The Centenary Award

Signalling to eIF4E in cancer

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Abstract

Translational control plays a critical role in the regulation of gene expression in eukaryotes and affects many essential cellular processes, including proliferation, apoptosis and differentiation. Under most circumstances, translational control occurs at the initiation step at which the ribosome is recruited to the mRNA. The eukaryotic translation initiation factor 4E (eIF4E), as part of the eIF4F complex, interacts first with the mRNA and facilitates the recruitment of the 40S ribosomal subunit. The activity of eIF4E is regulated at many levels, most profoundly by two major signalling pathways: PI3K (phosphoinositide 3-kinase)/Akt (also known and Protein Kinase B, PKB)/mTOR (mechanistic/mammalian target of rapamycin) and Ras (rat sarcoma)/MAPK (mitogen-activated protein kinase)/Mnk (MAPK-interacting kinases). mTOR directly phosphorylates the 4E-BPs (eIF4E-binding proteins), which are inhibitors of eIF4E, to relieve translational suppression, whereas Mnk phosphorylates eIF4E to stimulate translation. Hyperactivation of these pathways occurs in the majority of cancers, which results in increased eIF4E activity. Thus, translational control via eIF4E acts as a convergence point for hyperactive signalling pathways to promote tumorigenesis. Consequently, recent works have aimed to target these pathways and ultimately the translational machinery for cancer therapy.

Introduction

mRNA translation is divided into three steps: initiation, elongation and termination [1–3]. Initiation is the rate-limiting step of translation and is subject to extensive regulation. A large body of evidence shows that translational control occurs predominately at the initiation step [4,5]. All nuclear transcribed mRNAs contain the cap structure or '5'-cap', m7GpppN (where m is a methyl group and N is any nucleotide), which is added very early at the

transcription elongation step [6]. Translation initiation in eukaryotes commences with the binding of the eukaryotic translation initiation factor 4F (eIF4F) complex to the 5'-cap (Figure 1) [6]. eIF4F consists of the cap-binding subunit, eIF4E, the RNA helicase eIF4A and the scaffolding protein eIF4G [4,5,7]. It is thought that eIF4A unwinds the secondary structure present in the 5'-UTR of the mRNA to promote the binding of the ribosome and its scanning of the 5'-UTR [4,5]. Several other helicases, such as Ded1 and DHX29 (DEAH box polypeptide 29), are also involved in this process [8]. eIF4G interacts directly with eIF4E, eIF4A, eIF3 and the poly (A)-binding protein (PABP) [4,5]. The interaction of eIF4G with the multi-component initiation factor eIF3 is required in mammals for the recruitment of the 43S pre-initiation complex (which consists of the 40S ribosomal subunit and associated initiation factors), via the direct binding of eIF3 to the 40S subunit [4,5]. Following assembly at the cap structure, the 43S pre-initiation complex traverses the mRNA 5'UTR in a 5' to 3' direction, until it encounters the initiation codon (usually AUG, but in rare cases a near-cognate AUG), where it stops and the 60S large ribosomal subunit joins to form the 80S ribosomal complex, which is followed by the translation elongation step [9]. The eIF4G interaction with PABP brings about the circularization of the mRNA,

Figure 1 | Model of cap dependent translation initiation

eIF4F binds to the m⁷G cap structure via the cap-binding protein eIF4E. eIF4G is a scaffold protein which also binds to the RNA helicase eIF4A and eIF3, which in turn recruits the 43S pre-initiation complex (PIC). The PIC consists of the 40S ribosomal subunit, eIF2-GTP-Met-tRNAi and several other initiation factors, which are indicated. eIF4A melts the secondary structure in the 5'-UTR and the PIC scans the mRNA until it encounters the AUG start codon where the 60S subunit joins, followed by peptide chain synthesis. eIF4G also binds to PABP which brings about the circularization of the mRNA allowing for efficient ribosome recycling.
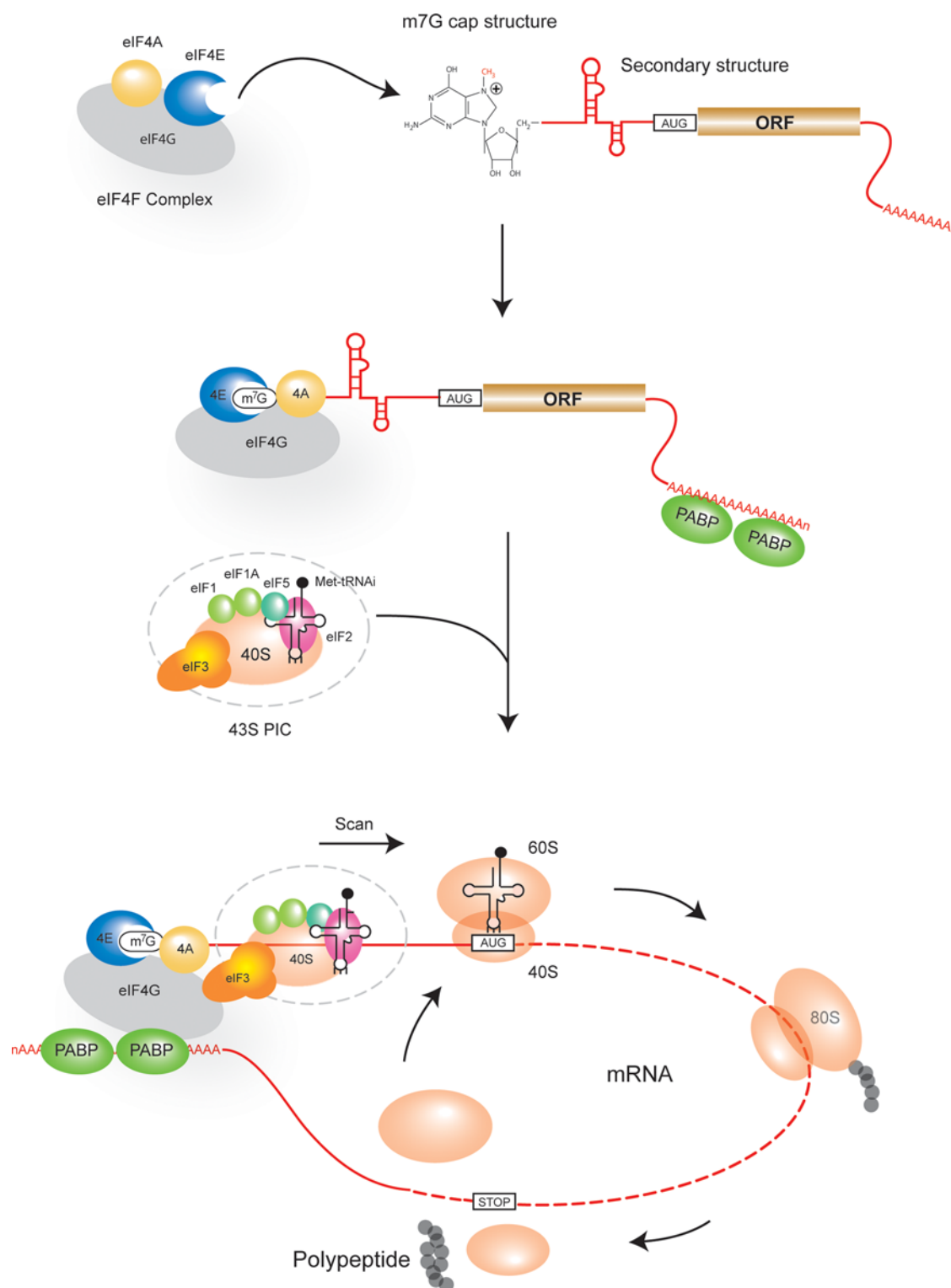
which is thought to facilitate the shunting of ribosomes from the termination codon to the mRNA 5′-cap to stimulate translation [10,11].

With the exception of mRNAs that translate via an internal ribosome entry site (IRES), the vast majority of eukaryotic mRNAs are translated in a cap-dependent manner [4,5]. Although eIF4E is necessary for cap-dependent translation, its requirement varies dramatically among mRNAs, as it preferentially stimulates the translation of a subset of mRNAs which are referred to as 'eIF4E-sensitive' [12]. These mRNAs mostly include those encoding proliferation and survival-promoting proteins such as cyclin D1 and D3, c-Myc, MDM2 (mouse double minute 2), VEGF (vascular endothelial growth factor), survivin and Bcl-2 (B-cell lymphoma 2) [13]. Considerable research efforts have been dedicated to the understanding of the mechanism that explains the preference for eIF4E-sensitive mRNAs. In general, mRNAs containing long G/C-rich 5′-UTRs, with the potential of forming stable secondary structures are feebly translated [14]. Reducing secondary structure by denaturing the mRNA enhances translation [15], whereas inserting sequences in the mRNA 5′-UTR that can form secondary structure impairs translation [16–20]. Strikingly, mRNAs with increased secondary structure are highly dependent on the cap structure for efficient translation [16]. Consistent with these studies, mRNAs with extensive secondary-structure in their 5′-UTRs are exceedingly dependent on eIF4E and eIF4A activity [21,22]. These observations can be mechanistically explained by recent findings showing that eIF4E exhibits a novel activity [23]. The eIF4E-binding region within eIF4G can inhibit eIF4A helicase activity when not bound to eIF4E. This inhibition is alleviated upon eIF4E binding to eIF4G. Thus, in addition to its canonical function as the 5'-cap-binding protein, eIF4E indirectly stimulates eIF4A helicase activity [23]. Recent reports have identified other motifs in mammalian mRNA 5′-UTRs that confer stimulation via eIF4E. These include terminal oligo pyrimidine (TOP) sequences at the mRNA extreme 5′-end and TOP-like mRNAs, which contain pyrimidine-rich sequences distal to the mRNA 5′end [24,25]; see other reports with additional conclusions [26,27]. The mechanisms by which eIF4E preferentially up-regulates the translation of TOP mRNAs are unknown.

eIF4E amounts and activity are regulated by multiple mechanisms. These include: (1) amplification of the gene encoding eIF4E; (2) transcriptional activation, particularly by c-Myc; (3) control of eIF4E activity by eIF4E-binding proteins (4E-BPs) and (4) post-translational modifications such as phosphorylation [12]. There are three 4E-BP homologues in mammals: 4E-BP1, 2 and 3 [7]. 4E-BPs and eIF4G share a common binding site for eIF4E [28]; thus, the interaction of 4E-BPs with eIF4E precludes the binding of eIF4E to eIF4G, causing impaired assembly of the eIF4F complex [29,30] (Figure 2). 4E-BPs are phosphoproteins, whose binding to eIF4E is determined by their phosphorylation status. The dephosphorylated forms of 4E-BPs bind avidly to eIF4E and therefore act as translational suppressors.
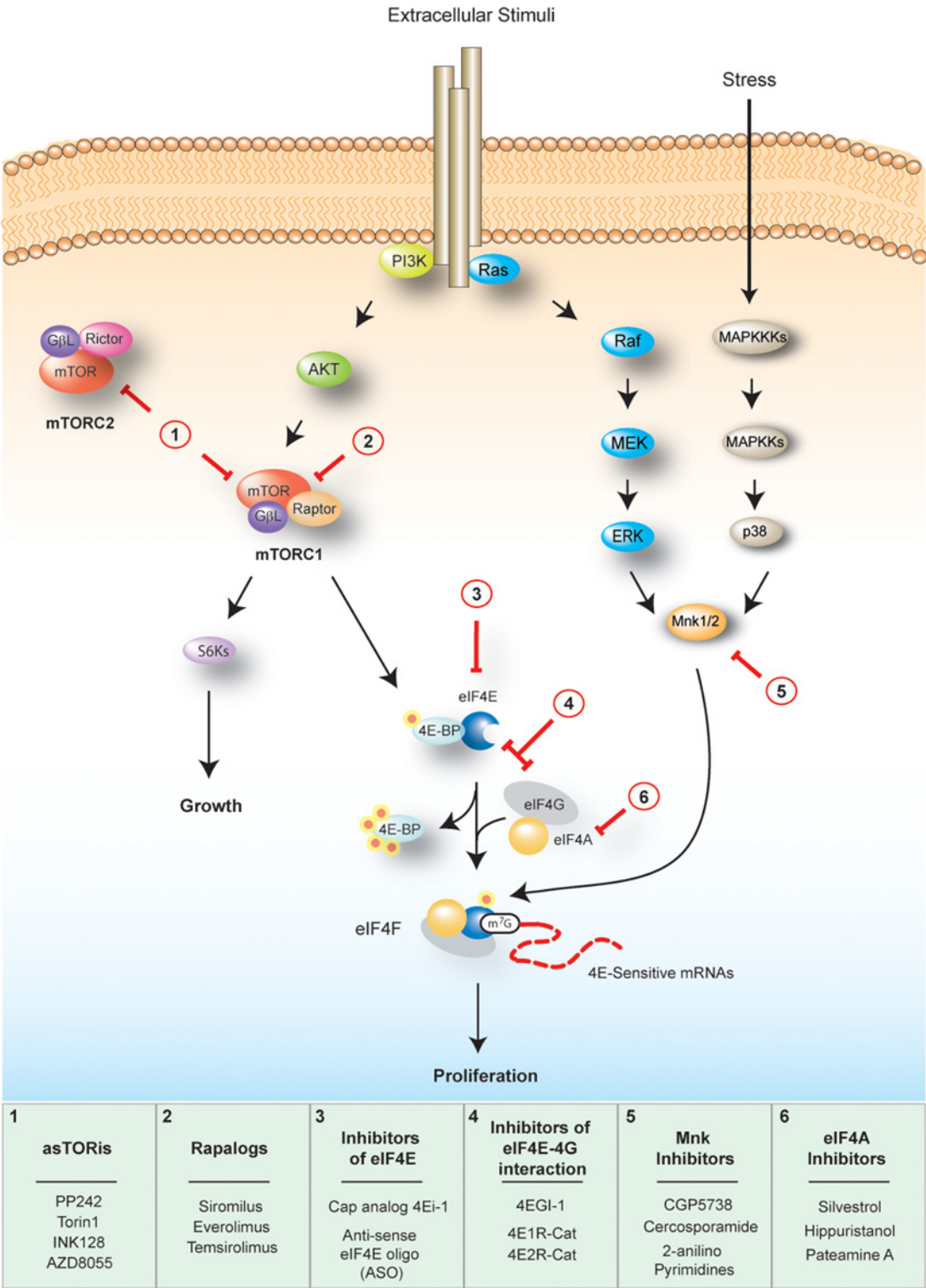
The mTOR pathway regulates eIF4E

The mechanistic/mammalian target of rapamycin (mTOR) is a highly evolutionarily conserved serine/threonine kinase that controls a staggering number of key cellular processes including protein synthesis, cell growth and proliferation, lipid metabolism, mitochondrial function, autophagy and cytoskeleton organization [31–33]. mTOR forms two separate complexes: mTOR complex 1 and 2 (mTORC1 and mTORC2) [32] [34] (Figure 2). Both mTOR complexes share the protein subunit LST8 (lethal with sec13 protein 8)/GβL (G-protein beta subunit like) [34,35]. mTORC1 contains the subunit Raptor that functions as an adaptor for the substrates of mTORC1, such as 4E-BPs, S6 kinases, PRAS40 (proline rich Akt substrate of 40 kDa) and Deptor [32,36]. mTORC2 contains the subunits Rictor, Sin1 (stress activated protein-kinase interacting protein) and Protor [32]. mTORC1 integrates a large number of inputs including extracellular stimuli signals such as growth factors, hormones and stress as well as intracellular cues, such as energy status, amino acid amounts and oxygen levels [37]. mTORC1 is activated via ordered serine/threonine phosphorylation and GTPase events, which constitute the PI3K (phosphoinositide 3-kinase)/Akt/mTOR signalling pathway. PI3K is a lipid kinase that phosphorylates phosphatidyl inositol 3,4 (PIP_2) to generate phosphatidyl inositol 3,4,5 (PIP_3), which activates the kinase Akt (also known as protein kinase B) [31,38]. The action of PI3K is antagonized by PTEN (phosphatase and tensin homologue on chromosome 10) a PIP_3 phosphatase [39,40]. PIP_3 binds to the pleckstrin homology (PH) domain of Akt to promote the translocation of Akt to the plasma membrane [41–43], where it is phosphorylated and activated by several kinases including mTORC2 [44]. Among its numerous targets, phosphorylation of tuberous sclerosis complex 2 (TSC2; a subunit of the tuberous sclerosis complex) by Akt stimulates mTORC1 activity [45–47]. Arguably, the best-studied and understood function of mTORC1 is its role in translation control. mTORC1 phosphorylates (inactivates) the 4E-BPs, leading to their dissociation from eIF4E which allows eIF4F formation and translation progression [48,49] (Figure 2). In our laboratory, we investigated the role of mTOR in promoting the translation of the 'eIF4E-sensitive' subset of mRNAs via the eIF4F complex [50,51].

Phosphorylation of eIF4E and cancer

eIF4E was suggested to act as an oncogene because it malignantly transformed NIH 3T3 murine fibroblasts [52] and subsequently rat primary embryo fibroblasts, in combination with c-Myc or adenovirus E1A [53] and human mammary cells [54]. It is remarkable that a relatively modest level of overexpression (∼2.5-fold) of eIF4E in NIH 3T3 was sufficient to cause transformation [55]. This is highly

Figure 2 | Signal transduction pathways converging on eIF4E

The PI3K pathway is activated in response to many extracellular stimuli resulting in the activation of the downstream serine/threonine kinase, mTOR. mTOR is a multi-domain protein which associates with several binding partners to form two different complexes mTORC1 and mTORC2. mTORC1 phosphorylates the translation repressors 4E-BPs, which then dissociate from eIF4E, allowing eIF4F formation and thus promoting translation. Mitogenic and stress signals activate components of the MAPK pathway including the ERK and p38 MAP kinase. Both converge to activate Mnk1/2, which bind to eIF4G and phosphorylate eIF4E at Ser[209]. Both pathways are hyperactivated in the majority of human malignancies. Drugs targeting, these signalling pathways and translation initiation factors are shown in red.

1	2	3	4	5	6
asTORis	**Rapalogs**	**Inhibitors of eIF4E**	**Inhibitors of eIF4E-4G interaction**	**Mnk Inhibitors**	**eIF4A Inhibitors**
PP242 Torin1 INK128 AZD8055	Siromilus Everolimus Temsirolimus	Cap analog 4Ei-1 Anti-sense eIF4E oligo (ASO)	4EGI-1 4E1R-Cat 4E2R-Cat	CGP5738 Cercosporamide 2-anilino Pyrimidines	Silvestrol Hippuristanol Pateamine A

significant, because in human cancers the magnitude of eIF4E increase is in this range [56]. Consistent with these results, depletion of eIF4E significantly reduced Ras-mediated tumorigenesis [57]. The conclusions from the *in vitro* experiments were bolstered by subsequent *in vivo* work in mice, which showed that overexpression of eIF4E augmented Eμ-Myc-driven lymphomas [58] and engendered cancers in a multitude of organs, when expressed from the β-actin promoter [59]. eIF4E activity is also regulated via the MAPK (mitogen-activated protein kinase) pathway through direct phosphorylation by the MAPK-interacting kinases (Mnk1 and Mnk2) at a single residue, Ser[209] [60,61]. Phosphorylation of eIF4E plays an important role in cancer development and progression [62–65]. Ectopic expression of the eIF4E[S209A] mutant protein failed to cause neoplastic transformation in NIH 3T3 cells and in the Eμ-Myc lymphoma mouse model [62,63]. Engineered knockin mice, in which the wild-type allele of eIF4E was replaced by the eIF4E[S209A] allele, were crossed with mice in which PTEN was deleted in the prostate. This deletion causes early onset of prostate intraepithelial neoplasia (PIN) and invasive carcinoma [66]. However, strikingly, the eIF4E[S209A] mutant mice were resistant to PIN and invasive carcinoma [64]. These results are highly relevant to human prostate cancer, inasmuch as eIF4E amounts and phosphorylation are gradually elevated in the progression of prostate cancer from PIN through hormone-sensitive and hormone-resistant forms [64]. In more recent studies, the mutant eIF4E[S209A] mouse was also shown to be resistant to polyoma middle-T driven mammary tumours [65]. Availability and phosphorylation of eIF4E promote metastasis in mice [67,68]. Translation of a subset of mRNAs, encoding several pro-metastatic proteins, such as MMP-3 (matrix metalloproteinase-3) and MMP-9, was reduced in the mutant eIF4E[S209A] mouse. MMPs cleave constituents of the extracellular matrix and promote migration and invasion [69]. eIF4E phosphorylation stimulated the translation of *Mmp3* and *Snail* mRNAs whose proteins promote invasion and epithelial-to-mesenchymal transition (EMT), which is required for metastasis [64]. Indeed, tumour growth factor β (TGFβ), which is an established inducer of EMT [70], promotes the phosphorylation of eIF4E via activation of ERK (extracellular signal regulated kinase) and p38 MAPK, which phosphorylate Mnk [71]. Strikingly, the phosphorylation of eIF4E by MNK1 is required for TGFβ-induced EMT [65].

Strategies for targeting eIF4E in cancer therapy

In light of the idea that eIF4E is a convergence point for the major cancer related signalling pathways [72,73] (Figure 2) and that eIF4E is activated or overexpressed in a large number of tumours, there has been considerable effort to target eIF4E directly or indirectly for cancer therapy. eIF4E activity in cancer can be targeted indirectly by inhibitors of the PI3K/Akt/mTOR pathway, which cause the dephosphorylation of 4E-BPs and inhibition of eIF4E. Some of these compounds, prominently rapamycin derivatives (rapalogues) are in use in the clinic for certain cancers, but many more are in clinical trials, particularly PI3K inhibitors and active-site mTOR inhibitors (asTORi); the latter inhibiting both mTORC1 and mTORC2 [74,75]. A highly pertinent question is whether eIF4E is a pivotal target that mediates the therapeutic activity of these inhibitors in cancer. Some affirmative answers to this question were obtained recently showing that cells in culture, which develop resistance to these drugs exhibit amplified eIF4E. Cells that became resistant to NVP-BEZ235, which is a dual PI3K/mTOR inhibitor, exhibited amplified c-Myc and eIF4E genes [76] and cells which acquired resistance to AZD8055, an asTORi, had amplified eIF4E [77]. These results support earlier findings from our laboratory that the ratio of eIF4E/4E-BP is a prime predictor of the efficacy of asTORi in reducing tumour growth in mice [78]. Moreover, asTORi inhibit cell proliferation, but not cell growth via inhibition of 4E-BP phosphorylation and subsequent suppression of translation of 'eIF4E-sensitive' mRNAs [79].

One of the first attempts to target eIF4E directly was undertaken by Graff et al. [80] by developing an anti-sense oligonucleotide (ASO) against eIF4E, which preferentially inhibited the translation of eIF4E-sensitive mRNAs encoding proteins, such as VEGF, cyclin D1, survivin, c-Myc, and Bcl-2, in cultured cells. Most striking was the observation that intravenous administration of ASO selectively reduced eIF4E expression in human tumour xenografts and dramatically suppressed tumour growth. eIF4E ASO reduced eIF4E levels in the mouse (80 % in the liver), but importantly, had no effect on body weight, organ weight or liver transaminase levels [80]. The puzzling question as to why a dramatic reduction in eIF4E did not significantly impair translation but rather caused only minimal deleterious effects in the mouse is most probably explained by results obtained from cells in culture in which shRNA was used to deplete eIF4E [81]. When eIF4E is dramatically reduced, the non-phosphorylated 4E-BPs, which no longer have a binding partner, undergo ubiquitination and subsequent degradation [81]. Thus, depletion of eIF4E also causes a reduction in the amounts of its inhibitory 4E-BPs and therefore, the potential deleterious effects of eIF4E depletion are significantly ameliorated.

As described above, phosphorylation of eIF4E plays an important role in cancer progression. Thus, another strategy to diminish eIF4E activity has been to develop drug-candidate compounds that target Mnks to prevent eIF4E phosphorylation [82]. These include CGP57380 [83], cercosporamide [84] and 5-(2-(phenylamino) pyrimidin-4-yl)thiazol-2(3H)-one derivatives [85]. As double 'knockout' Mnk1/2 mice develop normally and appear healthy [86], compounds that acutely inhibit Mnk activity are highly attractive candidates to treat cancer.

Another class of promising inhibitors comprises compounds that prevent eIF4E–eIF4G interactions [87] (Figure 2). These compounds disrupt the formation of the eIF4F complex, and therefore impair translation of eIF4E-sensitive mRNAs. The archetype of this family of compounds,

Figure 3 | Targeting eIF4E in tumour heterogeneity

A schematic diagram illustrating a simplified version of diversity within a hypothetical heterogeneous tumour. Sub-populations of cells expressing HER2 (green), ERα (blue) or presenting BRCA1/2 mutations (red) are shown. In contrast, all tumour cells contain elevated eIF4E. The tumours containing the indicated targets are treated in the clinic by the corresponding drugs listed in the figure. We hypothesize that direct eIF4E inhibitors will target all types of tumour cells, regardless of their genetic make-up.
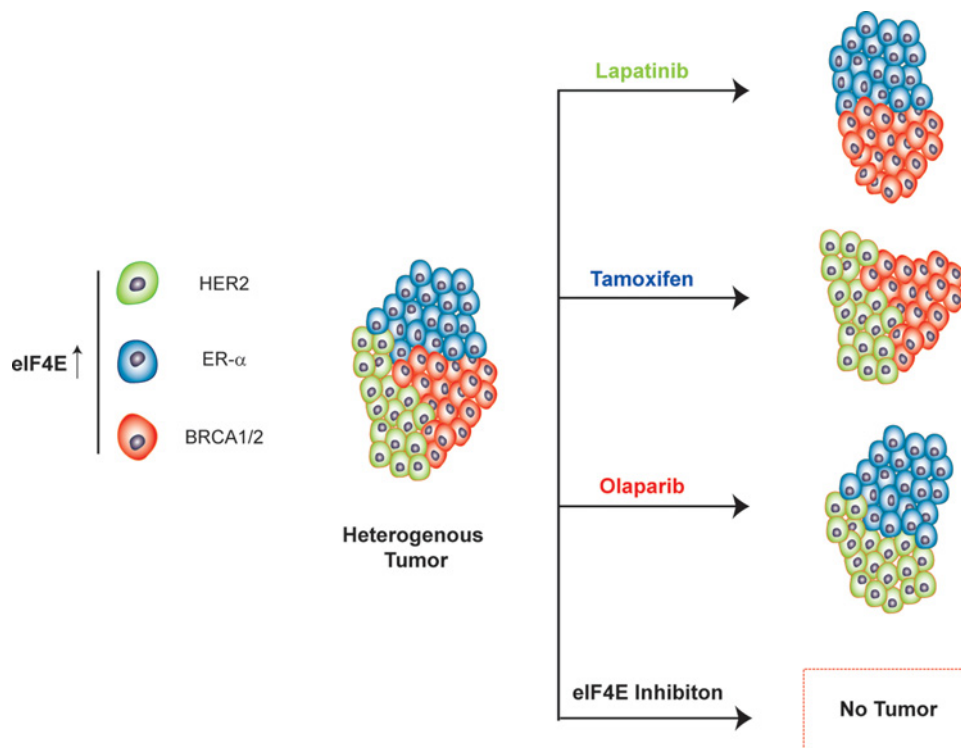
4EGI-1, suppressed the growth and induced apoptosis of multiple myeloma and lung cancer cells *in vitro* and inhibited myeloma and breast cancer xenografts without apparent toxicity *in vivo* [88–92]. Accordingly, current efforts are focused on developing more potent analogues of 4EGI [93–95]. Mechanistically, 4EGI-1 binds to a region of eIF4E that is distant from the eIF4E-eIF4G interface and induces an allosteric inhibition of the interaction between the two-subunits [87]. In addition to preventing eIF4G–eIF4E interaction, 4EGI-1 also stabilizes the 4E-BP binding to eIF4E, which exacerbates the translation inhibition of eIF4E-sensitve mRNA [87]. This observation was initially surprising and suggested that eIF4G and 4E-BPs may have some non-overlapping interaction sites with eIF4E. Indeed, recent structural studies have shown that 4E-BP2 contains a non-canonical motif (conserved in all 4E-BPs), which contacts a region of eIF4E that is not used by eIF4G [96,97]. Further structural characterization of eIF4E complexes revealed unique binding elements in different eIF4E binding partners that could be fused into a chimeric '4E-BP-like' mimic and dampen translational activity [98]. NMR studies using the full-length 4E-BP revealed that phosphorylation of 4E-BP results in a transition from a binding-competent disordered state to a folded state that cannot bind to eIF4E [99]. The latter findings raise for the first time the intriguing possibility of targeting a regulator of eIF4E. It is proposed that small molecules could be designed to alter the stability of 4E-BPs and thus alter its affinity for eIF4E [99]. Taken together, these detailed structural studies will aid in the rational design of more potent treatments such as 4EGI-like compounds, 4E-BPs mimics or small molecules that stabilize 4E-BP-eIF4E interactions.

As eIF4E's role in tumorigenesis depends on its ability to bind the 5′-cap, a key strategy has focused on developing bioavailable 5′-cap analogues which directly target eIF4E [63,100]. For instance, numerous atomic structures of eIF4E have been studied in great detail, with and without different 5′-cap structures [87], which have aided in the design of 5′-cap-mimetics such as 4Ei-1 [101]. 4Ei-1 inhibited cap-dependent translation and exhibited anti-neoplastic activities with minimal toxic effects [101–103]. High throughput screening efforts for more effective 5′-cap mimetics are ongoing [104].

The bevy of inhibitors of eIF4E and also eIF4A (see below) [105] provides an intriguing opportunity to cope with the pernicious problem of tumour heterogeneity in

targeting cancer. It is well established that most cancers consist of heterogeneous tumour types with different genetic aberrations [106,107]. Consequently, personalized medicine/targeted monotherapies are effective only for relatively short times (less than 1 year) in the majority of cancers, as they lead to the selection of pre-existing intrinsically resistant sub-population of cells (Figure 3) [107]. For example, mammary tumours are considered clinically HER2 (human epidermal growth factor receptor 2)-positive when immunohistochemical analysis identifies as few as 30 % of HER2-expressing cells [108], whereas the cut-off for EGFR (epidermal growth factor receptor) positivity is only 1 % [109]. Therefore, only a small fraction of tumour cells are required to strain positive by IHC (immunohistochemistry) in order to be considered for clinically targeted therapy [110]. Although the treatment of cancer with the EGFR/HER2 inhibitor Lapatinib provides initial benefit, the presence of tumour cells having acquired EGFR/HER2 independence or contain other mutations (i.e. PI3K mutation, TSC2 loss, PTEN loss, eIF4E amplification, etc.) will eventually lead to resistance to treatment. One possible solution is to employ a combination of drugs directed against two or more targets which can lead to enhanced efficacy; however, this approach also generally leads to additive or even synergistic toxicity [111,112].

In general, many therapeutic cancer targets display similar heterogeneous expression patterns; however, Ramon et al. [113] obtained IHC data demonstrating a homogenous distribution of elevated eIF4E and 4E-BP and their phosphorylated forms in breast cancer. If this striking observation were to be repeated in other tumours, this would provide an intriguing opportunity to use inhibitors of eIF4F as monotherapies for cancer. Consistent with this idea, recent studies have found that an inhibitor of eIF4F suppressed B-Raf (B-Raf proto-oncogene serine/threonine-protein kinase) resistant melanoma [114]. In addition to the inhibitors of eIF4E described above, several anti-eIF4A natural compounds were discovered, which exhibit potent inhibition of proliferation and anti-tumorigenic activities [105]. These compounds include, silvestrol, hippuristanol and pateamine A. Notably, Pelletier's group showed that multiple myeloma cancer cells which are resistant to standard therapy could be eliminated by the eIF4A inhibitor silvestrol [105]. Taken together, these experiments imply that a highly promising application of single eIF4F inhibitors for cancer treatment is to combat drug resistance.

In addition to cancer, eIF4E has been implicated in neurodevelopmental diseases, in which translation is dysregulated, such as autism and fragile-X syndrome (FXS). Notably, drugs that are being developed for cancer, such as 4EGI, were shown to correct autistic-like deficits in mice [115] and cercosporamide reversed many of the FXS-like symptoms in mice [116]. In summary, the cap-binding protein eIF4E plays a critical role in cell homoeostasis in health and disease. It is hoped that some of the currently tested candidate drugs or their derivatives that inhibit eIF4E activity will prove effective as anti-cancer drugs. It is also clear that the accumulated exhaustive knowledge of the structure and function of eIF4E and its regulators will prove to be instrumental in designing effective drugs against cancer.

Acknowledgements

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