specific mRNA classes. Importantly, many of these mRNAs code for proteins that potently regulate critical cellular processes such as cell growth and proliferation, enhanced cell survival, and cell migration that ultimately impinge on several hallmarks of cancer, including increased angiogenesis, deregulated growth control, enhanced cellular survival, epithelial-to-mesenchymal transition, invasion and metastasis. By being positioned as the molecular nexus downstream of key oncogenic signaling pathways (e.g. Ras, PI3K/AKT/TOR, and Myc), eIF4F serves as a direct link between important steps in cancer development and translation initiation. Identification of mRNAs particularly responsive to elevated eIF4F activity that typifies tumorigenesis underscores the critical role of eIF4F in cancer and raises the exciting possibility of developing new-in-class small molecules targeting translation initiation as anti-neoplastic agents.

The Eukaryotic Initiation Factor 4F Complex

There have been tomes written on the subject of translational control under normal and pathophysiological conditions (1). In this review, we focus on the role that the "cap binding complex", eukaryotic initiation factor (eIF) 4F, plays in mRNA discrimination and in

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driving tumorigenesis. We also discuss strategies aimed at therapeutically inhibiting eIF4F activity.

A salient hallmark of eukaryotic cytoplasmic, non-organellar, mRNAs is the 5′ terminal cap, a structure added to nascent mRNA templates shortly after initiation of transcription. Although the cap has been implicated in several nuclear events (splicing, polyadenylation, and nuclear/cytoplasmic transport) and plays a protective role against 5′ exonucleolytic degradation, its best documented function is in facilitating the recruitment of 43S preinitiation complexes to mRNA templates (2). Initial pioneering studies elucidating a role for the cap in translation uncovered an important conceptual point – *in vitro* its presence is facilitative in nature but *in vivo* it is absolutely essential (3).

A second important finding that emerged from these early experiments was the existence of an inverse relationship between secondary structure within the 5′ untranslated region (UTR) of mRNAs and translational efficiency. This link was deduced from experiments reporting on the translational efficiency of mRNAs with differing secondary structure, on the ATP requirement of initiation factors involved in cap recognition, and the varying degree of inhibition by cap analogues on initiation of mRNAs with differing secondary structure (4–9). An understanding of the basis of this relationship was afforded when the cytoplasmic mammalian cap binding protein eIF4E was identified and purified (10) and shown capable of stimulating translation of capped mRNA in HeLa cell extracts (11). eIF4E was subsequently found to be a component of the hetero-trimeric eIF4F complex which also contains a large ~220 kDa scaffolding protein (eIF4G) and the ATP-dependent RNA helicase, eIF4A (12).

A Molecular Commitment – Recruiting the Ribosome to the mRNA

Cap-Dependent Ribosome Recruitment

eIF4E is the least abundant of the initiation factors, present at 0.2–0.3 molecules/ribosome in reticulocytes and HeLa cells, rendering it rate-limiting for translation (13, 14). However, whether eIF4E levels are limiting at the organismal level across all cell types and cancer cells *in vivo* remains an outstanding question. In contrast to eIF4E, eIF4A is the most abundant initiation factor – present at ~3–6 molecules/ribosome and is solely cytoplasmic (13, 14). In mammals, there exist two highly related eIF4A homologs; eIF4AI (DDX2A) and eIF4AII (DDX2B) (the human proteins are 90% identical) (15, 16), with eIF4AI generally being the more abundantly expressed (14, 17). The majority (~90%) of eIF4A exists as a free form (eIF4A$_f$) whilst a small proportion is present as an eIF4F subunit (eIF4A$_c$) (18–20). There are also two homologs of eIF4G, eIF4GI and eIF4GII, that share 46% identity, with eIF4GI being more abundant (21). eIF4G interacts with eIF4E and eIF4A through defined domains and provides the scaffold upon which other factors important for the initiation process assemble (22). In mammals there are two separate eIF4A interacting domains on eIF4G and it is generally thought that the two domains interact with different regions of the same eIF4A molecule (23). Given that eIF4AI and eIF4AII are interchangeable in the eIF4F complex (16) it would appear that mammalian cells can generate four different eIF4F complexes, the functional consequences of which remain unknown.

Although the involvement of eIF4B and eIF4H in translation initiation is well established, their precise roles need to be better characterized. eIF4B and eIF4H are RNA binding proteins that stimulate eIF4A helicase activity, enabling eIF4A to unwind more stable duplexes (24–27). Their interaction with eIF4A is mutually exclusive, as the two proteins share a common binding site (28). eIF4B and eIF4H modulate the affinity of eIF4A for ATP or ADP (29, 30) and RNA (31) with the interaction of eIF4B near the 5′ cap structure being ATP (and presumably eIF4A)-dependent (6), and inhibited by secondary structure (7). Through their RNA-recognition motifs, eIF4B and eIF4H may also stabilize single-stranded regions in the 5′UTR to prevent re-annealing following unwinding by eIF4A (Fig. 1). eIF4B is obligatory for 48S initiation complex formation on mRNAs possessing even modest levels of 5′ UTR complexity (32) and its depletion results in reduced proliferation rates, cell survival and enhanced sensitivity to camptothecin-induced cell death (33). These results implicate eIF4B function in controlling the translation of mRNAs critical for cell proliferation and survival.

mRNA Discrimination

eIF4F is the long-sought discriminatory factor responsible for differences in translation rates among many mRNAs. Two criteria are required for this to hold true – firstly, eIF4F had to be limiting (see above) and different mRNAs had to exhibit distinct affinities (or requirements) for recruitment of eIF4F – a feature that was linked to the degree of mRNA 5′ UTR secondary structure (34, 35). Consistent with eIF4F discriminating between different mRNAs based on secondary structure, initiation on simple, unstructured model mRNAs (containing $[CAA]_n$ as 5′ UTR) does not require eIF4F, the cap, or ATP (36). As well, a dominant-negative mutant of eIF4A capable of inhibiting eIF4F activity exhibits less potent inhibition towards translation of mRNAs with a lower degree of secondary structure compared to transcripts harboring more structure (37).

How the location of secondary structure within the 5′ UTR determines whether a particular transcript will be a "weak" or "strong" competitor is not well defined, but the net consequences may reflect the accumulated effects on various steps of the ribosome recruitment process. Binding of eIF4E to the cap structure is the first step in loading of the 43S pre-initiation complex onto the mRNA– an interaction mediated by base stacking of the positively charged N-7 methylguanosine with two tryptophans (W56 and W102) and auxiliary interactions (38–40). Three RNA-binding sites on eIF4G, necessary for efficient translation initiation, stabilize this interaction (41–43) (Fig. 1). Structure proximal to the cap (secondary structure or protein-mRNA interactions) can influence the interaction between eIF4E and mRNA in a negative manner (44–48). On the other hand, structure located distal from the cap exerts little effect on eIF4E-cap interaction but can interfere with eIF4F mediated unwinding - inhibiting the interaction of eIF4A and/or eIF4B with mRNA (7, 49). Since $eIF4A_f$ has bi-directional helicase activity, its delivery to the 5′ end of the mRNA provides forced directionality to this enzyme. Although $eIF4A_f$ has weak helicase *in vitro* (24, 50), its presence in the eIF4F complex leads to a ~20-fold increase in helicase activity (24, 51, 52). This has been attributed to eIF4E since its binding to eIF4G masks an eIF4A autoinhibitory domain on eIF4G (53). Hence, eIF4E is capable of promoting the helicase activity of eIF4A and increasing translation rates by a mechanism distinct from its cap-

binding function. The overabundance of eIF4A relative to eIF4E (13, 54), the elevated helicase activity of eIF4A$_c$ relative to eIF4A$_f$ (24, 51, 52), and the ability of eIF4A$_f$ to exchange with eIF4A$_c$ (16) has led to the proposal that eIF4A recycles through the eIF4F complex during initiation (55) (Fig. 1). The idea that eIF4A/eIF4B/eIF4H may polymerize on the mRNA is consistent with the observed cap-dependent crosslinking of these factors downstream of the cap (56). As noted by Kapp and Lorsch (22), such a polymerization model is also consistent with the kinetics of unwinding at low concentrations of eIF4A where a lag in activity is often observed.

One additional interaction that impacts translation initiation, and where 5′ UTR may also exert a negative effect, is between eIF4G and the poly(A) binding protein, PABP (57). This interaction is thought to circularize the mRNA (57) and has been associated with stimulation of 48S pre-initiation complex formation (58, 59), 60S subunit joining (59), increased eIF4F cap-binding and ATPase activity (60–63), and stabilization of eIF4F-mRNA interactions *in vivo* (64). It is not known whether eIF4E needs to remain bound to the mRNA or is released and available for formation of new eIF4F complexes (Fig. 1). Consistent with the latter possibility is the finding that the addition of cap analogues to cell extracts after commencement of translation fails to block cap-dependent translation (65). Whereas the PABP-eIF4G interaction is stimulatory but dispensable in yeast cells (64, 66), it appears critical for translational control of maternal mRNAs during Xenopus development (67). Whether mRNAs with elevated structural barriers are less efficiently circularized or require *de novo* cap-recognition by eIF4E at every translational attempt is unknown, but could be an additional step that renders "weaker" mRNAs at a disadvantage over their more robust counterparts. Collectively, these studies support the notion that not all mRNAs are equally "translatable" and that the successful translation of certain mRNAs (i.e. "weak" mRNAs) is dictated by multiple and cumulative properties mostly associated with that mRNA's 5′UTR. Importantly, translation of these "weak" mRNAs, which typically encode for the potent growth and survival factors that drive the hallmarks of cancer, is suppressed except under conditions of enhanced eIF4F activity, that is during malignant progression.

Elevated eIF4F activity selectively upregulates translation of a subset of mRNAs

eIF4E over-expression in experimental cell models elicits only small increases in overall protein synthesis rates while enabling a substantial, disproportionate and selective increase in translation of a subset of mRNAs. Early attempts at identifying these eIF4E-responsive mRNAs focused on examining candidates on a one-by-one basis. These studies revealed that the production of housekeeping proteins (e.g. actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] was not altered by changes in eIF4E levels, but that the translation of mRNAs harboring long, highly-structured 5′UTRs (e.g. ODC, c-Myc) was profoundly impacted by fluctuations in eIF4E levels (68–70). Subsequent genome-wide analyses of changes in translation revealed that 5′ UTR structure was one determinant of eIF4E or eIF4F sensitivity, but also uncovered a more complex situation in that eIF4E responsive mRNAs did not always appear to possess complex 5′ UTRs. Inhibition of mTOR activity (and therefore eIF4F assembly) by rapamycin (for 4 hrs) in Jurkat T cells, followed by polysome profiling, revealed that the translation of only ~6% of expressed genes was inhibited (71). Similarly, polysome analyses of NIH 3T3 cells transiently overexpressing

eIF4E revealed that mRNAs encoding ribosomal proteins were prominently eIF4E-responsive (72). Interestingly, some of these mRNAs contain a distinct element in their 5′ UTR known as a 5′ TOP (5′ terminal oligopyrimidine tract) and recent evidence suggests that the RNA binding protein, LARP1, modulates the translation of this class of mRNAs (73). The relationship between LARP1 and eIF4E responsiveness remains to be fully explored.

More recently, these finding have been corroborated by ribosome profiling analyses, a methodology that relies on deep sequencing of ribosome-protected mRNAs to quantify the translation of mRNAs on a codon-by-codon resolution (74, 75). These studies further reveal a new regulatory element termed the PRTE (pyrimidine-rich translation element), which interfaces with eIF4E activity and is essential to control the translation of a subset of mRNAs encoding key proteins involved in critical steps of cancer initiation and progression (74). However, the mechanism by which PRTE elements act to cooperate with eIF4E to regulate the translation of specific mRNAs is still under investigation. Additional global approaches probing changes in mRNA distribution in polysomes as a consequence of altered eIF4F levels or activity revealed profound effects on the translation of mRNAs implicated in immune response, cell cycle progression, and metabolism (76, 77). Similar to increased eIF4E expression, eIF4E phosphorylation does not significantly increase global protein synthesis but stimulates the translation of pro-survival (e.g.- MCL) and pro-invasion mRNAs (e.g. MMP3 and Snail) (78–80). Since the cellular translatome at any given time is affected by upstream regulatory processes, relative mRNA abundance, and mRNA complexity the subset of mRNAs responsive to eIF4F can be expected to vary across tumor types and in response to different physiological stimuli.

Critical oncogenic signaling pathways converge to regulate eIF4F assembly and activity

Feed-forward loops drive malignancy - MYC and eIF4F

The MYC transcription factor is one of the most frequently activated oncogenes in human cancers (155) and has been repeatedly associated with poor prognosis and decreased patient survival (156–158). A major consequence of elevated MYC activity is a dramatic elevation in protein synthesis due to increased transcription of ribosomal RNA (rRNA) genes and genes required for rRNA processing and assembly. This increase in translational output is a critical determinant for MYC oncogenic activity *in vivo* (81). Importantly, elevated MYC also increases transcription of the core eIF4F components - eIF4E, eIF4AI, and eIF4GI, (but not eIF4AII or eIF4GII) (159–161). Indeed, MYC levels are elevated in pre-lymphomatous, pre-B, and pro-B cells isolated from Eμ-Myc mice, and engender an increase in eIF4F levels (162). MYC, in turn, is one of the best characterized translationally-controlled mRNAs. Hence, MYC and eIF4F work in a feed-forward loop, each enhancing the expression of one another. Indeed, elevated eIF4E consequent to MYC activity has been deemed necessary to suppress apoptosis resulting from aberrant MYC activity (169–171). This intimate relationship affords an opportunity by which part of MYC's function can be interdicted by inhibiting eIF4F activity (162, 163).

mTOR - Regulating eIF4F assembly and activity

The assembly of the eIF4F complex is under the control of the mammalian target of rapamycin (mTOR), a serine/threonine kinase whose activity is perturbed in many human cancers (82). mTOR exists in two functionally distinct complexes. mTORC2 regulates cytoskeletal reorganization and cell survival, whereas mTORC1 controls translation initiation, ribosome synthesis, expression of metabolism-related genes and autophagy (82). mTORC1 controls eIF4F assembly by liberating both eIF4E and eIF4A from their respective inhibitory binding proteins, eIF4E binding proteins (4E-BPs) and programmed cell death 4 (PDCD4). 4E-BPs (there are three highly related proteins in mammals with the most well characterized being 4E-BP1) compete with eIF4G for a binding site on eIF4E (83), an interaction that is regulated by mTORC1-dependent phosphorylation of 4E-BP. Hypophosphorylated 4E-BP binds to eIF4E with high affinity (nanomolar range) and inhibits translation initiation, whereas hyperphosphorylated 4E-BP does not bind eIF4E, allowing eIF4F complexes to form (83).

Similarly, eIF4A availability is regulated downstream of mTORC1 by the tumor suppressor gene product PDCD4 (84–87). PDCD4 binds to eIF4A, inhibiting its helicase activity and preventing its binding to eIF4G. PDCD4 is regulated by S6 kinase which, when activated by mTORC1, phosphorylates PDCD4, marking PDCD4 for ubiquitin-mediated degradation which then leads to liberation of eIF4A and eIF4F assembly (84, 88).

As noted above, the role of eIF4B is critical to the overall helicase activity of eIF4A within the eIF4F complex. eIF4B activity is also controlled downstream of mTOR and Ras-ERK pathway signaling by p90 ribosomal S6 kinase (RSK) and p70 ribosomal S6 kinase (S6K) (89–92). Phosphorylation of eIF4B on Ser422 increases its interaction with eIF4A and eIF3 and is required for ribosome recruitment to mRNAs containing secondary structure (89–91). Moreover, recombinant eIF4B, which is presumably not phosphorylated, cannot substitute for native eIF4B in this assay. Hence, the phosphorylation status of eIF4B affects mRNA discrimination, possibly by influencing eIF4A activity.

Regulation by phosphorylation - Ras and MAPK signaling converge on eIF4F

Increased signaling flux through the Ras-Raf-Erk pathway is a frequent occurrence in many human cancer types often consequent to mutational activation of Ras or Raf oncogenes or activation of upstream receptor tyrosine kinases (e.g. EGFR). Constitutive activation of this pathway enhances the assembly and activity of the eIF4F complex in multiple ways. Activation of Erk- and p90RSK can phosphorylate TSC2 activating mTORC1, which would promote eIF4F assembly (93). Ras pathway signaling also directly impinges upon eIF4F activity. Early studies by Rinker-Schaeffer et al. ((94) revealed that oncogenic Ras dramatically increased the rate of eIF4E phosphorylation. Subsequent work has now shown that ras pathway signaling through ERK stimulates the MNK kinases to interact with eIF4G and phosphorylate eIF4E at serine 209 (95–99).

What are the consequences of eIF4E phosphorylation? At this point, we do not have a complete picture. Mouse knockout studies have revealed that MNK1 and MNK2 are dispensable for normal development (100) and eIF4E$^{S209A/S209A}$ knock-in mice display no

obvious phenotype (79). Several studies have documented increased translation as a consequence of eIF4E phosphorylation (79, 83, 96), yet phosphorylation of eIF4E lowers its affinity for the cap structure (101–104). This conundrum can be explained if one posits that the reduced cap affinity is associated with increased recycling of eIF4E once functional 43S pre-initiation complexes have been successfully recruited to the 5′ end (105). Regardless, the phosphorylation of eIF4E at serine 209 has been shown in multiple reports to be absolutely critical to the oncogenic function of eIF4E (78, 79, 106).

Elevated eIF4F function drives cellular transformation, tumorigenesis and malignant progression

eIF4E in cellular transformation and tumorigenesis

The landmark study by Lazaris-Karatzas et al. (107) was the first to demonstrate that ectopic over-expression of eIF4E was oncogenic in NIH-3T3 cells, driving cellular transformation (focus formation, soft agar colonization) and tumorigenesis. Further highlighting the importance of eIF4E function in cellular transformation and tumorigenesis, engineered reduction of eIF4E consequent to expression of antisense eIF4E RNA profoundly suppressed the transformed phenotype of highly aggressive, Ras oncogene-transformed fibrosarcomas, reducing soft agar colonization more than 90%, increasing tumor latency periods and reducing tumor growth rates (108). Furthermore, development of an eIF4E transgenic mouse established eIF4E as a *bona fide* oncogene *in vivo*. Indeed, *in vivo* constitutive overexpression of eIF4E alone leads to increased cancer susceptibility, demonstrated by the wide spectrum of tumors that develop in these mice, including lymphomas, angiosarcomas, lung carcinomas, and hepatomas (109). As well, *in vivo* overexpression of eIF4E within the B cell compartment cooperates with c-Myc in lymphomagenesis as eIF4E counteracts MYC-induced apoptosis, a critical barrier to tumor formation (110). These and other studies showed that elevated levels of eIF4E can recapitulate key oncogenic functions of Akt (110) and antagonize the pro-apoptotic activity of c-Myc (110–112).

The phosphorylation of eIF4E at Ser209 also plays an important role in eIF4E's oncogenic function (106). In MYC-driven lymphomas, eIF4E expression accelerates lymphomagenesis whereas overexpression of an eIF4E S209A mutant is incapable of accelerating disease in this model (78). Similarly, in a PTEN knockout model of prostate cancer, knock-in of the same non-phosphorylatable eIF4E S209A mutant delays progression of prostate cancer (79). Consistent with these data, inhibition of MNK activity delays tumor development and outgrowth of metastases (113, 114). Mice homozygously deleted for both MNK1 and MNK2 are viable (100) making the targeting of this activity attractive for the development of novel anti-neoplastic agents. Increased Ras signaling (115) or altered eIF4E phosphorylation (79, 80) enhances the translation of a subset of mRNAs, with some encoding functions in cell growth, proliferation, and metastasis (80).

The prevailing data from these studies indicate that the oncogenic effects of eIF4E are a result of activated eIF4F rather than a unique function of eIF4E overexpression (116, 117). Early work showed eIF4E-mediated transformation to be particularly dependent on the

enhanced translation of ornithine decarboxylase (ODC) and cyclin D1 (68, 118–120). Subsequent work has continued to highlight the role that eIF4E plays in driving cellular transformation - selectively enhancing the translation of a limited pool of mRNAs whose protein products play an integral role in malignancy - c-MYC, cyclin D1, ODC, VEGF, among others (121). Importantly, a few common themes have consistently emerged from this body of work. Chief amongst these is that very modest changes in expression of eIF4E are required to affect malignancy. Indeed, only 2–3 fold increases in eIF4E are sufficient to drive cellular transformation whereas only a 50–60% reduction in eIF4E expression is necessary to block tumor formation and growth (108, 109, 119, 122–124).

eIF4E as a central regulator of metastatic progression

Beyond the initial changes that enable cellular transformation and expansion of a primary tumor, metastatic progression requires that tumors acquire a wide range of phenotypic characteristics, including the ability to establish autocrine growth and survival signaling, escape from the primary tumor site, invade surrounding normal tissue, disseminate through the blood or lymphatic circulation, establish and survive within the foreign microenvironment of the distal tissue site and outgrow as a metastatic colony. Though diverse stimuli that regulate the transcription of the critical gene products that drive these phenotypes, the synthesis of these proteins is coordinately elevated by eIF4E and eIF4F (125, 126).

The first evidence supporting a role for eIF4E in the metastatic process was revealed by antisense RNA studies in highly aggressive ras-transformed fibroblasts. Depletion of eIF4E by ~50% in these cells reduced the number of metastases formed in the lungs of mice following tail vein injection by up to 90% (123). Moreover, when implanted under the renal capsule, these same cells failed to invade the kidney parenchyma. Importantly, when metastases derived from these cells were examined, levels of eIF4E had been restored to that of the parental Ras-transformed cells indicating a selection *in vivo* for enhanced eIF4E function (123). Similarly, in breast cancer models, pharmacologic suppression of mTOR activity, which limits eIF4E availability and reduces eIF4F complex levels, diminished invasiveness and migration, as well as the formation of pulmonary metastases, thus delaying breast cancer progression (127).

Early overexpression studies have also implicated elevated eIF4E levels in driving not only tumorigenesis but also metastasis. Rat embryo fibroblasts engineered to overexpress eIF4E formed lung metastases both spontaneously after subcutaneous tumor growth, and experimentally following tail vein injection (123). Moreover, cells derived from these tumors and lung nodules showed elevated eIF4E expression levels, again indicating a selection for enhanced eIF4E function with malignant progression *in vivo* (123). Collectively, these data strongly implicate eIF4E, and by extension eIF4F, as a critical driver of metastatic progression.

To survive and grow in the primary tumor site, and especially within the foreign microenvironment of the metastatic site, tumor cells acquire growth factor autonomy, often through the establishment of autocrine growth factor networks. In the rat embryo fibroblast model, eIF4E overexpression enabled the establishment of an autocrine stimulatory loop

involving enhanced signaling through the ERK pathway (125, 128). Importantly, the progressive selection *in vivo* for more aggressive tumor behavior (reduced tumor latency, enhanced metastatic potential, reduced mouse survival) selected for increased eIF4E expression as well as enhanced signaling through the ERK pathway (125, 128). In the NIH3T3 model, eIF4E overexpression was shown to drive tumorigenesis via the establishment of a Ras-dependent autocine loop (107).

Collectively, these studies indicate that eIF4E plays a key regulatory role in metastasis. Another aspect of metastatic progression is the ability of tumor cells to establish a new vascular network. In experimental models of breast and head and neck cancers, eIF4E overexpression was shown to promote the overexpression of the potent angiogenic factors VEGF (129) and FGF2 (130), in both cases by selectively enhancing translation of these mRNAs. Immunohistochemical surveys of both breast and head and neck cancers have further supported the link between eIF4E overexpression, VEGF overexpression and enhanced microvessel density (131, 132). These data suggest that eIF4E may indirectly govern tumor-related angiogenesis by enabling the enhanced translation of critical angiogenic factors from the tumor cell compartment. Indeed, treatment of nude mice bearing human breast or prostate cancer xenografts with an antisense oligonucleotide targeting eIF4E effectively reduced eIF4E expression in these tumors and profoundly reduced tumor vascularity. Importantly, in *in vitro* cord formation assays, depletion of eIF4E by antisense oligonucleotide transfection suppressed the ability of endothelial cells to form vessel-like structures suggesting for the first time that eIF4E may also directly govern the response of endothelial cells to angiogenic stimuli (124). Similar results were observed when the eIF4A helicase subunit of eIF4F was targeted with the small molecule inhibitor, silvestrol (133).

In addition to the establishment of a vascular network, metastatic progression requires persistent cellular survival, not only at the primary tumor site but also within the foreign microenvironment of a metastatic site. Numerous studies have linked enhanced eIF4E expression to the suppression of apoptosis. The earliest demonstration of this was that eIF4E upregulation was necessary in MYC-induced malignancies to overcome MYC-induced apoptosis (111). The mechanism could be explained by enhanced synthesis of BCL-X_L and blockade of mitochondrial cytochrome C release (134). Subsequent work showed that eIF4E regulated the translation of a network of mRNAs encoding anti-apoptotic proteins (135). Most prominent amongst these was osteopontin (135), a protein that has been repeatedly implicated in metastasis (136). Additional work also highlighted the translational regulation of additional anti-apoptotic proteins by eIF4E including BI-1, dad1 and survivin (72) as well as BCL-2 (124, 137, 138). In the Eμ-myc B cell lymphoma model, enforced expression of eIF4E clearly promoted tumor cell survival and chemoresistance (122), in part by upregulating translation of the anti-apoptotic protein Mcl-1 (78).

The ability of tumor cells to break from the primary tumor mass, invade surrounding normal tissues and ultimately disseminate to distal tissue sites requires the remodeling and degradation of the extracellular matrix. This process is driven by expression and secretion of protein-degrading enzymes, most notably the Matrix Metalloproteases (MMP). In Ras-transformed rat embryo fibroblasts, reduction of eIF4E levels by only ~50% resulted in a remarkable reduction in the expression of MMP-9, concomitant with a marked diminution in

invasiveness. Interestingly, the cells selected for increased aggressiveness *in vivo* regained eIF4E levels as well as MMP-9 expression levels (123). Similarly, in murine prostate carcinoma cells, MMP-9 is translationally controlled and associated with increased malignancy (139). More recently, in a murine model of prostate carcinoma progression, knock-in of the non-phosphorylatable eIF4E (S209A) mutant allele suppressed disease progression and specifically reduced the translation of a subset of mRNAs critical for progression including MMP-9 and MMP-3 (79). As well, heparanase production, an enzyme implicated in the metastatic process and angiogenesis due to degradation of heparin sulfate proteoglycans with subsequent destruction of the basement membrane, is eIF4E responsive and is decreased when eIF4E levels are reduced by antisense oligonucleotides (140). Further highlighting a role for eIF4E in tumor cell invasiveness, Robichaud et al (2014) have shown that eIF4E Ser209 phosphorylation plays a prominent role in regulating the TGFβ-induced epithelial-to-mesenchymal (EMT) transition by controlling the translation of a pool of mRNAs critical for EMT, including Snail and MMP-3 (80). Consistent with these genetic studies, pharmacologic inhibition of eIF4E phosphorylation profoundly suppressed the outgrowth of lung metastases in the B16 melanoma model (114).

Studies employing ribosome profiling have revealed that oncogenic eIF4E activity, downstream of mTOR signaling, has a striking effect on the translational landscape of the cancer genome, particularly in the context of metastasis (74). This study has functionally characterized a novel subset of translationally regulated mRNAs associated with cancer cell invasion and metastasis *in vivo.* These mRNAs include vimentin, MTA1 (metastasis associated 1), CD44, and YB-1 (Y-box binding protein 1; also called YBX1) and have critical roles in controlling cell migration, metastasis and EMT (141). Mechanistically, eIF4E regulates the translation of these mRNAs at least in part through the PRTE, a regulatory element that is present in their 5′ UTRs. Significantly, INK128, a clinical ATP-site inhibitor of mTOR, blocks the increased translation of these eIF4E sensitive mRNAs with therapeutic benefit at all stages of prostate cancer progression, including metastasis (74). Elevated expression of eIF4E is common in a wide array of human cancers (colorectal, breast, prostate, lymphoma) (69). Importantly, in many studies, elevated eIF4E expression has been linked to advanced disease and/or reduced survival (69, 142, 143). Recent work has now also shown that 4E-BP1 is hyperphosphorylated in human cancers (notably ovarian and prostate carcinomas) and also associated with reduced patient survival (69, 137, 142–144).

eIF4E and chemoresponsiveness

Altered eIF4E levels modify tumor cell drug sensitivity. Increased eIF4E levels have been associated with resistance to front line therapy (e.g. – doxorubicin) (122) and rapamycin (145) in the Eμ-Myc lymphoma model (122). Elevated eIF4E levels are also associated with resistance to PI3K/mTOR kinase inhibitors (146, 147). In a report documenting resistance to anti-BRAF and anti-MEK therapies, eIF4F levels correlated with drug response with increased levels associated with diminished efficacy (148). Increased eIF4E phosphorylation has been associated with expression of BRAFV600E in melanocytes (149). Targeting the eIF4E:eIF4G interaction or eIF4A activity synergizes with anti-BRAF therapy (148). A recent shRNA screen targeting the translatome in multiple myeloma identified all three subunits of eIF4F (eIF4E, eIF4AI, eIF4GI) as modifiers of dexamethasone response in this

tumor type. Inhibition of eIF4F by small molecules synergized with dexamethasone as well as resensitized previously unresponsive cells (150). It will be important to identify the translational landscape responsible for these effects so as to obtain mechanistic insight and to distinguish between effects due to synergy versus resensitization of a previously resistant phenotype.

Breaking Bad Addictions - Targeting eIF4F

Over the last 10 years there has been significant interest in targeting the activity of the eIF4F complex. The knowledge that eIF4F assembly is under mTOR control was the first stepping stone towards this. The finding that MYC is one of the most frequently amplified genes in human cancers (151), coupled with an appreciation of its regulatory relationship with eIF4F, helped further fuel this interest. Targeting translation as an anti-neoplastic approach is not new. Depletion of asparagine pools with asparaginase inhibits translation elongation and is used to treat acute lymphoblastic leukemia and pediatric acute myeloid leukemia (152). As well, homoharringtonine, an inhibitor of translation elongation has been approved by the FDA for treatment of chronic myeloid leukemia (153). A large body of biological data ranging from cell-based and pre-clinical models assays suppressing eIF4E, eIF4A, and eIF4F support the idea that targeting eIF4F activity would be anti-neoplastic (reviewed in (154, 155)).

Experiments targeting eIF4E using antisense RNA (108, 124, 156–158), or peptides to interfere with eIF4E/eIF4G interaction (159–161), demonstrated suppression of the oncogenic properties of transformed cell lines *ex vivo* and/or tumor growth *in vivo*. Most telling, the development of a mouse model in which eIF4E expression can be inducibly suppressed by shRNAs engineered to be under doxycycline responsiveness afforded an alternative, genetic approach to pharmacologically targeting eIF4F activity *in vivo* (162). Using this model to suppress eIF4E in pre-lymphomatous B cells in Eμ-Myc mice revealed significantly delayed tumor onset and demonstrated a tolerance for suppressed eIF4E levels at the organismal level, thus underscoring eIF4F's status as an important marker for tumor-specific vulnerability *in vivo* (162). A further key functional link between MYC and mTOR has been recently described, where MYC directs mTOR-dependent phosphorylation of 4EBP1, without affecting other mTOR substrates such as S6K (163). Taken together, these findings reveal a critical vulnerability for MYC overexpressing cancer cells that may rely on eIF4E availability for cancer cell survival (162, 163). Indeed, blocking 4E-BP phosphorylation either genetically or pharmacologically with the ATP-site inhibitor INK128, results in a dramatic reduction of lymphomagenesis in Eμ-Myc mice (225).

The eIF4F complex offers multiple possibilities for functional interdiction, some of which have been probed with small molecule inhibitors in high throughput screens. These include blocking eIF4E-cap interaction, interfering with eIF4E-eIF4G interaction, inhibiting eIF4A helicase activity, and suppressing eIF4E levels (164–166).

Targeting eIF4E function

Although cap analogues have been used to probe eIF4E-cap interaction since the late 1970s, their use has been limited to *in vitro* experiments since they are not readily cell permeable

(167, 168)[1]. Therefore, efforts have recently focused on developing pro-drugs, with modifications that would allow the nucleosides to enter the cell followed by conversion to active inhibitors. Accordingly, one compound, 4Ei-1 inhibited cap-dependent translation *in vitro* and *in vivo* when injected into zebrafish embryos (150, 172). 4Ei-1 chemosensitized breast and lung cancer cells to non-toxic levels of the cytotoxic drug gemcitabine (173). 4Ei-1 reduced proliferation and repressed colony formation in mesothelioma cells and sensitized these to pemetrexed, a folate antimetabolite (174). Several inhibitors of eIF4E:eIF4G interaction have also been discovered (4EGI-1, 4E1RCat, 4E2RCat) and shown to inhibit cancer cell growth *ex vivo*, breast cancer xenograft growth *in vivo*, and reverse chemoresistance in MYC-driven murine lymphomas (166, 175–177).

Antisense oligonucleotides (ASO) against eIF4E (LY2275796 [aka ISIS EIF4E Rx]) have been tested in cell lines *ex vivo* and in xenograft models with promising activity (124). Here, translation of known eIF4E-specific pro-growth and pro-survival gene products (c-Myc, cyclin D1, VEGF, Bcl-2, and survivin), were reduced by LY2275796, while global protein synthesis was only modestly affected. A phase I trial demonstrated that LY2275796 could be safely administered to patients and was effective at decreasing eIF4E mRNA and protein levels in tumor cells (178). In this study, 30 patients with stage 4 disease received LY2275796 for three consecutive days and then were maintained on this compound by weekly administration for 3 additional consecutive weeks. The most common drug-related cytotoxicities reported were fatigue (47%), nausea (33%), fever (27%), and vomiting (20%) (178). The compound was effective at reducing eIF4E mRNA *in vivo* by 80% in post-treatment biopsies (compared to pre-treatment biopsies) (178). Phase II clinical trials are now underway combining ISIS EIF4E Rx with carboplatin and paclitaxel for non-small cell lung cancer (NCT01234038) or with docetaxel and prednisone for castration-resistant prostate cancer (NCT01234025).

Inhibiting eIF4A activity

A high throughput screen based on the differential inhibition of translation of eIF4F-dependent versus HCV IRES-dependent reporter mRNAs identified three natural products (pateamine A [Pat A], hippuristanol, and silvestrol) that selectively target eIF4A (164, 179–181). The binding site for hippuristanol has been mapped to the carboxyl terminal domain of eIF4A, but the binding sites of Pat A and silvestrol are not known. Hippuristanol prevents eIF4A from binding RNA (182), whereas Pat A and silvestrol act as chemical inducers of dimerization and force a non-sequence specific engagement between eIF4A and RNA, resulting in depletion of eIF4A from the eIF4F complex (133, 181, 183). Pat A inhibits translation irreversibly (likely as the consequence of a Michael addition site on the molecule) whereas inhibition by hippuristanol or silvestrol is readily reversible. All compounds exhibit anti-neoplastic activity in various xenograft mouse tumor models as

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[1]Ribavirin has been reported to behave as a cap analogue *ex vivo* (169) However, this has been challenged (170, 171). Importantly, in over 100 antitumor screens involving over 10 different xenograft models, ribavirin failed to show any efficacy as a single agent (http://dtp.nci.nih.gov/dtpstandard/servlet/dwindex?searchtype=namestarts&chemnameboolean=and&outputformat=html&searchlist=ribavirin%0D%0A&Submit=Submit). Any biological outcomes observed with ribavirin are therefore unlikely to be the consequence of inhibiting translation.

single agents (133, 184–187). Hippuristanol and silvestrol reverse drug resistance in MYC-driven tumor models (181, 188).

Of the three eIF4A inhibitors, silvestrol and the related rocaglamide family members show the most favorable pharmacological properties for *in vivo* studies. Systemic availability for silvestrol when delivered intraperitoneally is 100% - with 60% of the parental compound remaining after 6 hr (189). Silvestrol does not cause weight loss, liver damage, or immunosuppression in mice (133). B-cells derived from chronic lymphocytic leukemia patients are more sensitive to silvestrol than B-cells from healthy individuals (187) suggesting preferential targeting of faster growing leukemic cells. The anti-proliferative properties of silvestrol appear to be mediated primarily through inhibition of eIF4A since silvestrol-resistant eIF4A mutants can rescue the effect (190). As expected, the translation of mRNAs with extensive secondary structure is more sensitive to inhibition by silvestrol (133, 191–193). A current barrier to the clinical development of silvestrol is that resistance can develop due to overexpression of the ABCB1/P-glycoprotein (191, 194).

Preventing eIF4E phosphorylation

Blocking eIF4E phosphorylation has been shown to prevent the oncogenic function of eIF4E in multiple experimental models (122, 123, 175) suggesting that pharmacologic inhibition of the Mnk kinases may be promising. Indeed, the Mnk inhibitors, CGP57380 and cercosporamide, have been shown to block eIF4E phosphorylation in cultured cells, limiting cellular proliferation in large part by inducing apoptosis (195–197). Similarly, (5-(2-(-(Phenylamino)pyrimidin-4-yl)thiazol-2(3H)-one derivatives have been shown to inhibit MNK2, reduce eIF4E phosphorylation and diminish Mcl-1 expression in cancer cells (198). In animal models, oral administration of cercosporamide suppressed eIF4E phosphorylation in normal mouse tissues and xenograft tumor tissues. Importantly, cercosporamide administration profoundly suppressed the outgrowth of B16 melanoma metastases (195). A more recent report also showed that cercosporamide treatment at doses which specifically inhibited eIF4E phosphorylation decreased the growth rate of AML xenografted tumors and suppressed colonization of freshly explanted AML patient samples (199). Importantly, the biologic effects of cercosporamide on AML cells reflected MNK inhibition (i.e. reduced eIF4E phosphorylation) and did not reflect inhibition of Jak3, a putative additional target of cercosporamide (199). The precise consequence of MNK inhibition on tumor cell behavior will require additional studies.

Not all Roads lead to Rome – The Differential Consequences of Targeting eIF4E, eIF4A, and eIF4G

Inhibition of eIF4E by TOR inhibitors (rapamycin, PP242, INK128) (200) or LY2275796 (124) does not dramatically reduce global protein synthesis. For example, LY2275796 causes a 80% reduction in eIF4E levels with only a modest impact on global protein synthesis (~20% change) (124). In contrast, inhibition of eIF4A has a much more profound effect on global protein synthesis (181). These results could be explained if the bulk of ongoing translation requires high concentrations of eIF4A, but not eIF4E, to be sustained. This is consistent with a model in which eIF4E recycles following the initial cap binding:

eIF4G:eIF4A dimers would then be sufficient to maintain multiple subsequent rounds of initiation (Fig. 1). In this scenario, acute inhibition of eIF4A is expected to have a more immediate effect on translation than a block in eIF4E activity.

Overexpression of eIF4GI in NIH 3T3 cells is oncogenic (201). This may be a consequence of its ability to stimulate IRES-mediated translation of transcripts encoding key oncogenic functions (70, 202, 203). This is of clinical relevance since inflammatory breast cancers (IBC) display high levels of eIF4GI (with little change in eIF4E or 4E-BP1 levels). This has been linked to IRES-mediated translation of VEGFA and p120 catenin, which are required for progression to metastasis (204). Thus, in the setting of IBC, it would make more sense to target eIF4GI (and possibly eIF4A) rather than eIF4E.

Consistent with reports that document differential translational responses to eIF4E versus eIF4A inhibitors is the finding of a unique translation/transcription regulatory element, called TISU (translation initiator of short 5′ UTR; $^{5'}$SAASATGGCGGC$^{3'}$, in which S is C or G) (205). TISUs are present in ~4.5% of protein-encoding genes, most of which have unusually short 5′ UTRs (~12 nt) (205). Translation of TISU-containing mRNAs is cap-dependent, but much less dependent on eIF4A (206). Perhaps, the extreme cap-proximal location of the AUG initiation codon on TISU mRNAs significantly diminishes the scanning requirement for eIF4A-dependent unwinding.

Conclusion – Pushing eIF4F out of the Goldilocks Zone

The Goldilocks principle posits that parameters to maintain a specific state need to fall within a certain margin, or zone, to favor a particular outcome. For example, the distance of the earth from the sun is within a "Goldilocks zone" and hence favorable to life as we know it. The same analogy can be applied to the relationship between eIF4F activity and tumor cell homeostasis. The translational output of a tumor cell needs to fall within a "Goldilocks zone" to optimally support its proteastasis (Fig. 2). Too much eIF4F activity is deleterious to the cell, too little eIF4F activity precludes cellular transformation. Indeed, the preponderance of experimental work has revealed that cellular transformation requires only 2–3 fold increased eIF4E expression whereas depletion of eIF4E activity by only ~50% can reverse the transformed and malignant phenotype (108, 109, 119, 122–124). Perhaps most important for the therapeutic potential of targeting the eIF4F complex is the consistent observation from many experimental studies that malignant cells seem to be tuned to this "Goldilocks zone" of eIF4F activity. That is, malignant cells have selected for a certain enhanced translational output (i.e. enhanced eIF4F activity) necessary for the manifestation of the varied phenotypes responsible for tumor formation and metastatic progression-growth factor autonomy, angiogenesis, enhanced cellular survival, invasiveness and metastatic outgrowth. The particular dependence of malignant cells on this "zone" of enhanced eIF4F activity makes these cells especially susceptible to eIF4F inhibition. Indeed, in multiple preclinical studies *in vivo*, inhibition of eIF4F activity (via inhibition of eIF4E, Mnk or eIF4A) profoundly impacted malignant cells, inducing tumor cell death, cessation of tumor growth, and repression of metastatic outgrowth without substantially impacting normal cellular and organismal function (114, 124, 133, 178). For example, intravenous administration of the eIF4E ASO dropped eIF4E levels in the liver of treated, xenograft-

bearing mice > 80% without affecting liver enzymes or body weight. Yet, in these same animals, a reduction in eIF4E expression in the xenografted tumor of only 50–60% was sufficient to robustly induce apoptosis and flat-line xenograft tumor growth (124).

As detailed above, the sustained translation of a specific subset of mRNAs is key to enabling tumor maintenance and metastatic progression- that is to allow for the enhanced, selective expression of the potent growth and survival factors that regulate tumor cell survival, angiogenesis, growth factor autonomy, invasiveness and metastasis. The question arises as to which eIF4E-responsive mRNA(s) is/are particularly critical and whether this is expected to vary among tumor types. If the latter were true, this would entail the development of robust biomarkers to inform on eIF4F dysregulation and inhibition unique to each tumor type – a truly daunting task.

However, the situation may not be this complicated. Firstly, high resolution analysis of copy number variations (CNV) from >3000 cancer samples representing largely 26 different cancer types has documented that among the top 20 most common amplifications are four genes encoding key oncogenic drivers and tumor maintenance factors, whose mRNAs are eIF4E-responsive: MYC, MCL1, BCL2L1(BCL-xL), and CCND1 (151). Although the expression of these proteins may be elevated due to amplification events, if the translation of their mRNAs remains eIF4E-dependent, inhibition of eIF4F (coupled with the naturally rapid turnover of the MYC, MCL1, and CCND1 proteins (207, 208)), is expected to cause a rapid depletion of these proteins and have a dramatic consequence on tumor cell homeostasis- shifting cells out of the Goldilocks zone. MYC, MCL1, and CCND1 have been difficult to "drug" directly and thus inhibiting their production at the translation level is one strategy to overcome this problem.

Targeting the eIF4F complex may potentially bode well for dealing with the intratumoral heterogeneity that drives malignant progression and treatment resistance (209). Intratumoral heterogeneity arises from the diverse selection pressures imposed upon the tumor cell population and may reflect genetic, epigenetic and/or cellular changes. The manifestation of this diversity must involve changes in translational output- i.e. in eIF4F activity. Indeed, the eIF4F complex sits at the junction of numerous, potent oncogenic pathways: Ras-Raf-ERK, Myc, and PI3K-TOR pathways, which in turn may also be activated by other divergent oncogenic stimuli (e.g. - receptor tyrosine kinase activation (EGFR)). As such, these divergent pathways- which reflect the underlying cellular, genetic and epigenetic heterogeneity of the tumor- are critically reliant upon the activity of the eIF4F node for the alterations to the proteome that give rise to phenotypic heterogeneity driving malignant progression and therapeutic resistance. Hence, targeting this complex - this critical node of convergence for so many divergent oncogenic stimuli- may provide a powerful means to address the intratumoral heterogeneity that plagues current cancer therapy.

We've come a long way since the discovery of the mRNA cap structure and fundamental studies that defined its biochemical and biological function. One could never have predicted that these fundamental studies would have led to such a profound interest in targeting the eIF4F complex, particularly for the treatment of cancer. The thrust to "translate" these findings to the clinic provides a substantial challenge and will continue to demand rigorous,

concerted scientific partnership between academia and industry to reveal the best opportunities to leverage and advance our current knowledge of the eIF4F complex in order that we might develop new therapies to inhibit the translation of mRNAs encoding oncogenic functions.

Acknowledgments

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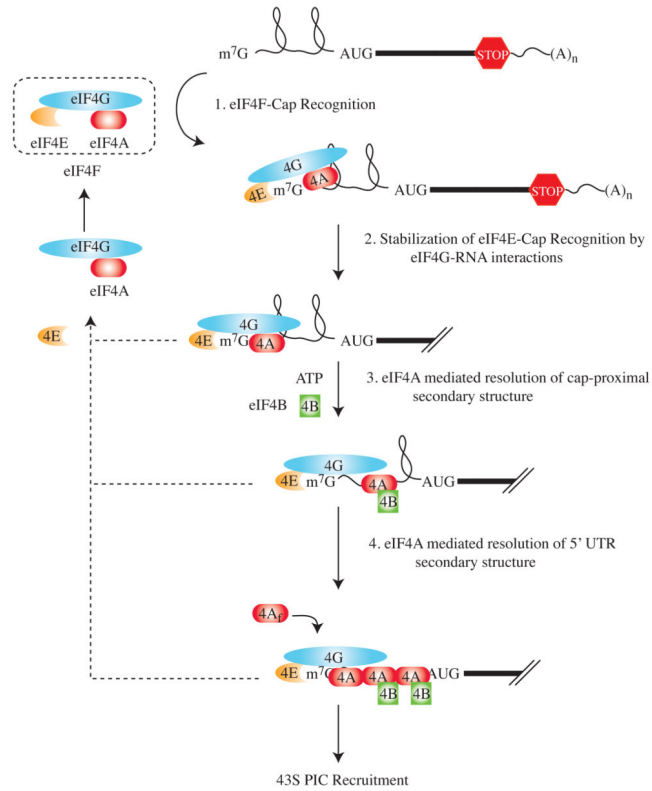
Figure 1.
Schematic model of cap-dependent binding of eIF4F-mRNA binding and subsequent destabilization of secondary structure. For clarity, the PABP-eIF4G interaction has not been recapitulated in this figure. Shown are four steps where mRNA structural barriers may impact on initiation efficiency. See text for details.
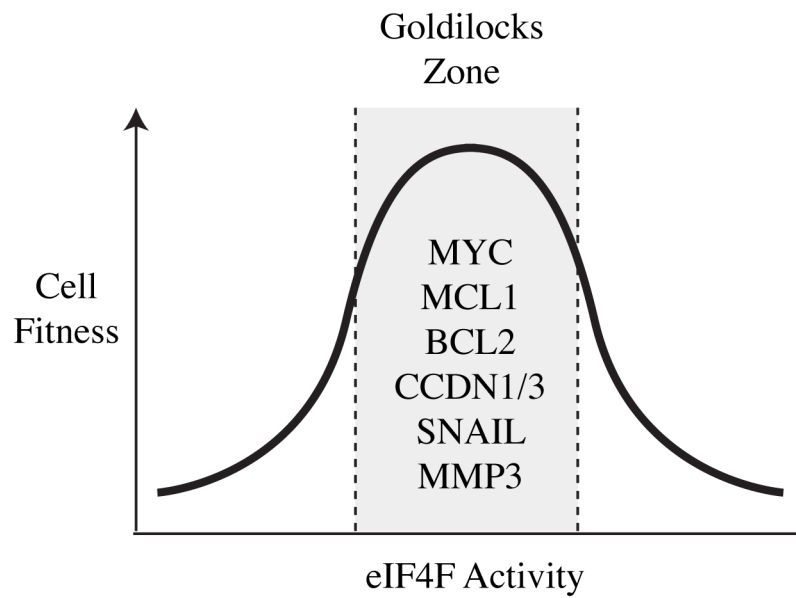
Figure 2.
Model illustrating eIF4F activity residing within a Goldilocks zone to sustain optimal tumor cell survival. Given that eIF4F activity is critical for sustained output of key players in tumor cell initiation, maintenance, and metastasis, altering this activity can dramatically impact tumor cell fitness. See text for details.