The Oncogene eIF4E: Using Biochemical Insights to Target Cancer

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The eukaryotic translation initiation factor eIF4E is overexpressed in many human malignancies where it is typically a harbinger of poor prognosis. eIF4E is positioned as a nexus in post-transcriptional gene expression. To carry out these functions, eIF4E needs to bind the m⁷G cap moiety on mRNAs. It plays critical roles in mRNA translation, mRNA export, and most likely in mRNA stability as well. Through these activities, eIF4E coordinately modulates the expression of many transcripts involved in proliferation and survival. eIF4E function is controlled by interactions with protein cofactors in concert with many signaling pathways, including Ras, Mnk, Erk, MAPK, PI3K, mTOR, and Akt. This review describes the eIF4E activity and provides several examples of cellular control mechanisms. Further, we describe some therapeutic strategies in preclinical and clinical development.

Overview

AS MULTIPLE LINES of evidence build that cancer cells are much like normal cells, it becomes increasingly critical to identify molecular targets that may act as an Achilles' heel for malignancy. One such target is the eukaryotic translation initiation factor eIF4E. eIF4E is a potent oncogene elevated in up to 30% of human malignancies, including carcinomas of the breast, prostate, lung, head, and neck as well as in many leukemias and lymphomas (Crew and others 1996; Nathan and others 1997a; De Benedetti and Harris 1999; Crew and others 2000; Berkel and others 2001; Seki and others 2002; Lee and others 2005; Matthews-Greer and others 2005; Culjkovic and Borden 2009; Borden and Culjkovic-Kraljacic, 2010). Increased eIF4E expression is associated with poor prognosis in patients [reviewed in (Culjkovic and Borden 2009)]. Furthermore, its overexpression in cell culture and in animal models leads to oncogenic transformation and tumor formation, respectively (De Benedetti and Rhoads 1990; Lazaris-Karatzas and others 1990; Avdulov and others 2004). On the cellular level, eIF4E overexpression is characterized by increased proliferation and survival (De Benedetti and Rhoads 1990; Lazaris-Karatzas and others 1990; Berkel and others 2001; Li and others 2003; Polunovsky and others 1996; Tan and others 2000; Avdulov and others 2004). It is an important part of many growth factor and stress signaling pathways (Ras, Erk, Mnk, PI3K, Akt, and mTOR; Brunn and others 1996; Mendez and others 1996; Brunn and others 1997; Waskiewicz and others 1997; Gingras and others 1998). In this review, we will focus on biochemical activities of eIF4E and their dysregulation in cancer. We will also describe how

this knowledge has been translated into the clinic and discuss ways to further utilize knowledge of this target in advancing cancer therapy.

Molecular Functions of eIF4E

The best-described function for eIF4E is as a translation initiation factor involved in directing ribosomes to capped mRNAs (Fig. 1). During mRNA translation, eIF4E, a 25-kDa protein forms the eIF4F complex with the cofactors eIF4G, eIF4A, and eIF4B to recruit these to the ribosome (Sonenberg and Gingras 1998). To carry out this function, eIF4E relies on its ability to bind the 5' methyl-7-guanosine (m⁷G cap) found on transcripts (Filipowicz and others 1976). This promotes the more efficient translation of transcripts by loading more ribosomes per transcript (Sonenberg and Gingras 1998). Importantly, eIF4E differentially affects transcripts. For instance, overexpression of eIF4E does not lead to elevation of all proteins, as observed by ³⁵S-Met incorporation (Kaufman and others 1993). In fact, there was no detectable change in ³⁵S-Met incorporation between controls and eIF4E overexpressing cells highlighting the specificity (Kaufman and others 1993). Concomitantly, antisense-mediated knockdown of eIF4E does not lead to global decreases in protein levels (Graff and others 2007). Studies by the Sonenberg laboratory highlight this specificity. For example, eIF4E overexpression leads to increased translational efficiency of ornithine decarboxylase transcripts, but cyclin D1 and GAPDH transcripts are unaffected at the translation level (Rousseau and others 1996). Transcripts that are most sensitive to eIF4E at the translation level are typically characterized by highly

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FIG. 1. Model of 2 known eIF4E functions: mRNA export and mRNA translation. See text for details.

structured untranslated region (5'UTRs) (Sonenberg and Gingras 1998). For example, deletion of these structured elements in Pim1 transcripts substantially reduces their requirement for stimulation by eIF4E (Hoover and others 1997). Studying model transcripts, the Pestova and Hellen laboratories have shown that as the complexity of the 5'UTR is reduced, there is a loss of sensitivity for eIF4E in their translation (Pestova and others 2001). Thus, the complex 5'UTR sensitizes transcripts to eIF4E regulation, while housekeeping transcripts with short unstructured UTRs are generally not affected. Additionally, eIF4E may play novel functions in cap-assisted internal translation of histones, which is somewhat akin to a mixture of cap dependent and internal ribosome entry site mechanisms (Martin and others 2011). Here eIF4E can contact the RNA independently of the cap structure, although the cap is still required for translation (Martin and others 2011). Additionally, a cap-dependent translation can occur via the cap-binding complex both in the pioneer round of translation (Maquat and others 2010) and during stress responses, for example, (Garre and others 2011). Importantly, eIF4E is also found as a component of P-bodies and stress granules implying that it also plays a role in mRNA sequestration and/or turnover (Ferraiuolo and others 2005; Parker and Sheth 2007). Overall, the cytoplasmic function of eIF4E can be summarized as an enhancer of translation for a select group of transcripts particularly during periods of cellular stress and proliferation.

Aside from the cytoplasm, eIF4E exists in nuclear bodies and throughout the nucleoplasm in most cell types examined (Lejbkiewicz and others 1992; Iborra and others 2001; Topisirovic and others 2003b) (Fig. 1). In the nucleus, eIF4E acts to promote the export of a subset of growth promoting mRNAs, thereby increasing their cytoplasmic concentration (Rousseau and others 1996; Culjkovic and others 2005, 2006, 2007, 2008). Similar to the cytoplasm, eIF4E affects the export of only a specific subset of mRNAs encoding genes functioning in proliferation and survival. A ~50 nucleotide structural element in the 3'UTR of transcripts, denoted an

eIF4E sensitivity element (4E-SE), confers sensitivity to eIF4E at the export level (Culjkovic and others 2005, 2006, 2008). For instance, Lac-Z 4E-SE chimeras have their mRNA export promoted by eIF4E overexpression, whereas unmodified LacZ transcripts are unaffected. There may be other elements as well, but these have yet to be discovered. eIF4E-dependent mRNA export is independent of bulk mRNA export (Culjkovic and others 2005, 2006, 2008). Bulk mRNA export requires the association of export ribonucleoproteins (RNPs) with the TAP1 nuclear receptor, whereas eIF4E-dependent export uses the CRM1-Ran export pathway (Culjkovic and others 2006). In the nucleus, eIF4E associates with CRM1, a specific subset of transcripts and specific cofactors to facilitate export to the cytoplasm (Culjkovic and others 2006; Topisirovic and others 2009a). Interestingly, eIF4E overexpression alters the composition of the nuclear pore, which leads to increased mRNA export of sensitive transcripts (Culjkovic-Kraljacic and others 2012). In fact, the major constituent of the cytoplasmic fibrils of the nuclear pore RanBP2 is a suppressor of both the mRNA export and transformation activity of eIF4E (Culjkovic-Kraljacic and others 2012). This is consistent with spontaneous tumor formation observed in RanBP2 hypomorph mice (Chakraborty and others 2008). Here reduction in RanBP2 levels likely increases the efficiency of recycling export components back to the nucleus for subsequent rounds of export. Thus, eIF4E promotes gene expression of select groups of transcripts through both enhancement of nuclear export via the 4E-SE and through enhanced translation in the cytoplasm of transcripts with complex 5'UTR structures.

Combinatorial Changes in Gene Expression Underlie the Potent Biological Effects of eIF4E

The RNA regulon model postulates that groups of RNAs acting in the same biochemical pathways have their regulation coordinated post-transcriptionally to ensure that all factors necessary for a particular function are present at the correct time (Tenenbaum and others 2000; Keene and Tenenbaum 2002; Keene and Lager 2005; Keene 2007). For instance, eIF4E enhances nuclear export of transcripts involved in proliferation, including most of the cyclins because these contain 4E-SE elements. In the cytoplasm, many of the nuclear export targets are also translation targets of eIF4E because they also contain a highly complex 5'UTR (Culjkovic and others 2006). In this way, the production of these factors is coordinated enabling a robust biological effect. Thus, eIF4E via these 2 activities in mRNA processing can promote proliferation and survival (Culjkovic and others 2007) by ensuring adequate and balanced expression of critical regulators of these activities. Consistent with this idea, both the mRNA export and translation functions of eIF4E contribute to its effects in oncogenic transformation and apoptotic rescue in cell culture (De Benedetti and Rhoads 1990; Lazaris-Karatzas and others 1990; Polunovsky and others 1996; Tan and others 2000; Li and others 2003; Avdulov and others 2004; Culjkovic and others 2005, 2006, 2008).

Dysregulation of eIF4E in Cancer

eIF4E is highly elevated and dysregulated in many cancers, including those of the lung, bladder, colon, breast, prostate, cervix, ovary, thyroid, and squamous cell carcinoma of the head and neck and others (Crew and others 1996;

Nathan and others 1997a; Nathan and others 1997b; De Benedetti and Harris 1999; Crew and others 2000; Berkel and others 2001; Li and others 2002; Seki and others 2002; Lee and others 2005; Matthews-Greer and others 2005; Culjkovic and Borden 2009; Pettersson and others 2011). Further, eIF4E is dysregulated in many hematological malignancies, including acute myeloid leukemia (AML), the blast crisis stage of chronic myeloid leukemia, and some lymphomas (Topisirovic and others 2003b; Assouline and others 2009; Culjkovic and Borden 2009). Importantly, in many cases, eIF4E is only dysregulated in a subset of each cancer type. For instance, eIF4E is upregulated in M4 and M5 AML subtypes, but not generally in M1, M2, or M3 subtypes (Topisirovic and others 2003b; Assouline and others 2009). eIF4E is elevated in only a subset of breast cancers, but in ~98% of head and neck cancers [reviewed in (Culjkovic and Borden 2009; Pettersson and others 2011)]. Thus, an important question arises as to how eIF4E levels themselves are dysregulated and what is unique about this subset of tumors. Of note, it is unclear whether the eIF4E activity may be dysregulated in subsets of primary tumors where its expression is not elevated.

eIF4E transcript levels are known to be dysregulated by at least 3 mechanisms: gene amplification, transcriptional dysregulation, and mRNA stability [reviewed in (Culjkovic and Borden 2009)]. These mechanisms are not mutually exclusive and can occur coincidentally. Gene amplification has been observed in a subset of head and neck and breast cancers (Sorrells and others 1998; Sorrells and others 1999). The promoter of the eIF4E gene contains an E-box and is a direct transcriptional target of c-Myc (Schmidt 2004). Interestingly, c-Myc is an mRNA export and translation target of eIF4E suggesting a potential positive feedback mechanism (Culjkovic and others 2006, 2007). Transcriptional control goes beyond c-Myc as eIF4E is still produced in c-Myc null fibroblasts and its expression in these cells is still stimulated by addition of serum (Bush and others 1998). Other studies have implicated Sonic hedgehog signaling (Mainwaring and Kenney 2011) and p53 (Zhu and others 2005) in the control of *EIF4E* transcription, but these effects are likely mediated by the c-Myc interaction with the *EIF4E* promoter. The hnRNP protein binds the *EIF4E* promoter and its regulation of eIF4E contributes to oncogenic transformation (Lynch and others 2005). Recent studies demonstrate that eIF4E is also a C/EBP target (Khanna-Gupta and others 2012). eIF4E is also an NF κ B target where transduction of primary M4 and M5 AML cells with the I κ B-SR results in relocalization of eIF4E as described below and also reduction in mRNA levels (Topisirovic and others 2003b). Finally, the stability of eIF4E mRNA is increased by the HuR and TIAR proteins (Mazan-Mamczarz and others 2006; Topisirovic and others 2009b). In primary M4 and M5 AML, increased HuR levels and activated NF κ B coincident with the increased eIF4E expression are observed in these leukemias (Topisirovic and others 2009b). As with other observations of eIF4E, multiple mechanisms likely simultaneously alter transcript levels in human malignancies.

eIF4E-Cap Recognition

The defining biochemical feature of eIF4E is its specific association with the m⁷G cap on the 5' end of mRNAs (Filipowicz and others 1976). eIF4E binds unmethylated cap with 100-fold less affinity than the m⁷G cap although this

varies depending on the conditions used (Liu and others 2011). The m⁷G cap intercalates between 2 tryptophan residues of eIF4E (Marcotrigiano and others 1997, 1999). At pH 7.5, the m⁷G cap is a zwitterion and it is the positive charge component that is thought necessary for interaction with the negative pi electron clouds of the 2 tryptophans in the cap-binding site (Carberry and others 1990; Carberry and others 1991). However, crystal structures show that glycerol, a neutral molecule, binds eIF4E in the same pocket (Brown and others 2009) suggesting that other features can drive interactions within the cap-binding site. Interestingly, eIF4E also binds analogues with benzene or phenol substituted for the methyl group (Carberry and others 1990). The benzylate-7-G cap analogue binds eIF4E more tightly than m⁷G cap indicating that the cap-binding pocket is adjustable (Carberry and others 1990). Tri-methylated guanosine cap is found on many RNAs involved in splicing and also binds with about a 10-fold reduced affinity to human eIF4E relative to m⁷G cap. However, in nematodes, tri-methylated and m⁷G cap have similar affinities for eIF4E (Liu and others 2011). Despite the weaker affinity for eIF4E in humans, the tri-methylated cap still binds with a nearly 50-fold greater affinity than guanosine triphosphate (GTP). The crystal structures for eIF4E m⁷GTP or tri-methylated cap complexes are highly similar (Liu and others 2011). Thus, although m⁷G cap binding is the defining biochemical feature of eIF4E, it can bind other moieties adding to its list of known and possible functions.

Studies of other cap binding proteins can enhance our understanding of the critical structural elements of the eIF4E-m⁷G cap interaction. For example, although other m⁷G cap binding proteins, such as VP39 and CBP20, use the aromatic stacking model for cap recognition (Quirocho and others 2000), there are other cap binding proteins, which specifically bind the m⁷G cap that do not use this strategy. Reovirus polymerase lambda3 uses a pocket where the tryptophan is perpendicular rather than planar to the m⁷-guanosine moiety and also utilizes aliphatics for recognition to bind m⁷G cap (but not guanosine) (Tao and others 2002). Similarly, the decapping scavenger enzyme, DcpS, binds m⁷G cap and not guanosine through a half aromatic sandwich model whereby there is 1 tryptophan present, but for the other side, aliphatic residues are packed onto the ring (Gu and others 2004). A third member of the eIF4E family, eIF4E-3, has only 1 tryptophan with a cysteine in the other traditional aromatic position (Osborne and others, in press). Here the cap is recognized by the conserved tryptophan and sandwiched with backbone residues (Osborne and others, in press). Consistently, mutation of the conserved tryptophan 102 to leucine in eIF4E only modestly reduces m⁷G cap binding, while mutation to alanine completely abrogates these effects (Osborne and others, in press). Thus, principles for cap recognition are not restricted to the aromatic sandwich, and even in eIF4E, there is much to still learn about ligand recognition.

These findings have important implications for drug design and understanding the molecular basis of competitive inhibitors of cap binding, such as ribavirin. Ribavirin and its active metabolite ribavirin triphosphate (RTP) bind eIF4E with similar affinity to the m⁷G cap in a phosphate buffer pH 7.5 as measured by fluorescence quenching, cap chromatography, circular dichroism, mass spectrometry, and NMR (Kentsis and others 2004; Kentsis and others 2005; Borden and Culjkovic-Kraljic 2010; Volpon and others, submitted). NMR studies with RTP indicate that the ligand binds in the

same cap binding site as m⁷G cap, but its exact location in the pocket is likely slightly different (Volpon and others, submitted). Further, ³H ribavirin immunoprecipitated with endogenous eIF4E in live cells (Borden and Culjkovic-Kraljacic 2010). It may be feasible to generate more potent inhibitors of eIF4E binding to the m⁷G cap although whether such inhibitors would be safe for human therapy is unclear.

Direct Regulators of eIF4E

Regulation of eIF4E is extremely complex, as one would expect for a protein that modulates gene expression so potently. There are, to date, 3 classes of direct regulators of eIF4E described: those that contain an eIF4E consensus binding motif (YXXXXLΦ), those with zinc binding really interesting new gene (RING) domains, and a class of viral proteins that contain neither of these motifs (summarized in Table 1). All of these proteins bind the dorsal surface of eIF4E, which is about 35 Å away from the m⁷G cap-binding site. They mediate changes to activity by either blocking the ability of eIF4E to bind other factors, such as eIF4G, and/or mediate allosteric effects. These regulators can be present in the nucleus and/or cytoplasm suggesting that the biochemical basis for control is similar between compartments. The overall effect of these diverse regulators in specific tissues and organisms is incompletely understood; however, the plethora of regulators suggest that fine-tuning of the diverse activities of eIF4E is critical.

The first class of regulators is characterized by a consensus-binding motif first identified in eIF4G and eIF4E-BP1 (Mader and others 1995). The motif is YXXXXLΦ where X is any residue and Φ is any hydrophobic residue (Mader and others 1995). Structural studies indicate that the motif forms a helix and a reversed L-shape structure that interacts with the dorsal surface of eIF4E (Marcotrigiano and others 1999; Tomoo and others 2005). New structural studies demonstrate that BP1 and CUP, two proteins that use this motif, utilize the consensus sequence in concert with another part of the protein, in fact, having 2 distinct binding sites for eIF4E (Kinkelin and others 2012).

BP1 is the best-studied regulator of eIF4E. Its association with the dorsal surface competes for association with eIF4G, and thereby inhibits translation by blocking formation of the translation initiation complex (Haghighat and others 1995). Additionally, binding BP1 increases affinity of eIF4E for the cap through allosteric changes (Ptushkina and others 1998; Ptushkina and others 1999; Tomoo and others 2005). This is consistent with the model postulated by the McCarthy laboratory whereby BP1 inhibits protein translation by not only

blocking binding of eIF4G, but also by sequestering the RNA bound to eIF4E from active translation complexes (Ptushkina and others 1998; Ptushkina and others 1999; Tomoo and others 2005). Phosphorylation of BP1, which can occur via multiple pathways (see *Targeting eIF4E at multiple levels in the clinic*), leads to unfolding of the recognition helix and thus impairs BP1 binding to eIF4E (Tait and others 2010). Of note, BP1 is found in both the nucleus and the cytoplasm indicating that it likely modulates mRNA export as well as translation (Rong and others 2008). BP1 overexpression suppresses the ability of eIF4E to transform cells. Highlighting the importance of other regulators of eIF4E, BP1 null mice do not have a substantial phenotype (Blackshear and others 1997) although there is some evidence of adipocyte tissue reduction (Tsukiyama-Kohara and others 2001). There are 3 homologues of BP1: BP1, BP2, and BP3, the latter of which has a very limited tissue distribution. The double knockout of BP1 and BP2 leads to diet-induced obesity and insulin resistance (Le Bacquer and others 2007). Interestingly, and highlighting the complex regulation of eIF4E, BP1 or BP1/2 null mice do not get cancers more readily than littermate controls (Tsukiyama-Kohara and others 2001; Le Bacquer and others 2007). Of note, BP1 is not conserved in *Saccharomyces cerevisiae*, which use Caf20p and Eap1p to regulate translation. These proteins have no apparent sequence homology outside of the consensus binding motif, but they can still inhibit human eIF4E activity (Altmann and others 1989). Caf20p and Eap1p regulate the translation of specific transcripts suggesting that binding proteins can specifically regulate a subset of eIF4E-sensitive transcripts (Cridge and others 2010). These observations showcase the importance of other regulators to the control of eIF4E activity and specificity of these regulators.

In cancer, there are many surprising results with regard to BP1. BP1 levels are elevated in breast cancer where increased levels and phosphorylation are correlated with a more advanced stage of the disease (Armengol and others 2007). This correlation is actually stronger than the correlation with eIF4E levels themselves (Armengol and others 2007). Tissues from esophageal cancers have more eIF4E-BP1 complexes than adjacent normal tissue (Salehi and Mashayekhi 2006). Similar results are seen in primary M4 and M5 AML specimens that have highly elevated eIF4E (3–10-fold) (Assouline and others 2009) and similarly highly elevated BP1 and phospho-BP1 (Culjkovic-Kraljacic and Borden, unpublished). The basis for this increased expression of BP is not clear, but may reflect compensation within the tumor cells for increasing eIF4E levels. This and other observations make it challenging to come up with single variable models correlating eIF4E expression with tumor growth.

Other proteins that use the consensus motif to bind to and regulate eIF4E are described in a wide variety of functional classes of proteins that can both stimulate and repress eIF4E activity. For instance, about 200 homeodomain containing proteins include the consensus-binding site. Of these, HoxA9, Hex/PRH, Hox11, Bicoid, Emx-2, and Engrailed 2 are confirmed to directly bind eIF4E (Niessing and others 2002; Topisirovic and others 2003a; Nedelec and others 2004; Brunet and others 2005; Topisirovic and others 2005). HoxA9 is demonstrated to promote the mRNA export and translation activities of eIF4E (Topisirovic and others 2005). By contrast, Hex/PRH suppresses the nuclear function and does not modulate translation presumably due to the fact it is

TABLE 1. CLASSES OF EIF4E REGULATORS^a

<p>Class I: Consensus YXXXXLΦ eIF4G, BP1,^b CUP,^b Neurogadin, CYF1F1F, LRPPRC, GEMIN5, 4E-T, DDX3 Homeodomain subset: Bicoid, PRH/Hex, HoxA9, Emx-2, En2 (only a subset given, over 200 potential to date)</p> <p>Class II: RING motif PML, arenaviral Z, HHARI</p> <p>Class III: Potyviral genome linked VPg- amphipathic helix VPg from potyviruses</p>
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^aOther classes may also exist.

^bAdditional binding sites act with the consensus site.

restricted to the nucleus (Topisirovic and others 2003a). Interestingly, in M4 and M5 AML, Hex/PRH is substantially downregulated and mislocalized suggesting that loss of this level of eIF4E regulation could contribute to leukemogenesis (Topisirovic and others 2003a). The RNA helicase DDX3 also uses a consensus-binding site to directly associate with eIF4E and modulates translation, and potentially functions in P-bodies and in mRNA export (Shih and others 2008; Topisirovic and others 2009a; Shih and others 2011). The mRNA export cofactor LRPPRC associates with eIF4E in the nucleus using the consensus site (Topisirovic and others 2009a). In fact, LRPPRC overexpression enhances eIF4E-dependent mRNA export and mutation of the consensus binding site abrogates this (Topisirovic and others 2009a). Other factors in this class include Gemin5, the translational repressor CYF1F1, Neurogadin, and many others (Culjkovic and Borden 2009; Rhoads 2009). Taken together, the consensus-binding motif is utilized in likely hundreds of regulators of eIF4E in tissue- and context-specific fashions.

A structurally distinct class of eIF4E regulators utilize their RING domains to interact with eIF4E and modulate its function (Kentsis and others 2001; Kentsis and others 2002a, 2002b; Volpon and others 2010; Culjkovic-Kraljacic and others 2012). To date, the promyelocytic leukemia protein (PML), and the arenaviral Z proteins from lymphocytic choriomeningitis virus and lassa fever virus have been shown to directly bind eIF4E via their RING domains, while human homologue of ariadne (HHARI) binds an eIF4E homologue 4EHP (Kentsis and others 2001; Kentsis and others 2002a; 2002b; Tan and others 2003; Volpon and others 2010). Biochemical and solution structure studies indicate that the RING domains of PML and Z directly bind the dorsal surface in a region that overlaps with, but is distinct from, the area bound by consensus site containing factors (Kentsis and others 2001; Kentsis and others 2002a, 2002b; Volpon and others 2010). The association of the RING with eIF4E leads to allosteric changes in the cap-binding site that correlated with up to a 100-fold reduction in cap binding. Cap binding is required for eIF4E-dependent mRNA export (Culjkovic and others 2005, 2006). This is consistent with previous observations that PML, which is almost entirely a nuclear protein suppresses the mRNA export functions of eIF4E (Cohen and others 2001). In fact, PML overexpression represses eIF4E-mediated oncogenic transformation through this interaction (Cohen and others 2001; Culjkovic and others 2006). Addition of purified PML RING or the viral Z protein in reticulocyte lysates leads to repression of translation, suggesting that PML and Z could impair translation when localized to the cytoplasm (Kentsis and others 2001). In the context of viral infection, Z binds other viral factors that likely impact on its eIF4E effects (Kentsis and others 2001). Importantly, PML, Z, and HHARI do not alter eIF4E/4EHP protein levels suggesting that these functions are independent of any functions they may play in ubiquitination or related pathways (Kentsis and others 2001; Kentsis and others 2002a, 2002b; Tan and others 2003).

The third class of known eIF4E binding proteins, which come from the plant potyviruses, comprise the potyviral genome-linked proteins VPg, which directly bind eIF4E and reduce its affinity for the m⁷G cap by about 15-fold (Michon and others 2006; Roudet-Tavert and others 2007; German-Retana and others 2008). VPg contains neither a consensus motif nor RING domain. Mapping studies suggest that VPg

uses an amphipathic helix to associate with eIF4E and, importantly, does not bind to the same surface as the consensus binding proteins (Michon and others 2006; Roudet-Tavert and others 2007; German-Retana and others 2008). In support of this, VPg increases the affinity of eIF4E for eIF4G, a protein that does use the consensus-binding motif. Further structural studies will elucidate the molecular underpinnings of this interaction and will likely enable one to identify mammalian proteins that may use the same or similar strategies to associate with and regulate eIF4E.

Taken together, it is clear that the regulation of eIF4E is complicated with many context- and tissue-specific factors in play. Redundancy of regulation is the likely key for the normal control of both the nuclear and cytoplasmic functions of eIF4E. Further, in malignancies, it is likely that the overall level of eIF4E activity is critical and not simply the level of protein expression.

The Subcellular Localization of eIF4E

eIF4E is found throughout eukaryotic cells (Culjkovic and Borden 2009). It is found in the cytoplasm and can be distributed both diffusively through the cytosol or it can be found associated with P-bodies and stress granules under appropriate conditions (Ferraiuolo and others 2005; Parker and Sheth 2007). In the nucleus, eIF4E is found in both nuclear bodies and diffusely throughout the nucleoplasm, but is excluded from the nucleolus (Lejbkiewicz and others 1992; Cohen and others 2001). A subset of nuclear bodies colocalize with those formed by PML. This latter localization is dependent on the RING domain of PML (Cohen and others 2001; Topisirovic and others 2003a). As noted above, PML is a potent repressor of eIF4E-dependent mRNA export and, subsequently, eIF4E-mediated oncogenic transformation (Cohen and others 2001). The relative cellular distribution of eIF4E is context and tissue-type dependent (Culjkovic and Borden 2009). Obviously, its subcellular distribution will have a profound impact on its function, but it is incompletely understood how its cellular localization is regulated.

Studies by the Borden laboratory have demonstrated one example of how eIF4E subcellular localization is dysregulated in malignancy. In primary leukemic blast cells isolated from patients with the M4 and M5 subtypes of AML, and in the M5 AML cell line THP-1, eIF4E is not only highly elevated, but also concentrated in the nucleus (Topisirovic and others 2003a, 2003b; Kraljacic and others 2011). By contrast, normal hematopoietic stem and progenitor CD34⁺ cells have discrete nuclear bodies and a cytoplasmic diffuse staining found in many other normal tissue types (Topisirovic and others 2003a; Topisirovic and others 2003b; Kraljacic and others 2011). Increased nuclear accumulation in these AML cells is associated with increased eIF4E-dependent mRNA export and, thus, this dysregulation contributes to oncogenesis (Topisirovic and others 2003b; Kraljacic and others 2011). In primary AML, the distribution of eIF4E is controlled, at least in part, by NFκB. Introduction of the NFκB repressor, IκB-SR, leads to a remarkable redistribution of eIF4E so that its localization is indistinguishable from normal CD34⁺ stem and progenitor cells, that is, there are discrete nuclear bodies with a diffuse cytoplasmic localization (Topisirovic and others 2003b). The effects of NFκB in this context are likely indirect. As described below, studies with ribavirin have shown activity in the therapy of AML

patients with highly elevated nuclear eIF4E levels, and these molecular studies suggest that targeting mRNA export function can be clinically useful (Assouline and others 2009).

Basic laboratory studies have demonstrated that some direct regulators of eIF4E also modulate the subcellular distribution of eIF4E. The 4E-Transporter protein (4E-T) utilizes its consensus-binding site to bind eIF4E. 4E-T overexpression leads to relocalization of eIF4E to the cytoplasm where it is found in a subset of P-bodies (Ferraiuolo and others 2005; Cargnello and others 2012). Hex/PRH overexpression, again via its consensus binding site, causes relocalization of nuclear eIF4E to the cytoplasm (Topisirovic and others 2003a). This substantially impairs its mRNA export activity and its ability to transform cells (Topisirovic and others 2003a). LRPPRC utilizes its consensus-binding site to compete for PML RING binding to eIF4E in the nucleus (Topisirovic and others 2009a). Here LRPPRC overexpression leads to reduced PML-eIF4E association at nuclear bodies and more nucleoplasmic eIF4E. This correlates with increased mRNA export activity of eIF4E (Topisirovic and others 2009a). These studies demonstrate clearly that it is not simply nuclear versus cytoplasmic localization of eIF4E that is critical, but the exact distribution of the protein within the nucleus or cytoplasm that affects the overall function of eIF4E in modulating nuclear mRNA export, mRNA translation, and stress responses.

The molecular work above has led to the identification of inhibitors of eIF4E function. For example, treatment of cells with a cap analogue m⁷GpppG leads to disruption of eIF4E nuclear bodies (Dostie and others 2000; Cohen and others 2001). Similarly, and importantly, treatment with a competitive inhibitor of the m⁷G cap, ribavirin, redistributes nuclear eIF4E to the cytoplasm without altering other subnuclear organelles (Kentsis and others 2004; Assouline and others 2009). Ribavirin is a food and drug administration approved antiviral drug. The parent compound is actually a prodrug that is metabolized into RTP (Glue 1999). Leptomycin B, the CRM1 inhibitor, leads to a nuclear accumulation of eIF4E (Topisirovic and others 2009a). Thus, both ribavirin and leptomycin B treatment lead to reduced mRNA export by eIF4E; one by displacing eIF4E to the cytoplasm and the other by inhibiting its export function, which is CRM1 dependent (Kentsis and others 2004; Culjkovic and others 2006).

In summary, eIF4E localization within and between the nucleus and cytoplasm can be modulated by many factors. Ultimately, modulating eIF4E trafficking can have profound effects on its mRNA export, mRNA sequestration, and translation roles. Given that eIF4E only affects the expression of certain transcripts at one of these levels, for instance, vascular endothelial growth factor is a translation, but not an export target (Culjkovic and others 2006), this leads to differential effects on the proteome. Thus, modulators of the subcellular distribution of eIF4E differentially effect gene expression thereby modulating the biological effects of the cells tailoring responses to given stimuli or stresses.

Targeting eIF4E at multiple levels in the clinic

As described above, eIF4E can also be dysregulated at the functional level by post-translational modifications. Such modification can occur downstream of activation of a num-

ber of common signaling pathways, including RAS, mitogen-activated kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and Mnk kinases. A full description of the regulation of these pathways and their diverse effects is beyond the scope of this review, and we have selected a few critical points for discussion here. Conceptually, 3 steps regulate the eIF4E function—its phosphorylation, its binding to regulatory cofactors, and its binding to cap complexes. Under normal cellular conditions, eIF4F complex assembly is limited as eIF4E is sequestered from eIF4G by binding to BP1 and other regulators. Stimulation of the PI3K/AKT/mTOR pathway leads to hierarchical 4EBP phosphorylation, dislodging 4EBP from eIF4E and enabling assembly of the eIF4F complex. This process is regulated by the PI3K/mammalian target of the rapamycin (mTOR) pathway and other signaling networks. Many clinical studies have used mTOR inhibitors as cancer therapeutics and it is assumed, but not proven that they work in part through inhibition of mRNA translation. One difficulty in interpreting *in vivo* work with rapalogs is the difficulty of assessing 4EBP1 phosphorylation, eIF4E activity, or mRNA translation in primary tissue. Thus, phosphorylation of the highly abundant ribosomal S6 protein is the typical surrogate for mTORC1 inhibition (Perl and others 2012). Interesting work in AML is consistent, however, with the hypothesis that rapamycin is an incomplete inhibitor of cap-dependent translation suggesting that the inhibition of eIF4E activity through rapamycin-dependent inhibition of mTORC1 is likely less than complete (Tamburini and others 2009). This and other data suggest that combination of drugs that feed into mRNA translation regulation would have a strong rationale in the clinic. Consistent with this idea, recent work has shown that combinations of rapamycin with Mnk inhibition can augment cytotoxic effects in T cell lymphoma (Marzec and others 2011). New families of drugs that can inhibit both mTORC1 and mTORC2 are currently being tested with hopefully more potent effects.

eIF4E can also be dysregulated via its enhanced phosphorylation. eIF4E is phosphorylated at S209, although the necessity of this event for function of eIF4E is not entirely clear and may be different in normal and malignant tissues. Mnk kinases phosphorylate eIF4E when it is bound to eIF4G and perhaps other factors. The effect of phosphorylation on eIF4E cap binding affinity is controversial with some studies suggesting an increase in affinity, while others suggest reduced affinity (Scheper and Proud 2002). Mnk1 was identified by homology to other serine threonine kinases and quickly recognized as a target of both Erk and p38 MAP kinases. It was also demonstrated to phosphorylate eIF4E on S209 (Waskiewicz and others 1997). This phosphorylation event increases cap-dependent translation in some, but not all systems. As noted, in normal cells, S209 phosphorylation may not be critical as a Mnk1/Mnk2 deficient mouse strain has a normal phenotype despite the complete lack of detectable eIF4E S209 phosphorylation (Ueda and others 2004). On the other hand, mutation of this residue completely abrogates its ability to transform cell lines and lead to tumors in animal models (Topisirovic and others 2004; Wendel and others 2004). Here as elsewhere, the suggestion is that highly elevated eIF4E expression and function are associated with malignant transformation, but not normal mRNA translation/metabolism regulation. Such specificity again suggests that Mnk kinases may be appropriate targets for cancer

therapy. A small molecular inhibitor (Tschopp and others 2000; Knauf and others 2001) was selected from a drug library and demonstrated to potently inhibit Mnk kinases, although nonspecifically. The compound, CGP57380, can inhibit transformation in cells overexpressing eIF4E (Topisirovic and others 2004), the growth of BCR/ABL-dependent cell lines (Zhang and others 2008), and survival of breast cancer cell lines (Wheater and others 2010). An orally available Mnk kinase inhibitor has recently been described. The compound inhibits the growth of melanoma and colon cancer xenografts without significant toxicity providing proof of principle that Mnk targeting is a promising avenue of investigation for cancer therapeutics (Konicek and others 2011). Interestingly, inhibition of Mnk kinase may be a double-edged sword. Elegant work by the Platanias laboratory has revealed that Mnk kinase plays a critical role in the growth inhibitory function of interferons (Joshi and others 2009; Altman and others 2010; Joshi and others 2011). Indeed these effects have been suggested to play a role in normal hematopoiesis (Joshi and others 2011).

Given these many associations with human malignancy, there has been substantial interest in targeting eIF4E both for therapeutic purposes and to increase understanding of its function. Studies dating back to 1991 by the De Benedetti and Zimmer laboratories demonstrated that antisense-mediated repression of eIF4E expression would slow cell growth in cancer cell lines (De Benedetti and others 1991; Rinker-Schaeffer and others 1993; Graff and others 1997). Graff and others (2007) took a substantial step forward in application of this approach with development of an eIF4E targeting antisense molecular containing a phosphorothioate backbone core (to promote RNase H-mediated degradation) flanked with five 2-methoxyethyl-modified (MOE-modified) bases to improve potency, nuclease resistance, and tissue half-life. Animal studies demonstrated that intravenous administration of this modified antisense oligonucleotide molecule decreased expression of eIF4E in multiple organs by nearly 80% and decreased growth of xeno-transplanted tumor cells without inducing toxicity (Graff and others 2007). This study demonstrated proof of principle that eIF4E targeting strategies would have a therapeutic index that would allow targeting to the molecule in cancer cells without the induction of toxicity (Graff and others 2007). This molecule (now designated LY2275796) has been taken forward into Phase I clinical trials and results were recently described (Hong and others 2011). In this trial, LY2275796 was given over an initial 3-day loading dose followed by weekly maintenance. The drug was well tolerated with only mild to modest toxicities observed. Importantly, the authors were able to obtain pretreatment and post-treatment biopsies and document slight decreases in eIF4E expression in most patients, but not nearly as extensive as observed in their animal models. No clinical responses were seen, perhaps due to inefficient knockdown of eIF4E or to synthetic lethal relationships with other pathways. There was no clear increase in efficacy at decreasing eIF4E between the top 2 doses and a dose of 1000 mg was chosen as both the maximum tolerated dose and the biologically effective dose. The authors conclude that administration of LY2275796 is feasible. However, the decrease in eIF4E expression in tumors was modest and it may be inadequate to provide therapeutic benefit by itself in humans. Combination studies of LY2275796 with chemotherapeutic agents are planned.

A more recent effort has involved characterization of a small molecular inhibitor, 4EGI-1 (Moerke and others 2007). Moerke and others initially identified this molecule and demonstrated that it could inhibit growth of cancer cell lines. Tamburini and others (2009) extended these results and demonstrated that 4EGI-1 inhibits the growth of primary AML cells. Of note, the authors primarily focused on the role of eIF4E in modulating mRNA translation and whether 4EGI-1 alters transcript export in AML cells is unknown. Relatively few other studies with this inhibitor have been published, although they are consistent with the data that 4EGI-1 can inhibit growth of cancer cells in myeloma, melanoma, and breast tumors (Pettersson and others 2011; Chen and others 2012; Descamps and others 2012). Subsequent work suggested that the molecule is not potent enough for *in vivo* work and may have other mechanisms of action, including effects on TRAIL (Blagden and Willis 2011).

Peptide inhibition using a novel tissue-specific targeting approach also suggests proof of principle that eIF4E is a potential target in ovarian cancer (Ko and others 2009). These studies demonstrate an elegant targeting mechanism by fusion of BP1 with a steroid agonist so that only ovarian cancer cells are targeted. These approaches, however, are not robust for clinical translation leading to a search for other inhibitors.

The Borden laboratory initially described the inhibitory effects of the antiviral, ribavirin, on eIF4E function (Kentsis and others 2004; Kentsis and others 2005; Assouline and others 2009). Direct binding studies indicate that ribavirin binds eIF4E in specific conditions, including at physiological pH and salt concentrations (Kentsis and others 2004; Kentsis and others 2005; Volpon and others, submitted). Studies have shown activity of ribavirin in inhibiting growth of breast cancer cell lines in an eIF4E-dependent manner (Pettersson and others 2011). Ribavirin is a nucleoside analog that also inhibits cellular inosine monophosphate dehydrogenase (IMPDH) and leads to a decrease in cellular GTP levels (Hong and Cameron 2002). Thus, descriptions of its anticancer activity may reflect diverse mechanisms of action, a favorable characteristic of the drug. Importantly, a ribavirin analogue that is a potent IMPDH inhibitor, tiazofurin, has no effect on eIF4E activity in translation or mRNA export and is completely unable to repress eIF4E-mediated transformation (Tan and others 2008). Further, mycophenolic acid, another potent IMPDH inhibitor, does not modulate eIF4E activity (Culjkovic-Kraljic and Borden, unpublished observation). Ribavirin is used for treatment of viral infections and its safety profile is well known. Investigators at McGill have led a multicenter trial studying ribavirin as a single agent for treatment of patients with AML of the M4/M5 subtype (Assouline and others 2009). Patients were refractory, relapsed, or unable to undergo chemotherapy. Among 11 evaluable patients, there were 1 complete remission and 2 partial remissions (overall 3/11 or 27% response rate in patients with high-risk disease). Importantly, ribavirin-induced relocalization of nuclear eIF4E to the cytoplasm and reduction of eIF4E levels were associated with a clinical response demonstrating a pharmacodynamics correlation with results described *in vitro* (Assouline and others 2009). Lack of response or relapse coincided with continued or renewed nuclear localization of eIF4E (Assouline and others 2009). Ribavirin similarly targeted the eIF4E activity in an ongoing combination study with low-dose ara-C, where

prolonged remissions have been observed [ClinicalTrials.gov NCT01056523 & (Assouline and others 2011)]. Indeed, ribavirin combined well with standard chemotherapies in primary AML specimens *ex vivo* (Kraljacic and others 2011]. Development of an eIF4E inhibitor that can safely be used in cancer therapy is an important goal and this proof of principle experiment suggests that this widely used drug may be an important agent in cancer therapy although it is unlikely to be highly active as a single agent.

Conclusions

To summarize, multiple lines of evidence suggest that control of post-transcriptional mechanisms of gene expression is a critical node for regulation of cancer cell growth. This node is largely regulated through the diverse functions of eIF4E. Although many studies have focused on the role of eIF4E in regulation only of mRNA translation, its regulation of cell growth occurs at multiple levels, only some of which are elucidated. Regulation of eIF4E itself is complex and initial studies summarized above show that small molecule inhibitors of some of the functions of eIF4E may be promising cancer therapeutics. Although inhibitors of Mnk and mTOR kinases are also being used as approaches to inhibiting mRNA translation, these approaches will not inhibit all of the functions of eIF4E. Future studies focusing on drug combinations of ribavirin or other eIF4E inhibitors with signal transduction inhibitor studies should be of great interest.

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