Therapeutic Opportunities in Eukaryotic Translation

Jennifer Chu¹ and Jerry Pelletier^{1,2,3}

¹Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada

²Department of Oncology, McGill University, Montreal, Quebec H3G 1Y6, Canada

³Rosalind and Morris Goodman Cancer Research Center, McGill University, Montreal, Quebec H3G 1Y6, Canada

Correspondence: jerry.pelletier@mcgill.ca

The ability to block biological processes with selective small molecules provides advantages distinct from most other experimental approaches. These include rapid time to onset, swift reversibility, ability to probe activities in manners that cannot be accessed by genetic means, and the potential to be further developed as therapeutic agents. Small molecule inhibitors can also be used to alter expression and activity without affecting the stoichiometry of interacting partners. These tenets have been especially evident in the field of translation. Small molecule inhibitors were instrumental in enabling investigators to capture short-lived complexes and characterize specific steps of protein synthesis. In addition, several drugs that are the mainstay of modern antimicrobial drug therapy are potent inhibitors of prokaryotic translation. Currently, there is much interest in targeting eukaryotic translation as decades of research have revealed that deregulated protein synthesis in cancer cells represents a targetable vulnerability. In addition to being potential therapeutics, small molecules that manipulate translation have also been shown to influence cognitive processes such as memory. In this review, we focus on small molecule modulators that target the eukaryotic translation initiation apparatus and provide an update on their potential application to the treatment of disease.

A large collection of chemical probes targeting various steps of prokaryotic and eukaryotic translation has been identified and characterized over the last ~50 years (Pestka 1977; Vazquez 1979; Pelletier and Peltz 2007; Malina et al. 2012). Early efforts in this endeavor were primed by the realization that prokaryotic and eukaryotic translations differ sufficiently to enable selective targeting, from which emerged some of the most effective antimicrobial therapies (aminoglycosides, tetracyclines, macrolides, chloramphenicol, clindamycin, spectinomycin,

streptogramins, lincosamides, oxazolidinones, etc.). In the last 15 years, there has been a resurgence of interest in targeting translation driven by a number of medical needs. One is the multidrug resistance crisis currently facing the field of medical microbiology and prompting an urgent need to identify new antibiotics. Another is the realization that targeting the translation apparatus in parasites, such as malaria, offers a broad therapeutic window (Baragaña et al. 2015). A third is the recognition that translational control is frequently usurped in human can-

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cers and represents a druggable vulnerability (Silvera et al. 2010; Stumpf and Ruggero 2011; Bhat et al. 2015; Pelletier et al. 2015; Chu et al. 2016a). Our goal in this review is to visit ongoing developments aimed at targeting the two main regulatory nodes of eukaryotic translation initiation: the eIF4F-mediated ribosome recruitment step and the modulation of eIF2 activity. eIF4F and eIF2 link translational output to changes in extra- and intracellular cues and are critical actors in maintaining cellular homeostasis. For a detailed review of the roles of eIF4F and eIF2 in translation, the reader is referred to Merrick and Pavitt (2018).

THERAPEUTIC TARGETS IN TRANSLATIONAL CONTROL

Messenger RNA (mRNA) Activation by eIF4F

Cap-dependent translation is mediated by eIF4F, a heterotrimeric complex comprising eIF4E, eIF4A, and eIF4G, with the essential eIF4E cap-binding protein being rate-limiting for translation (Fig. 1). Under conditions of growth restriction, the bulk of eIF4E is sequestered away from eIF4F into translationally inactive complexes by eIF4E-binding proteins (there

are three isoforms), leading to reduced cap-dependent translation (Pause et al. 1994). The phosphoinositide 3-kinase (PI3K)/mechanistic target of rapamycin (mTOR) pathway is critical to cell growth and survival and responds to a multitude of extra- and intracellular cues. Increased signaling flux leads to elevated 4E-BP phosphorylation and dissociation of eIF4E:4E-BP complexes. Because 4E-BPs and eIF4G share a common binding site on eIF4E, 4E-BP phosphorylation leads to increased eIF4F complex formation. This results in elevated translation of some mRNAs, several of which have been implicated in tumor onset and maintenance (e.g., c-MYC, cyclins, ornithine decarboxylase [ODC], BCL-2, MCL-1, BCL-X_L, osteopontin, survivin, vascular endothelial growth factor [VEGF], fibroblast growth factors [FGF]) (Colina et al. 2008; Mills et al. 2008; Petroulakis et al. 2009; Dowling et al. 2010; Bhat et al. 2015; Chu et al. 2016a). There are a number of strategies that have been explored to abrogate eIF4F function, including disruption of the eIF4E:eIF4G association, perturbation of the eIF4E:cap interaction, and inhibition of eIF4A and eIF4G activity (Fig. 1).

In mammals, eIF4E activity is also regulated through phosphorylation of Ser 209 by MNK1

Figure 1. Schematic diagram outlining the regulation of eIF4F assembly by mechanistic target of rapamycin (mTOR), eIF4E phosphorylation by MNK1/2, and messenger (mRNA) recruitment by eIF4F. The targets of known inhibitors are denoted. mTOR complex 1 (mTORC1) is a complex composed of mTOR, Raptor, MLST8, PRAS40, and DEPTOR and links nutrient/redox/energy levels to translation initiation.

(mitogen activated protein kinase [MAPK]-interacting kinases) and MNK2, two kinases that function downstream from the p38 MAPK and MEK-ERK (MAPK/ERK kinase-extracellular signal-regulated kinase) signaling cascades (Waskiewicz et al. 1999). In response to stress (p38 MAPK) or mitogens (MEK/ERK) (Cargnello and Roux 2011), the MNKs bind to eIF4G and phosphorylate eIF4E (Pyronnet et al. 1999). This causes preferential translation stimulation of a subset of mRNAs—several of which are known to be required for tumor maintenance (e.g., MCL1, MMP3, SNAIL) (Wendel et al. 2004; Furic et al. 2010; Robichaud et al. 2015).

The association and activity of eIF4A, the helicase subunit of eIF4F, is also affected by mTOR signaling flux through the S6 kinases (S6K1 and S6K2). Activation of S6K1 by mTOR leads to phosphorylation and degradation of programmed cell death 4 (PDCD4), an eIF4A-binding partner that represses translation (Fig. 1) (Yang et al. 2003a). Loss of PDCD4 results in increased eIF4A assembly into the eIF4F complex. S6K also phosphorylates the eIF4A auxiliary factor, eIF4B, which is an RNA-binding protein that promotes initiation complex formation and stimulates eIF4A helicase activity (Raught et al. 2004; Holz et al. 2005).

Ternary Complex Formation

The second major regulatory checkpoint in translation initiation is ternary complex (TC) formation (Fig. 2). Here, the initiator Met-tRNA^{Met}, eIF2, and guanosine triphosphate (GTP) form a TC that is recruited to 40S ribosomal subunits. Upon initiation codon recognition, GTP is hydrolyzed and eIF2-guanosine diphosphate (GDP) is released from the ribosome in complex with eIF5. The eIF2B guanine nucleotide exchange factor is required to exchange GDP for GTP as well as to displace eIF5 from eIF2 to enable its participation in subsequent rounds of initiation (Jennings and Pavitt 2014). In response to stress, the eIF2 α subunit is phosphorylated at Ser51 and this increases its affinity for the limiting amounts of eIF2B, leading to sequestration of the latter and reduced eIF2-GDP/eIF2-GTP recycling (Jennings and Pavitt 2014). Decreasing TC availability hinders global translation, while permitting stimulation of translation of a set of mRNAs encoding regulators of the stress response (e.g., GCN4 in yeast, ATF4 in humans), which is an event dependent on appropriately positioned short, upstream open reading frames (ORFs) (see Wek 2018). There are four known kinases that phosphorylate eIF2 α : (1) GCN2

Figure 2. Schematic diagram outlining the regulation of eIF2 recycling and the different steps known to be modulated by small molecules.

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(general control nonderepressible), (2) HRI (heme-regulated inhibitor), (3) PKR (double-stranded RNA-dependent protein kinase), and (4) PERK (PKR-like endoplasmic reticulum kinase). Dephosphorylation of eIF2 α is mediated by protein phosphatase 1 (PP1) containing either GADD34 or CReP (Fig. 2). Small molecules targeting the eIF2 regulatory node have been identified that interfere with TC formation, modulate eIF2 α kinase activity, block dephosphorylation of phospho-eIF2 α , and perturb eIF2B activity (Fig. 2).

TRANSLATIONAL CONTROL AND DISEASE

Cancer

The discovery that translation is dysregulated in cancer cells emerged from early studies documenting a small (~6%) change (increase and decrease) in unique mRNA species associated with polyribosomes from resting compared with growing cells (Williams and Penman 1975; Getz et al. 1976). The subsequent demonstration that eIF4E overexpression could drive tumor initiation (Lazaris-Karatzas et al. 1990) put translational control on the cancer research map and opened a new therapeutic avenue. Subsequent studies highlighted the pervasive extent to which deregulated translation contributes to tumor initiation and maintenance (Robert and Pelletier 2009). First, changes in expression levels and activity of a number of initiation factors have been documented in human cancer cells and tumors, many of which are associated with tumor progression and poor prognosis (Silvera et al. 2010; Chu et al. 2016a). The non-overlapping nature of the associated changes in mRNA translation suggests a complex layer of gene-expression regulation that remains to be systematically explored. Second, eIF4F activity is often altered by signaling pathways found activated in cancer (PI3K/mTOR, MEK-ERK, p38 MAPK) and this event has been linked to increased cell proliferation and survival (Bhat et al. 2015). Third, the quintessential c-MYC proto-oncogene regulates transcription of the genes encoding eIF2 α , eIF4E, eIF4A1, and eIF4G1 (Rosenwald et al. 1993; Lin et al. 2008).

Because c-MYC is a well-characterized eIF4F-responsive mRNA (Lin et al. 2012; Chu et al. 2016b), this establishes a positive feedback loop. Given that MYC occupies a pinnacle position in the cellular gene expression hierarchy and is among the top amplified genes across all human cancer types (Beroukhi et al. 2010), one would expect the MYC-eIF4F loop to be tightly regulated in normal cells. Fourth, mRNA sequence variations that alter translational efficiency have been associated with tumor initiation or maintenance. These include point mutations in the 5' leader region of c-MYC, BRCA1, and CDKN2A that affect protein output, as well as usage of alternative splice sites or transcription start sites that lead to significant nucleotide changes in the 5' leader region (Meric and Hunt 2003). Last, increased eIF4F activity, altered eIF4E/4E-BP1 ratios, and persistent eIF4F complex levels can impart resistance to chemotherapy (e.g., doxorubicin [Wendel et al. 2004], dexamethasone [Robert et al. 2014], cisplatin, and antimetabolic microtubule stabilizers [Zhou et al. 2011]) and to therapies targeting the PI3K/mTOR (Mallya et al. 2014) and MAPK pathways (Ilic et al. 2011; Zindy et al. 2011; Alain et al. 2012; Boussemart et al. 2014; Cope et al. 2014; Mallya et al. 2014). Collectively, these studies indicate that changes in translational control can have profound consequences for normal cellular physiology.

A number of in vitro studies have highlighted the value of targeting the eIF4E and eIF4A subunits to curtail tumor cell survival. (1) Early experiments using antisense approaches to suppress eIF4E expression in cell culture showed that this would block the growth of transformed cells, reduce soft agar colonization, increase tumor latency periods in xenograft models, reduce metastatic potential, and enhance chemosensitivity in vitro (Rinker-Schaeffer et al. 1993; Graff et al. 1995; Thumma et al. 2015). (2) Sequestration of eIF4E by ectopic expression of 4E-BP1 or by peptides harboring the eIF4E-binding site was shown to inhibit tumor cell proliferation (Rousseau et al. 1996; Herbert et al. 2000) and block eIF4E- and MYC-driven transformation ex vivo and in vivo in xenograft models (Jiang et al. 2003; Lynch et al. 2004). A fusion peptide

containing the eIF4E-binding site linked to a gonadotropin receptor agonist displayed antineoplastic activity when delivered by intraperitoneal injection to an ovarian xenograft model, with no detectable toxicity (Ko et al. 2009). (3) Antisense suppression of eIF4A or sequestration of eIF4A by ectopic expression of PDCD4 in tumor cells reduced cellular proliferation (Eberle et al. 2002), blocked transformation (Yang et al. 2003b), and delayed tumor onset in a chemically induced murine skin tumor model (Jansen et al. 2005). Delivery of an expression vector driving expression of PDCD4 to H-Ras12V mice with liver cancer significantly reduced tumor burden (Kim et al. 2013).

Manipulating TC availability as an anticancer approach has not been as extensively assessed. One problem is that eIF2 α phosphorylation can have either prosurvival consequences or trigger cell death, depending on context (Bi et al. 2005; Koromilas and Mounir 2013). For example, expression of a nonphosphorylatable eIF2 α mutant (S51A) is capable of transforming NIH 3T3 cells (Donze et al. 1995). Phosphorylation of eIF2 α is observed in response to treatment with different proteasome inhibitors and is associated with an apoptotic response (Jiang and Wek 2005a). However, phosphorylation of eIF2 α in response to ultraviolet (UV) irradiation (mediated by GCN2) facilitates nuclear factor (NF)- κ B activation and leads to a prosurvival response (Jiang and Wek 2005b). Thus, the issue of context has to be better understood to rationalize how best to manipulate this pathway to achieve a proapoptotic response when applied to the treatment of cancer cells.

Cognitive Functions

eIF2B exists as a dimer of two pentamers of five subunits (α – ϵ) (Kashiwagi et al. 2016). The α , β , and δ subunits make up the regulatory subcomplex, whereas the γ and ϵ subunits are responsible for the catalytic activity. Mutations in any of the five eIF2B subunits can lead to a progressive loss of brain white matter known as leukoencephalopathy with vanishing white matter (VWM) or childhood ataxia with central nervous system hypomyelination (CACH) (Kashiwagi et al. 2016). These mutations impact on eIF2B assembly and/or affect eIF2B: eIF2 interactions, both of which diminish TC formation and reduce translational output. This disorder highlights the critical role that eIF2B plays in nervous system development and maintenance.

Perturbing TC formation through modifying eIF2 α phosphorylation status can also affect cognitive function. In eIF2^{+/S51A} mice in which eIF2 α phosphorylation is reduced, long-term potentiation and memory are enhanced (Costa-Mattioli et al. 2007). Genetic deletion of PERK or GCN2 in mouse models of Alzheimer's disease (AD) alleviates deficits in protein synthesis, synaptic plasticity, and memory (Ma et al. 2013). Compounds that alter eIF2 α phosphorylation in the brain also impact cognitive functions. Minocycline (a semisynthetic tetracycline) can attenuate eIF2 α phosphorylation in an AD mouse model and this is associated with improved learning and memory (Choi et al. 2007). Trazodone hydrochloride and dibenzoylmethane, two compounds identified for their ability to reverse eIF2 α phosphorylation, can block neurodegeneration, restore memory deficits, and improve survival in prion-disease mice (Halliday et al. 2017). These and additional studies have implicated the eIF2 α kinases in cognitive functions and offer new and exciting avenues to potentially treat neurodegenerative disorders as well as alter cognitive functions (Trinh and Klann 2013).

Dysregulated translation has also been implicated as a core deficit in fragile X syndrome, with hyperactivation of mTORC1 and MAPK signaling associated with behavioral issues, learning deficits, and developmental delays (Hou et al. 2006; Gkogkas et al. 2014). Remarkably, metformin treatment of Fmr1^{-/-} mice ameliorated the core deficits in this model and this effect was associated with normalization of ERK signaling and reduced eIF4E phosphorylation (Gantois et al. 2017).

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IS TARGETING TRANSLATION INITIATION SAFE?

Targeting translation as an antineoplastic approach is already in the clinic, as exemplified

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by the use of omacetaxine mepesuccinate, a semisynthetic formulation of homoharringtonine (HHT), against tyrosine kinase inhibitor-resistant chronic myeloid leukemia (CML). HHT inhibits formation of the first peptide bond (Garreau de Loubresse et al. 2014) and, therefore, is quite different from a targeted therapy in which only one tumor cell dependency is blocked. A significant percent of patients with CML show a major cytogenetic response (i.e., reduction in Philadelphia chromosome-positive cells) when administered omacetaxine mepesuccinate (Alvandi et al. 2014). Several adverse reactions were reported (Alvandi et al. 2014), including some that were considered serious but manageable. HHT will block synthesis of all proteins in transformed and normal cells and the selectivity of HHT for CML versus normal cells likely stems from inhibition of synthesis of short-lived oncoproteins to which tumor cells have become addicted, such as MCL-1 and c-MYC (Chen et al. 2006; Robert et al. 2009a).

Unlike the global inhibitory effects on translation exerted in cells treated with HHT, suppression of eIF4E is expected to lead to a more selective reduction in translation of a subset of mRNAs. The preclinical data to date suggest that partial suppression of eIF4E at the organismal level is well tolerated. In mice engineered to express inducible eIF4E short hairpin RNAs (shRNAs) (*sh4E/rtTA*) in all tissues, transient eIF4E suppression affected only a subset of normal regenerating cells (e.g., immature/undifferentiated intestinal crypts), but was otherwise well tolerated and the aforementioned effects were rapidly and completely reversible (Lin et al. 2012). Systemic suppression of *sh4E/rtTA* mice also carrying an Eμ-Myc translocation significantly delayed B-cell tumor onset (Lin et al. 2012). Similarly, eIF4E^{+/-} mice showed no developmental defects and had normal body weights and survival (Truitt et al. 2015). When eIF4E^{+/-} mice were crossed to a KRas^{G12D}-driven lung cancer model, eIF4E^{+/-}/KRas^{G12D} mice showed reduced tumor burden compared with eIF4E^{+/+}/KRas^{G12D} mice (Truitt et al. 2015). Telling are the consequences of suppressing eIF4E expression systemically using antisense oligonucleotides (ASOs). Here, an eIF4E ASO that

targets the 3' untranslated region (LY2275796; GenBank: M15353, nucleotides 1285–1304) was developed and tested in vivo (Graff et al. 2007). When administered at 50 mg/kg thrice weekly in an MDA-MB-231 breast cancer xenograft model, a 64% reduction in eIF4E protein levels was documented in tumors and was associated with a pronounced suppression of tumor outgrowth. At 25 mg/kg thrice weekly (after an initial loading dose of 50 mg/kg) in a PC3 prostate cancer xenograft model, a 56% reduction in eIF4E protein levels was documented in tumors and was associated with a significant reduction in tumor outgrowth. No change in mean body weight was observed in the MDA-MB-231 or PC3 models. Lower concentrations of LY2275796 (5 mg/kg) had no effect on PC3 tumor outgrowth and failed to reduce eIF4E proteins in the tumors. Normal mice dosed with LY2275796 (40 mg/kg twice weekly; 80 mg/kg/wk) showed an 80% decrease in eIF4E protein levels in the liver (the site where ASOs preferentially accumulate) with no appreciable change in liver and spleen histology, body weight, or liver transaminase levels detected (Graff et al. 2007).

Two clinical trials with LY2275796 have been undertaken but they failed to satisfactorily address the issue of effectiveness of eIF4E suppression. A phase I trial primarily sought to determine an appropriate delivery dose for LY2275796 in patients (Hong et al. 2011). In this study, LY2275796 was delivered for 3 consecutive days followed by weekly maintenance doses (Hong et al. 2011). The maximum tolerated dose was designated as 1000 mg based on clinical, pharmacokinetic, and pharmacodynamics observations with patients having manageable toxicities (grade 1/2). This dose was reported to cause an 80% reduction in eIF4E mRNA levels (as determined using a branched DNA assay), but this could not be attributed to a specific effect of LY2275796 because expression of housekeeping genes was also reduced (by 64%). In pre- and posttreatment biopsy samples, cytoplasmic eIF4E protein levels were assessed by immunohistochemistry and reported to be reduced by ~25%. Cytoplasmic VEGF and nuclear cyclin D1 protein levels (two eIF4F-responsive mRNAs) were unaffected or reduced

~25%, respectively. The best response observed was seven patients out of 22 that achieved stable disease for a minimum of 6 weeks. We note that in this study, in which the median patient age was 58.5 years (and based on an average weight of 76 kg (see shortsupport.org/Research/Papers/NHANES.pdf), patients received ~52 mg/kg/wk (first week) and 13 mg/kg/wk (maintenance dose) of LY2275796, which is significantly lower than the doses used to achieve an antitumor response in the aforementioned mouse xenograft studies.

A second clinical trial looked at combining the 4E-ASO with irinotecan in solid tumors and irinotecan-refractory colorectal cancer (Duffy et al. 2016). The combination of LY2275796 (1000 mg/wk; 13 mg/kg/wk) and irinotecan was not well tolerated because of chronic low-grade toxicities (Duffy et al. 2016) and appearance of more severe toxicities (grade 3/4). This likely stemmed from an LY2275796: irinotecan drug interaction that increased the half-life of irinotecan and its metabolites leading to an increased drug exposure. Analysis of post- versus pretreated tumor biopsies found a reduction (~10%–75%) in eIF4E mRNA levels in 5/9 of patients, but no change in eIF4E protein levels (as assessed by immunohistochemistry). Overall, although the preclinical studies are encouraging, we cannot make general conclusions regarding the effectiveness of suppressing eIF4E and more robust and reliable approaches are needed to inhibit eIF4E/eIF4F activity in the clinical setting.

INHIBITING eIF4E

Targeting the eIF4E–Cap Interaction

Cap analogs have been used for the last 35 years to inhibit eIF4E cap-binding activity (primarily in *in vitro* biochemical assays), but assessment of their potential as antineoplastic agents is hindered by poor cell permeability and *in vivo* stability (Wagner et al. 2000). Cocrystal structures of eIF4E with cap analogs have defined the key features critical for cap recognition (Marcotrigiano et al. 1997, 1999; Niedzwiecka et al. 2002; Tomoo et al. 2002). These include the

delocalized positive charge of the 7-methyl guanosine ring, which forms cation- π stacking interactions with Trp56 and Trp102, a hydrogen-bonding interaction between the guanine carbonyl group and the backbone amide of Trp102, hydrogen bond engagement of guanine N1 and N2 with Glu103, and a hydrogen bonding network between phosphates, and mediated through water, with Arg112, Arg157, and Lys162 (Fig. 3A). This latter feature explains the higher binding affinity of m⁷GTP for eIF4E relative to m⁷GDP (39-fold) and m⁷GMP (407-fold). The eIF4E cap-binding pocket has three cavities that can accommodate extensions from the N7 position, and this explains why compounds that harbor bulky N7 substituents, like N7-benzyl GMP and 7-(*p*-fluorobenzyl)-GMP, still function as cap analogs (Brown et al. 2007). This insight was used to design a novel guanine analog (Cmpd #33) that showed a 147-fold increase in binding affinity for eIF4E compared with m⁷GMP (Fig. 3B) (Chen et al. 2012b). Unfortunately, Cmpd #33 suffers from poor cellular permeability.

To overcome this liability, Wagner and colleagues (Wagner et al. 2000) developed 4Ei-1, an N7-benzyl GMP tryptamine phosphoramidate pronucleotide (Fig. 3B) that is cell permeable and converts to the biologically active compound, 7-benzyl guanosine monophosphate in cells (Ghosh et al. 2009). 4Ei-1 has been shown to block the epithelial-to-mesenchymal transition in zebrafish (Ghosh et al. 2009) and in lung epithelial cells by inhibiting translation of Snail1 (Smith et al. 2015). It also chemosensitizes lung cancer cells to gemcitabine (Li et al. 2013) and mesothelioma cells to pemetrexed (Chen et al. 2014).

Targeting the eIF4E:eIF4G Interaction

Two independently conducted high throughput screens led to the discovery of three eIF4E:eIF4G interaction inhibitors: 4EGI-1, 4E1RCat, and 4E2RCat (Fig. 3C) (Moerke et al. 2007; Cencic et al. 2011a). Compound 4EGI-1 (which exists as two interconverting isomeric forms, E and Z) binds to eIF4E at a location removed from the eIF4G-binding site and causes local conforma-

Figure 3. Small molecule inhibitors of eIF4E. (A) Interactions between m⁷GTP and eIF4E are denoted. (B) Chemical inhibitors of eIF4E:cap interaction. (C) Chemical inhibitors of eIF4E:eIF4G interaction.

tional changes that lead to disengagement of eIF4G from eIF4E (Papadopoulos et al. 2014). An unexpected property of 4EGI-1 is that it increases 4E-BP1 binding to eIF4E in cells (Moerke et al. 2007). 4EGI-1 inhibits expression of known eIF4F-responsive mRNAs, such as cyclins D1 and E, c-MYC, and BCL-2 (Moerke et al. 2007), and blocks the hypoxia-induced gene-activation program (Yi et al. 2013). It selectively kills transformed cells more than nontransformed cells (Moerke et al. 2007; Tamburini et al. 2009) and shows efficacy in several cancer models, including U87 glioma, breast, and melanoma xenograft models (Chen et al. 2012a; Descamps et al. 2012; Wu et al. 2016) and against multiple myeloma (Descamps et al. 2012) and melanoma cell lines (Mahalingam et al. 2014). 4EGI-1 also impairs mitochondrial function (Yang et al. 2015). This compound has been used in studies to probe the role of eIF4F in memory, autism, and viral replication (Hoeffler et al. 2011; McMahon et al. 2011; Gkogkas et al. 2013; Santini et al. 2013). Rigidified variants of 4EGI-1 have been synthesized and characterized, with some capable of better inhibiting the eIF4E:eIF4G interaction than the parental compound (Fig. 3C) (Mahalingam et al. 2014).

4E1RCat and 4E2RCat prevent the association of both eIF4G and 4EBP1 with eIF4E (Fig. 3C) (Cencic et al. 2011a). 4E1RCat dampened protein synthesis by ~30% in Jurkat cells (Cencic et al. 2011a). 4E1RCat is pharmacologically active in mice and was shown to synergize with doxorubicin in a mouse lymphoma model, prolonging tumor-free survival compared with single-agent-treated cohorts (Cencic et al. 2011a). 4E1RCat blocks mitosis-restricted protein synthesis (Shuda et al. 2015) and decreases MCL-1 levels in HL-1 cardiomyocytes (Arnold et al. 2014). 4E1RCat and 4E2RCat have been used to block coronavirus replication (Cencic et al. 2011b).

Targeting eIF4A

There are two related eIF4A homologs in mammals, eIF4A1 and eIF4A2, which share 90% identity at the amino acid level (Nielsen and

Trachsel 1988; Galicia-Vazquez et al. 2012). Accurate quantitation of the two proteins has not been extensively performed across a large number of cell or tissue types. At the mRNA level, eIF4A1 is generally more abundantly expressed with notable exceptions being in fetal brain, heart, and kidney and adult brain, ovary, and skeletal muscle (Galicia-Vazquez et al. 2012). eIF4A is an abundant factor (about three to six molecules/ribosome) with the majority (~90%) existing outside of the eIF4F complex. Both eIF4A1 and eIF4A2 can assemble into the eIF4F complex. eIF4A1 suppression is not well tolerated, whereas eIF4A2 can be completely eliminated in NIH 3T3 fibroblast cells using CRISPR/Cas9 with no detectable effects on protein synthesis rates, proliferation, and viability. An analysis of the viability of 398 cancer cell lines in which eIF4A1 or eIF4A2 expression was suppressed using shRNAs (21 shRNAs targeting eIF4A1 and 19 shRNAs targeting eIF4A2 were used in this study) is consistent with eIF4A1, but not eIF4A2, being essential (McDonald et al. 2017). Three natural products, pateamine A (PatA), hippuristanol, and rocaglate family members have been identified as selective and potent eIF4A inhibitors.

Pateamine A

PatA is a naturally occurring metabolite isolated from the marine sponge *Mycale hentscheli* and was originally found to have potent cytotoxic activity against P388 leukemia cells (half-maximal inhibitory concentration, $IC_{50} = 0.27$ nM) (Northcote et al. 1991). Initial efforts to identify the molecular target of PatA involved affinity chromatography of total cell extracts prepared from HL60 cells using immobilized PatA, in which cytokeratin, tubulin, eIF4A1, eIF4A2, and eIF4A3 (DDX48) were retained (Bordeleau et al. 2005). In a separate set of experiments, also using immobilized PatA, eIF4A1 and serine/threonine kinase receptor associated protein (STRAP) were captured from RKO colorectal carcinoma cell extracts (Low et al. 2005). Overexpression of eIF4A1, but not STRAP, in HeLa cells increased the PatA IC_{50} fivefold suggesting that inhibition of eIF4A is primarily responsible

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for PatA's ability to block cell proliferation (Low et al. 2005). In addition to inhibiting translation through targeting eIF4A1 and/or eIF4A2, PatA also impedes nonsense-mediated mRNA decay by targeting eIF4A3, a core component of the exon junction complex (Dang et al. 2006). PatA is not a pan-helicase inhibitor as it does not block Ded1p helicase activity, in vitro splicing reactions, or cellular DNA and RNA synthesis (Bordeleau et al. 2005). Moreover, PatA does not inhibit prokaryotic translation (Bordeleau et al. 2005).

With respect to its mechanism of action, PatA increases the ATP hydrolysis rate and RNA-binding ability of free eIF4A (Bordeleau et al. 2005; Low et al. 2005), but not if eIF4A is present in the eIF4F complex (Bordeleau et al. 2005), suggesting that binding of eIF4A to eIF4G might occlude PatA binding. Exposure of cells to PatA reduces the levels of eIF4A1 found within the eIF4F complex (Low et al. 2005). On the other hand, high concentrations of PatA (10 μ M) have been reported to stimulate the interaction between eIF4A and eIF4B (Low et al. 2005), but this may be an indirect effect mediated by a bridging RNA fragment (Bordeleau et al. 2006b). Taken together, the current body of evidence depicts PatA as a compound that forces nonspecific eIF4A-RNA engagements, depleting eIF4A from the eIF4F complex with concomitant inhibition of cap-dependent translation (Bordeleau et al. 2006b). PatA blocks 48S complex formation in vitro and PatA-exposed cells have reduced polysomes (Bordeleau et al. 2005). The hepatitis C virus internal ribosome entry site (HCV IRES), which does not require any of the eIF4F subunits for initiation, is significantly more resistant to inhibition by PatA (Bordeleau et al. 2005; Low et al. 2005). As an inhibitor of translation, PatA also induces the assembly of stress granules (Dang et al. 2006; Mazroui et al. 2006). Although the PatA-binding site on eIF4A has yet to be determined, it may be located within the eIF4A amino-terminal domain (NTD) because a carboxy-terminal deletion of eIF4A (Δ 246–406) is still capable of binding PatA (Low et al. 2007).

The total chemical synthesis of PatA has been reported and second-generation analogs

have been prepared (Romo et al. 2004; Kuznetsov et al. 2009; Low et al. 2014). One analog, DMDA-PatA, has comparable cytotoxicity and activity toward eIF4A as PatA. PatA is an irreversible inhibitor of translation, possibly caused by the presence of a Michael addition site on this compound (Bordeleau et al. 2005; Low et al. 2005) and analogs lacking this feature are no longer cytotoxic (Low et al. 2014). PatA and DMDA-PatA inhibit proliferation and induce apoptosis in many tumor cell lines at nanomolar concentrations ex vivo as well as in melanoma xenograft models (Northcote et al. 1991; Hood et al. 2001; Low et al. 2005; Kuznetsov et al. 2009). DMDA-PatA is not a substrate for multidrug resistance protein 1 (MDR1) (Kuznetsov et al. 2009), a protein in which overexpression is associated with intrinsic and acquired drug resistance (Hodges et al. 2011).

Hippuristanol

Hippuristanol is a polyoxygenated steroid first isolated from the gorgonian *Isis hippuris* (Higa and Tanaka 1981; Higa et al. 1981). It prevents eIF4A from interacting with RNA without affecting ATP binding (Bordeleau et al. 2006a). The binding site for hippuristanol resides in the eIF4A carboxy-terminal domain (CTD) and involves amino acids spanning, and adjacent to, motifs V and VI, which are two regions that are conserved among DEAD-box RNA helicases and that participate in RNA and ATP binding (Bordeleau et al. 2006a; Lindqvist et al. 2008). The amino acids flanking motifs V and VI are not conserved among other DEAD-box helicases, providing an explanation for the specificity of hippuristanol toward eIF4A.

Hippuristanol has been used to link eIF4A-dependent initiation events to long-term synaptic potentiation (Ran et al. 2009; Hoeffler et al. 2013), herpes simplex virus 1 host shutoff (Dauber et al. 2011; Radtke et al. 2013), and influenza virus mRNA translation (Yángüez et al. 2011). Hippuristanol shows single-agent activity against DBA/MC fibrosarcoma cells (Higa et al. 1981), multiple myeloma cells (Robert et al. 2014), Burkitt lymphoma cells (Cencic et al. 2013), adult T-cell leukemia cells in vitro

and in vivo (Tsumuraya et al. 2011), and against lymphocytic leukemia P-388 tumors in mice (Higa et al. 1981). Combining hippuristanol with vemurafenib (anti-B-Raf therapy) (Boussemart et al. 2014), ABT-737 (Cencic et al. 2013), doxorubicin (Cencic et al. 2013), or dexamethasone (Robert et al. 2014) overcomes resistance to the initial therapy, an effect that is also obtained with rocaglates (see below), implying a role for eIF4A-dependent translation in drug resistance.

Several synthetic routes to hippuristanol have been reported (Li et al. 2009; Ravindar et al. 2010, 2011; Somaiah et al. 2014). These provided some insight into structure–activity relationships (SAR) with respect to translation inhibition (Li et al. 2009; Somaiah et al. 2014) and cellular cytotoxicity (Li et al. 2009). However, our understanding of hippuristanol SAR is still limited because of an inability to synthetically access functionality at a large number of locations on this molecule. To date, no analog has been made that has equivalent or greater potency toward inhibiting translation than hippuristanol.

Rocaglates

Rocaglates originated from plants of the *Aglaia* genus. They interact directly with eIF4A as determined by affinity chromatography (Chambers et al. 2013), differential scanning fluorimetry (Chu et al. 2016b), and cellular thermal shift assay (CETSA) (Chu et al. 2016b). A significant body of work has shown that rocaglates possess single-agent activity in a variety of cancer models, reverse the chemoresistance phenotype in a number of settings (including toward PI3K/mTOR and RAS/MAPK pathway inhibitors), and also affect tumor-supportive processes such as angiogenesis (reviewed in Bhat et al. 2015; Chu and Pelletier 2015). Importantly, through the generation of an eIF4A rocaglate-resistant allele using CRISPR/Cas9, the in vivo antiproliferative property of this class of compounds toward tumor cells has been shown to be a direct consequence of eIF4A1 inhibition (Chu et al. 2016b).

There has been significant interest in identifying mRNA features that modulate rocaglate

sensitivity. Ribosome profiling analysis of MDA-MB-231 breast cancer cells treated for 2 h with 25 nM silvestrol yielded modest repression of global translation, with only 3.3% of translating mRNAs being markedly inhibited (Rubio et al. 2014). These mRNAs had 5' leaders with longer, structured sequences and relatively low overall guanine-cytosine (GC) content compared with nonresponsive mRNAs (Rubio et al. 2014). Ribosome profiling analysis of KOPT-K1 T acute lymphoblastic leukemia cells exposed to 25 nM silvestrol for 45 min also identified 5' leader length as a silvestrol responsive feature (Wolfe et al. 2014). In addition, ~1/3 of silvestrol-responsive mRNAs harbored a putative G-quadruplex, a feature previously shown to confer rocaglate responsiveness (Cencic et al. 2009). A third study examined the consequences on translation in HEK 293 cells treated with the rocaglate, RocA (Iwasaki et al. 2016), and found that RocA-responsiveness did not correlate with the thermodynamic stability of the 5' leader or the presence of G-quadruplexes (Iwasaki et al. 2016). Using a Bind-n-Seq approach, a high-throughput method by which to identify in vitro protein–RNA interactions, revealed that RocA leads to clamping of eIF4A onto RNA-containing polypurine sequences (4–6 nts in length). In vitro experiments revealed that this clamping, when it occurs on mRNAs, can lead to reduced initiation events at start codons residing downstream from the polypurine stretch, suggesting impaired ribosome scanning. Rocaglates can also block eIF4F activity because cells exposed to these have reduced levels of eIF4A in the eIF4F complex, an effect that will block 48S pre-initiation complex formation (Bordeleau et al. 2008; Cencic et al. 2009).

Targeting eIF4G

eIF4G remains a largely unexplored drug target. Virtual docking experiments aimed at identifying novel inhibitors of AKT led to the discovery of BI-69A11, a compound that unexpectedly was also found to bind to eIF4G1 following mass spectrometry analysis of proteins from UACC903 cells retained using biotinylated BI-69A11 (Gaitonde et al. 2009; Feng et al. 2015).

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The pursuit of analogs with better pharmacokinetic properties led to the development of SBI-0640756 (SBI-756) (Feng et al. 2015). Isolation of eIF4F by cap-analog affinity chromatography from mouse embryonic fibroblast (MEF) cell extracts revealed partial loss of eIF4G in SBI-756-treated cells. SBI-756 inhibits AKT and NF- κ B activity and blocks growth of NF1-mutant, NRas-mutant, and BRAF-resistant melanoma cells (Feng et al. 2015). Nontransformed fibroblasts are threefold less sensitive than A375 or UACC903 melanoma cells to the cytotoxic effects of SBI-756. It will be important to determine to what extent these biological effects are a consequence of eIF4G perturbation versus other activities that this compound may have.

INHIBITING eIF2

Perturbing eIF2 Activity

Blocking Dephosphorylation of Phospho-eIF2 α

Ser/Thr protein phosphatase 1 can be recruited to dephosphorylate eIF2 α by either GADD34 or CReP, the two proteins shown to blunt the endoplasmic reticulum (ER) stress response (Novoa et al. 2001; Jousse et al. 2003). Because GADD34 enhances the toxicity of ER stress and GADD34^{-/-} mice develop normally (Marciniak et al. 2004), it was postulated that GADD34 inhibition might be of therapeutic benefit. To this end, a screen was undertaken in search of compounds (19,000 molecules tested) that could protect rat PC12 cells against ER stress-induced apoptosis (Boyce et al. 2005). Salubrinal, and a more potent and soluble derivative (Sal003) (Robert et al. 2006), were found to prevent the dephosphorylation of phospho-eIF2 α , likely by blocking the conserved PP1-binding domain of GADD34 and CReP (Boyce et al. 2005; Fullwood et al. 2012). These compounds have been used to show protective effects in a number of neurodegenerative disease models driven by ER stress that include (1) amyloid β -protein aggregation (Hoozemans et al. 2009; Lee et al. 2010); (2) mutant Huntingtin aggregation (Reijonen et al. 2008); (3) misexpression of α -

synuclein seen in Parkinson's disease (Smith et al. 2005); and (4) polyubiquitinated protein build up in a transgenic model of amyotrophic lateral sclerosis (Saxena et al. 2009). These results are difficult to reconcile with those of genetic experiments in which loss of PERK in mice expressing a familial AD-related mutation in APP and PSEN1 prevented deficits in synaptic plasticity and spatial memory associated with this model (Ma et al. 2013). It is possible that the differences are caused by salubrinal having additional activity (which has not been systematically assessed), to the timing of changes in eIF2 α phosphorylation status (e.g., loss of PERK before onset of AD pathology versus maintenance of phospho-eIF2 α levels during ER stress), or to differences in the models used. Salubrinal has been shown capable of resensitizing multiple myeloma cells to bortezomib (Schewe and Aguirre-Ghiso 2009). Current compound liabilities include low solubility and poor bioavailability (Long et al. 2005).

Guanabenz is a drug used to treat hypertension and acts as an α 2-adrenergic agonist. It binds to GADD34, but not CReP, and disrupts its interaction with PP1 α . Guanabenz is capable of rescuing cells from protein misfolding stress (Tsytler et al. 2011) and blocks the proliferation, survival, and invasion of 4T1 and MDA-MB-231 mammary tumor cells (Hama-mura et al. 2014). These results are exciting given the clinical experience with Guanabenz and its known safety profile and are encouraging for future strategies aimed at pharmacologically modulating ER stress.

Blocking eIF2 α Function

Another manner by which eIF2 function can be blocked is highlighted by experiments with NSC119889 and NSC119893, two fluorescein derivatives that prevent the binding of Met-tRNA^{Met} to eIF2 (Robert et al. 2006). These compounds were used to show a reduced requirement of the HCV IRES for TCs (Robert et al. 2006); the HCV IRES can use eIF5B, eIF3, and eIF2A for translation initiation under stress (Terenin et al. 2008; Kim et al. 2011).

Targeting eIF2B

Using a luciferase reporter-based screen, Sidrauski et al. (2012) identified ISRIB (integrated stress response inhibitor), a compound that inhibits PERK-mediated activation of ATF4 translation. The *trans*-isomer ISRIB-A1 is 100-fold more potent than its *cis*-isomer (Sidrauski et al. 2015a). ISRIB-A1 does not diminish PERK phosphorylation (if anything, an increase is observed) nor does it affect eIF2 α phosphorylation in ER-stressed cells (Sidrauski et al. 2015a). Yet, global translation rates are sustained in ISRIB-treated cells in the face of ER stress or when the eIF2 α S51D phosphomimetic allele is introduced into cells. In fact, ISRIB blocks the integrated stress response (ISR) triggered by all eIF2 α kinases (Sidrauski et al. 2012). Memory consolidation and enhancement are limited by the ISR and ISRIB-treated mice display enhanced spatial and fear-associated learning (Sidrauski et al. 2012). ISRIB completely reverses the translational effects elicited by eIF2 α phosphorylation and induces no major change in mRNA translation or levels in treated cells (Sidrauski et al. 2015b). At the molecular level, ISRIB likely overcomes the effects of eIF2 α phosphorylation by acting on eIF2B. ISRIB causes dimerization of eIF2B, enhances the thermostability of the eIF2B δ subunit in the CETSA assay, and enhances the GEF activity of eIF2B (Sidrauski et al. 2015a). Amino acid changes within the amino terminus of the eIF2B δ subunit confer ISRIB resistance (Sekine et al. 2015). Analogs with higher potencies are currently sought after and, one such compound, ISRIB-A17, has been found to be 10-fold more potent than the parental ISRIB-A1 compound (Sidrauski et al. 2015a; Hearn et al. 2016).

Modulating eIF2 α Kinase Activity

Many compounds have been identified that cause an increase in phospho-eIF2 α levels, but data are lacking regarding the specificity of these compounds and there is a paucity of genetic information linking their biological activity to changes in phospho-eIF2 α levels. For example, the flavonoids, genisten, and quercetin, are cy-

totoxic to tumor cells in vitro and induce eIF2 α phosphorylation at high concentrations (100 μ M) (Ito et al. 1999). However, supporting genetic data are needed to link changes in phospho-eIF2 α levels to the biological activity of these (and other) compounds, especially because genisten (Yan et al. 2010) and quercetin (Boly et al. 2011) are known tyrosine and serine/threonine kinase inhibitors and exert profound effects on the phosphoproteome.

1. Heme-regulated inhibitor (HRI). There are two known modulators of HRI activity: aminopyrazolindane and *N,N'*-diarylureas. Aminopyrazolindane was identified as an inhibitor from a screen reporting on HRI kinase activity (Garcia-Villegas et al. 2009). This compound suffers from low bioavailability and very rapid clearance in rats (Garcia-Villegas et al. 2009). A screen of 102,000 small molecules identified *N,N'*-diarylureas as a class of compounds capable of activating HRI and inducing eIF2 α phosphorylation (Chen et al. 2011; Denoyelle et al. 2012). *N,N'*-diarylureas interact directly with HRI, as determined by nuclear magnetic resonance (NMR) and by drug affinity-responsive target stability (DARTS), an assay that assesses small molecule binding to target by monitoring protection against proteolytic degradation (Chen et al. 2011). *N,N'*-diarylureas reduce proliferation of tumor cells, which is an effect linked to enhanced eIF2 α phosphorylation because ectopic expression of eIF2 α^{S51A} rescued the proliferative block. A subsequent screen of a ~1900 member *N,N'*-disubstituted urea library identified a compound (*N*-aryl, *N'*-cyclohexylphenoxyurea) that significantly increased eIF2 α phosphorylation and CHOP synthesis (Chen et al. 2013). We note that because the diarylurea scaffold is a widely used framework for the design of receptor tyrosine kinase (RTK) inhibitors (Shan et al. 2016), it will be important to link the biological activity of new compounds to changes in eIF2 α phosphorylation.
2. PKR. There have been a number of PKR inhibitors described in the literature. The compound 2-amino purine was the first reported

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inhibitor of PKR, but is neither very potent nor selective (Huang and Schneider 1990). Another inhibitor (Cmpd #16) was isolated by the Beal Laboratory (Jammi et al. 2003) from a collection of ATP site inhibitors and shown to be capable of rescuing a translational block imposed in vitro using a recombinant glutathione *S*-transferase (GST) fusion containing the PKR kinase domain. This compound is able to rescue translation inhibition caused by PKR in cells (Eley et al. 2007). In a separate study, a collection of kinase inhibitors was screened for their ability to block PKR activity (Bryk et al. 2011). Pharmacophore modeling of these hits (and of those from the Beal Laboratory screen [Jammi et al. 2003]), followed by an in silico screen of 15 million compounds identified several novel compounds that showed activity toward PKR, although their selectivity for PKR over other kinases remains to be established.

3. GCN2. An autophosphorylation assay was used to identify GCN2 kinase domain inhibitors. From a collection of kinase inhibitors, three compounds (indirubine-3'-monoxime, SP600124, and staurosporine) were identified (Robert et al. 2009b). Evaluation of 14 different indirubine-3'-monoxime analogs identified Syk1 (spleen tyrosine kinase inhibitor) as having equipotent activity as the parental compound. Inhibition of GCN2 in cells with these compounds showed a protective effect against UV-induced eIF2 α phosphorylation (Deng et al. 2002).
4. PERK. A phenotype-based screen was implemented based on ectopic expression of a CHOP-luciferase fusion mRNA in CHO cells designed to report on ISR induction (Harding et al. 2005). Two hits were identified from this screen, TGD31 and TGD45, both of which were subsequently benzoylated to improve cell permeability, leading to the generation of TGD31 and TGD45BZ. Treatment of cells with TGD45BZ causes ER stress and activation of PERK, but not of GCN2 or PKR (HRI activation could not be assessed because of its low expression level in fibro-

blasts). The ISR induced by these compounds is thought to be promoted by activation of PERK as well as additional contributing mechanisms, because ISR markers were also induced in TGD45BZ-treated PERK^{-/-} MEFs (Harding et al. 2005).

Structure-guided in silico docking was used to identify a different set of compounds with activity toward PERK, but kinome-wide assessment of their activities is lacking (Wang et al. 2010). In an assay assessing eIF2 α phosphorylation by GST-PERK, a theinopyrimidine-containing compound was identified from a collection of kinase inhibitors and inhibited PERK with an IC₅₀ of 11 nM (Axten et al. 2012). A series of analogs was generated, and informed by PERK-inhibitor cocrystal structures, GSK2606414 was developed. Kinome profiling showed extremely high selectivity for PERK and xenograft studies showed that this compound could reduce pancreatic tumor growth with no notable side effects (Axten et al. 2012). Medicinal chemistry optimization led to the development of GSK2656157, displaying an IC₅₀ of 0.9 nM against PERK and impressive selectivity; among 300 kinases tested against GSK2656157, the most potent off-target activity was against HRI (IC₅₀ of 460 nM) (Atkins et al. 2013; Axten et al. 2013). This compound reduced tumor outgrowth in xenograft settings established from pancreatic and multiple myeloma cells, as well as reduced angiogenesis and vascular perfusion. GSK2606414 and GSK2656157 have recently been shown to inhibit RIPK1 and induce RIPK1-kinase dependent cell death in a PERK-independent manner, indicating an off-target biological activity (Rojas-Rivera et al. 2017).

FUTURE PERSPECTIVE

A number of exciting opportunities remain for the discovery of small molecules targeting translation initiation. The interaction between eIF4A and eIF4G and the association between eIF4G and MNK1/2 have not been exploited for compound discovery. In addition, compounds that discriminate between eIF4A1, eIF4A2, eIF4G1,

and eIF4G3 activity would be useful to tease out potential differences among these factors in the translation process.

Recently, eIF2A, a factor that binds Met-tRNAi (but not GTP) and interacts with 40S ribosomes in a codon-dependent manner, has been identified as an essential factor required for tumor initiation in squamous cell carcinoma (Sendoel et al. 2017). It is thought that eIF2A mediates initiation at upstream ORFs (uORFs), imparting a profound effect on the cellular proteome. eIF2A^{-/-} mice are viable (Golovko et al. 2016), indicating that eIF2A inhibitors might affect normal cells minimally and produce few or minor side effects, while significantly impacting on tumor cell survival.

New compounds targeting other translation factors would be enormously helpful as tools to define whether these play critical roles in normal and abnormal physiology. For example, the multifactorial complex eIF3 has been implicated as a translational activator or repressor by binding to specific RNA elements, has cap-binding activity, promotes reinitiation, and overexpression of some of its subunits is oncogenic (Zhang et al. 2007; Roy et al. 2010; Hershey 2015; Lee et al. 2015, 2016; Kumar et al. 2016). A better understanding of the role of eIF3 in the initiation process may provide insight into the molecular basis of different translation initiation pathways. Likewise, the role that eIF4B plays in preinitiation complex recruitment, in stimulating eIF4A helicase activity, and unraveling its putative eIF4A-independent function in translation still remain to be elucidated. Hence, future progress in this field holds promise to shed light on the fundamental process of translation and offers the exciting possibility of uncovering novel therapeutic strategies and compounds.

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