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Eukaryotic initiation factor 4F — sidestepping resistance mechanisms arising from expression heterogeneity

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There is enormous diversity in the genetic makeup and gene expression profiles between and within tumors. This heterogeneity leads to phenotypic variation and is a major mechanism of resistance to molecular targeted therapies. Here we describe a conceptual framework for targeting eukaryotic initiation factor (eIF) 4F in cancer — an essential complex that drives and promotes multiple Cancer Hallmarks. The unique nature of eIF4F and its druggability bypasses several of the heterogeneity issues that plague molecular targeted drugs developed for cancer therapy.

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The problem of tumor heterogeneity

Cancer is a disease with genetic and phenotypic variation present between patients with the same tumor type (inter-tumor) and within a single tumor bed (intra-tumor) [1••]. While there are over 250 different types of malignant tumors, intra-tumor diversity is much more significant at the molecular and cellular level. This heterogeneity is a consequence of cell-to-cell variation in mutation burden, gene and proteomic expression signatures, extent of tumor bed infiltration by host components (e.g. immune cells, lymphovascular infiltration) as well as environmental conditions (e.g. hypoxia, nutrient deprivation, oxidative stress). It has long been known that within cancers affecting the same site, one can find distinctly different neoplastic diseases classified under a common name with several histological subtypes. Molecular studies including detection of genetic alterations and expression profiling have proven crucial for the subclassification of these diseases. For example, breast cancers are subdivided into luminal A, luminal B, human epidermal growth factor receptor (HER-2) positive, claudin-low or basal-like breast cancer [2]. These distinctions are critical to make since cancer subtypes will often differ with respect to progression and therapeutic response and stratification optimizes therapy choice and improves treatment [3].

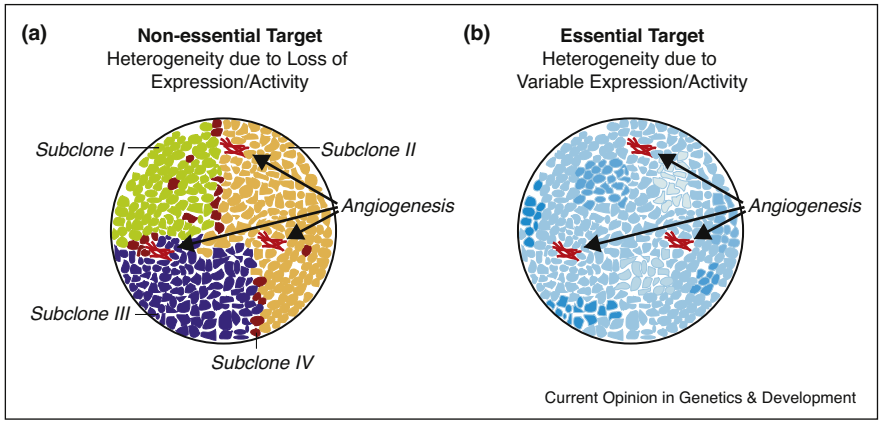
Complex epigenetic and mutational heterogeneity has been well documented at the cellular level within the same tumor bed [4]. Single-cell gene expression profiles, as exemplified by a study assessing ~6000 genes in 430 cells from resected human glioblastomas [5] revealed variability in gene expression between cells that extended to multiple processes, including signalling, proliferation, immune response, and hypoxia [5]. The resulting gene expression mosaicism was not restricted to transcriptional differences but also extended to variations in splicing patterns [5]. In a separate study, three colon cancer cells isolated from a primary tumor showed significant heterogeneity in the phosphorylation status of multiple signalling components [6,7]. Taken together, these studies attest to the complex nature of heterogeneity and that it extends to multiple layers in the gene expression pathway.

The heterogeneity problem is not restricted to tumor cells but also extends to the tumor microenvironment. The microenvironment itself will significantly vary depending on its intra-tumor location and can also be a driver of heterogeneity. For example, variations in distance from the vascular network means that tumor cells may find themselves in diverse milieus differing in pH, nutrient availability, and oxygen tension. This can have dramatic effects on tumor cellular genotype by selecting for cells that have disrupted apoptotic programs (e.g. p53 mutations) and are more refractory to chemotherapy [8].

Heterogeneity impacts on therapy response

Current therapies and drug discovery pipelines treat cancer as a homogenous disease but tumor heterogeneity constitute a severe shortcoming to the development of

Figure 1



Schematic representation of intra-tumor expression heterogeneity. **(a)** In the case of a non-essential target, loss of expression or activity can lead to resistance to molecular targeted therapies. **(b)** In the case of an essential target, expression heterogeneity may exist, but expression or activity can never be eliminated. If tumor cells have become dependent on processes regulated by said target, then this may be considered a therapeutic target.

effective molecular targeted therapies. Many compounds are still tested in xenograft models where the tumor is derived from an isogenic cell line. Patient-derived tumor xenograft (PDX) models overcome some of these limitations, but their size restriction and variability in transplant success implies that they are only able to capture a fraction of the total heterogeneity present in the primary human specimen.

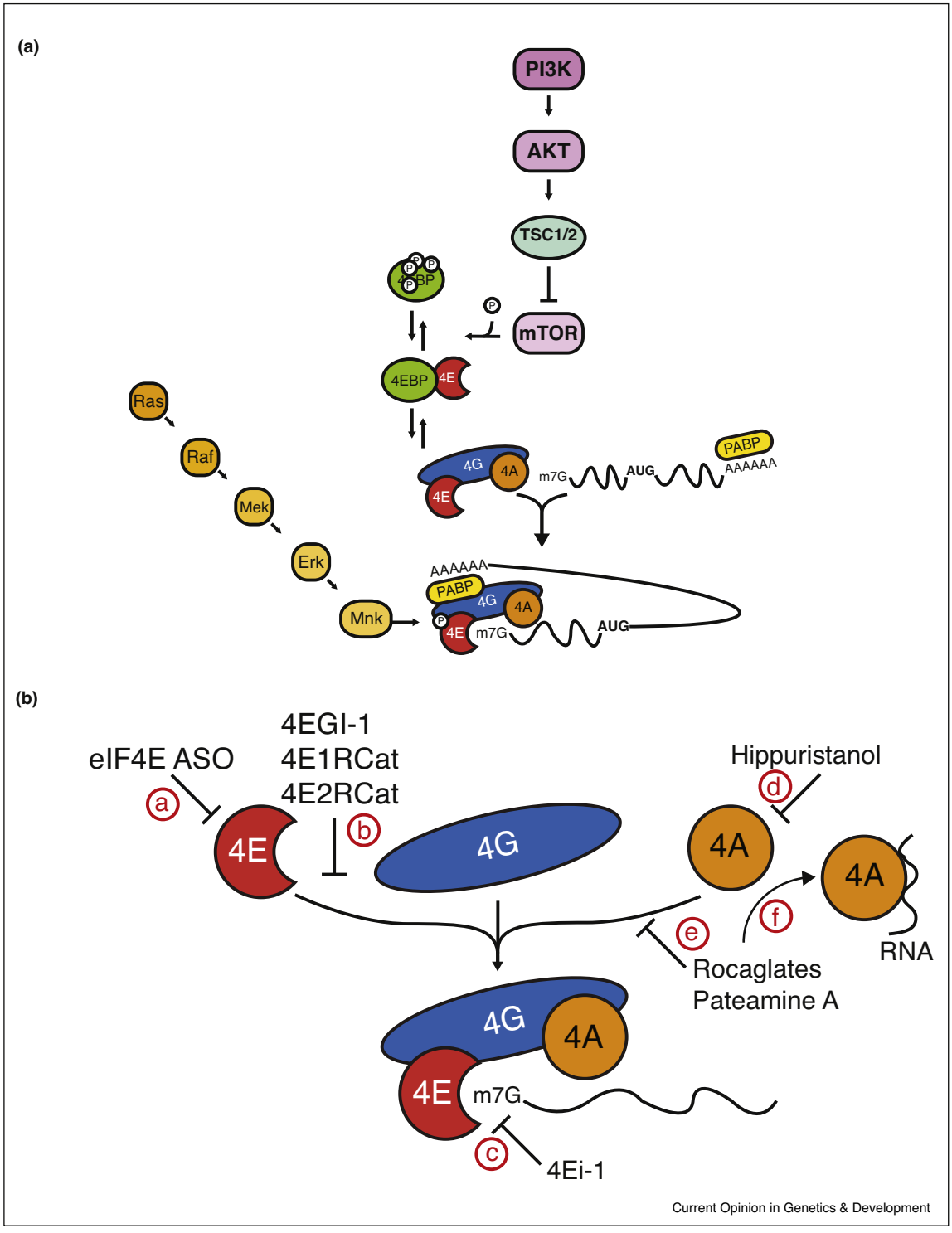
Variation in gene expression networks in tumors plays out at multiple levels to affect therapy response. Firstly, there is the issue of protein expression heterogeneity (Figure 1a). For example, it is sufficient for only ~10% cells of a breast cancer specimen to stain positive for human epidermal growth factor receptor 2 (HER2) for it to be classified as $HER2^+$ [9]. These patients are then treated with Herceptin (Trastuzumab), an antibody that targets HER2 and although this leads to remission, it is invariably followed by the re-emergence of drug-resistant tumor clones. Secondly, there is the problem of pathway redundancy. For example, treating patients with melanomas harboring BRAF mutations with BRAF inhibitors (Vemurafenib and Dabrafenib) leads to an impressive initial response, but this is then followed by relapse. Among the mechanisms that can lead to BRAF resistance, one route is via activation of downstream MEK [10] — essentially bypassing the original BRAF dependency of the tumor cells. A similar theme has been noted in some $BCR\text{-}ABL^+$ leukemias where recurrence after treatment with imatinib (which targets ABL) arises due to loss of dependency on the translocation fusion product [11,12]. This type of adaptive, compensatory response likely arises because the driver target is no longer essential and is replaced by another upon inhibition. Thirdly, there is the problem of redundancies in gene expression regulatory mechanisms that lead to the generation of resistant isoforms. This is observed in patients with B-cell malignancy treated with chimeric antigen receptor (CAR) T cells. When CD19 is targeted, ~30% of patients will relapse following an initial response. This is due to the expression of an alternatively spliced CD19 isoform which no longer retains the originally targeted epitope [13••]. Taken together, these cases exemplify how heterogeneity at multiple levels in tumor cells can curb anticipated drug responses.

Targeting eukaryotic initiation factor (eIF) 4F bypasses issues of heterogeneity arising from loss of expression or activity

Conceptually, one way to overcome the problems presented by target heterogeneity is to drug essential factors or processes to which tumor cells have become addicted. While these processes are essential to all cells, tumors tend to possess a heightened dependency on some of these and thus become more vulnerable to their inhibition — enabling a therapeutic window to be achieved. In fact, this is known to work well in several settings. Actinomycin D (ActD), an anticancer drug in use since 1954, is a DNA intercalator that blocks transcription. There is much evidence that cancer cells are also highly dependent on splicing functions, altered translational output, and on the ability to adapt to proteotoxic stress for their survival [14••,15,16]. Hence strategies that target appropriate factors in these essential processes to inhibit these altered dependencies may afford therapeutic approaches that will not be plagued by gene and protein expression loss. Within the domain of translational regulation, eIF4F represents such a compelling target (Figure 2).

eIF4F consists of three subunits: eIF4E, the cap binding subunit; eIF4A, a DEAD-box RNA helicase; and eIF4G,

Figure 2



Targeting eIF4F in cancer. **(a)** Schematic representation of the regulation imposed by the PI3K/Akt/mTOR and RAS/MAPK pathway on eIF4F. Increased signalling through PI3K/Akt/mTOR leads to phosphorylation of 4E-BP, reduced levels of 4E-BP:eIF4E complexes, and increased eIF4F formation. Activation of the RAS/MAPK pathway leads to MNK-mediated phosphorylation of eIF4E, which also increases translation initiation. **(b)** Molecules that target various components of the eIF4F complex are highlighted. These include: (a) anti-sense oligonucleotides to eIF4E, (b) compounds that inhibit eIF4E:eIF4G interaction, (c) inhibition of eIF4E: cap interaction, (d) inhibition of eIF4A RNA binding, and (e) blocking eIF4A: eIF4G association [15]. Rocaglates and pateamine have also been shown to cause eIF4A to clamp onto RNA (f) [15,52,53,], with rocaglates dictating a preference for polypurine stretches [51••].

a large scaffolding protein that recruits the 40S ribosome and associated factors (43S pre-initiation complex [PIC]). The eIF4E subunit is the least abundant translation factor — rendering PIC recruitment to mRNAs rate-limiting for translation. Cap accessibility and cap-proximal secondary structure are known to affect the ability of eIF4F to efficiently recruit PICs to mRNAs and this is attributed to reduced eIF4F:cap interaction and limitations imposed on the helicase activity of eIF4A [17]. eIF4F is thought to act as a discriminatory factor with distinct affinities towards different mRNAs, resulting in variable initiation rates and translational output. Regulation of eIF4F activity is thus an elegant way by which to exert selective translational control while sustaining the bulk rate of global translation. Given the presence of three major discrete mRNA abundance classes in cells — with the majority (37–49%, depending on the tissue) of mRNAs present in the very low abundance class (<30 mRNA copies/cell), subtle alterations in eIF4F activity are not expected to be easily detectable by bulk analysis approaches [18,19]. Rather, more sensitive approaches such as assessing RNA distribution across polysome profiles or ribosome footprinting approaches are required to document subtle changes in translation mediated by eIF4F [20••].

eIF4F activity is regulated by two major signalling networks — the PI3K/mTOR and RAS/MAPK (mitogen-activated protein kinase) pathways (Figure 2a) [15,17]. (I) eIF4E can be sequestered from the eIF4F complex by one of three eIF4E-Binding proteins (4E-BP). Since the structure of the eIF4E binding site on 4E-BP and eIF4G are identical, eIF4E can only be bound to one of these two partners. Increased signalling flux through the PI3K/mTOR pathway leads to mTOR phosphorylation of 4E-BP, which in turn releases eIF4E and allows it to enter the eIF4F complex [15,17]. The net consequence is a selective increase in the translation of a subset of mRNAs, some of which support cell survival, proliferation, cell cycle progression, and angiogenesis [15,17]. (II) eIF4F is also regulated by the RAS/MAPK pathway and this involves direct phosphorylation by MNK (MAPK-interacting serine/threonine kinase) 1 and MNK2 on eIF4E Ser209. This modification increases translation of selective mRNAs, although the mechanistic details as to how this discrimination occurs is not known [21,22••]. Neither MNK1 nor MNK2 are essential in the mouse [23], yet Ser209 phosphorylation appears critical for eIF4E-dependent cell transformation [24,25]. These properties make MNK1 and MNK2 very interesting targets for anti-cancer drug development.

In an extensive study involving 19 grade II astrocytomas, 25 anaplastic astrocytomas (grade III), 60 glioblastomas (grade IV), and 15 cases of reactive gliosis, the expression levels of EGFR, p-MAPK, 4E-BP1, p-4E-BP1, pS6, eIF4E, and p-eIF4E were assessed [26]. The levels of p-4E-BP1 and p-eIF4E were found to increase during tumor progression. Similar observations regarding p-4E-BP1 and p-eIF4E have been made in other cancers and are consistent with the hypothesis that elevated eIF4E levels are associated with poor prognosis [27–35]. Whereas HER-2, p-MAPK, p-mTOR, p-S6 and p-AKT display heterogenous distribution patterns in breast cancers when analyzed by immunohistochemistry, eIF4E, 4E-BP1, p-4E-BP1, and p-eIF4E staining is more diffuse and importantly, homogenous [36].

Given the essential nature of eIF4E [37,38••], targeting this factor in tumor cells makes sense and is expected to circumvent any possibility of expression extinction or loss of activity being a drug resistance mechanism. Experiments with mice engineered to systemically suppress eIF4E using an inducible shRNA [39], or in mice in which administration of an eIF4E antisense oligonucleotide (ASO) was used to suppress eIF4E at the organismal level [40], revealed that reductions in eIF4E levels in normal tissues can be transiently tolerated and that growth and survival of cancer cells are more susceptible than normal tissues to eIF4E depletion *in vivo*. Studies in which one allele of eIF4E has been ablated in mice have also shown that reduced eIF4E levels are systemically well tolerated [38••].

The situation with eIF4A and eIF4G is more complex since mammalian cells express two eIF4A (eIF4A1 and eIF4A2) and eIF4G (eIF4G1 and eIF4G3) gene products involved in cap-dependent translation. A compendium of cancer dependencies across 398 cancer cells revealed that eIF4A1 and eIF4G1 are essential to cancer cell survival [41••]. With respect to eIF4A, eIF4A1 and eIF4A2 are interchangeable as eIF4F subunits [42]. However, the two proteins may not play equivalent roles in cells, as eIF4A2 does not compensate for the suppression of eIF4A1 [43]. As well, NIH 3T3 cells can tolerate complete loss of eIF4A2 [44], but suppression of eIF4A1 in mouse lymphoma cells imparts a severe growth disadvantage [45]. As appears to be the case for eIF4E, targeting eIF4A1 represents another approach by which the issue of heterogeneity due to loss of expression or activity may be circumvented.

Targeting eIF4F activity

Rocaglates

Several molecules capable of blocking eIF4F activity have been identified, characterized, and extensively reviewed (Figure 2b) [15,46]. Among the most potent and selective of these are the rocaglates, a class of compounds that perturb the activity of eIF4A1 and eIF4A2. Rocaglates are characterized by a common cyclopenta[*b*] furan skeleton. Several studies have demonstrated that rocaglates exhibit potent anti-cancer activity in a variety of preclinical cancer models, reverse therapy resistance acquired to anti-BRAF and anti-MEK inhibitors in melanoma cells [47], and inhibit tumor supportive processes

such as angiogenesis (reviewed in Refs. [15,46]). Affinity chromatography experiments [48], differential scanning fluorimetry [49••], yeast-based genetic screens for resistant mutants [50], and CRISPR/Cas9 engineered mutations in NIH 3T3 cells [50] have identified eIF4A as the target of this compound class and have linked their antitumor activity to eIF4A inhibition.

Rocaglates exert their effects on translation in at least two ways. (I) They cause eIF4A to clamp onto polypurine sequences in RNA [51••]. When this occurs within the mRNA 5′ leader it can block scanning ribosomes, resulting in reduced translation initiation [51••]. (II) Increased RNA binding of eIF4A is not restricted to mRNAs, as it has also been documented to occur on high molecular weight RNA-protein complexes that co-fractionate with ribosomes and polysomes [52]. This global sequestration of eIF4A to RNA is associated with reduced eIF4A association in the eIF4F complex [52,53]. Depletion of eIF4A from the eIF4F complex is expected to reduce translation initiation rates — a scenario similar to what is observed with PDCD4, a negative regulator of translation that sequesters eIF4A from eIF4F [54]. The effects of rocaglates on gene expression have been extensively studied and one of the first rocaglate-responsive mRNAs identified was c-Myc [53]. Subsequent ribosome profiling experiments led to the identification of rocaglate-responsive (and hence eIF4A-dependent) mRNAs. Wolfe *et al.* [55] and Rubio *et al.* [56] both found that rocaglate-responsive transcripts have more complex 5′ leader regions — with length, ΔG values, and the presence of G-quadruplexes in the 5′ leader being important determinants of this response [55,56]. The presence of polypurine sequences in 5′ leader regions also imparts rocaglate-responsiveness [51••].

Rocaglates affect the activity of both eIF4A1 and eIF4A2, so compensation by eIF4A2 upon eIF4A1 inhibition is not a likely resistance mechanism to rocaglates. Rather, mutations in eIF4A1 can lead to rocaglate resistance [49••,50], while maintaining activity in translation, and it is likely that these could be selected out during drug treatment. This argues for the need to use combination therapy, either simultaneously targeting several eIF4F subunits or combining eIF4A inhibition with therapies against other targets to which tumor cells have evolved an addiction.

MNK1/2 inhibitors

As p-eIF4E levels appear quite uniform throughout tumor beds [36], and eIF4E phosphorylation is essential to its oncogenic activity [24,25], its inhibition should have significant anti-cancer activity. $eIF4E^{S209A/S209A}$ mice expressing an eIF4E mutant which cannot be phosphorylated [21], or mice homozygous null for MNK1 and MNK2 [57], show significantly attenuated tumorigenesis in the face of potent oncogenic driving events, such as PTEN loss. Cells express two isoforms of MNK1 and MNK2 with the major structural differences lying in the carboxy-terminal domain (CTD). MNK1a and MNK2a contain a MAPK-binding site making these, substrates for phosphorylation (but not MNK1b and MNK2b) and activation by p38 MAPK and ERK [58,59].

There is much interest in identifying selective pharmacological inhibitors of MNK, a number of which have already been reported in the literature. CGP57380 was developed by Novartis as an inhibitor of MNK1 and is effective at blocking eIF4E phosphorylation in cell culture but unfortunately is not stable *in vivo* [60]. Cercosporamide was identified by Lilly Research Laboratories following a chemical screen for MNK1 inhibitors and was used to suppress eIF4E phosphorylation *in vivo* in melanoma lung metastasis and colon cancer xenograft tumors and this lead to reduced tumor growth [61]. Cercosporamide also showed activity against primary leukemic progenitors from AML (acute myeloid leukemia) and in combination with low-dose cytarabine produced an anti-leukemic response more potent than obtained with either agent alone [62].

Using a structure-based drug design approach, Wu *et al.* [63] discovered a dual specificity BTK (Bruton's tyrosine kinase)/MNK inhibitor called QL-X-138 which exhibits covalent binding to BTK and noncovalent inhibition of MNK. QL-X-138 shows enhanced anti-proliferative activity towards a number of B cell cancer lines and AML and CLL primary patient cells, compared to either BTK or cercosporamide. A different structure-guided approach was used to convert resorcyclic acid lactones into covalent inhibitors of MNK1/2 by tuning an electron-withdrawing functionality to covalently bind to a cysteine residue in the MNK1/2 binding sites [64]. One of these compounds (Cmpd #20) inhibited both MNK1 and MNK2 (IC_{50} = ~9–13 μM, respectively) and blocked eIF4E phosphorylation in HeLa cells with an IC_{50} = 1.6 μM [64].

The retinamides were first developed as retinoic acid metabolism blocking agents and later found to also cause degradation of MNK1 and block eIF4E phosphorylation [65]. These compounds block cell growth, induce cell death, and exert anti-migratory and anti-invasive activities when tested *ex vivo* against MDA-MB-231 and MDA-MB-468 breast cancer cells [65]. Retinamide derivatives also enhance degradation of the androgen receptor (AR) and simultaneous inhibition of AR and MNK appears quite effective in androgen sensitive and castration resistant prostate cancer cells [66,67].

Effector therapeutics has developed a MNK1/MNK2 inhibitor called eFT508. It has been reported to be efficacious in preclinical models of diffuse Large B cell lymphoma (DLBCL) [68]. eFT508 is currently in two clinical trials, one in patients with solid tumors (NCT02605083) and the other in patients with lymphomas (NCT2937675).

Early reports indicate that the compound is well tolerated in humans and disease stabilization was seen following at least two cycles of therapy (42 days) in four of 11 patients (http://effector.com/2017/06/05/effector-therapeutics-presents-phase-1-data-on-eft508-its-lead-product-candidate-at-asco-2017/).

Concluding remarks

In the last decade there has been significant emphasis placed on defining the synthetic lethal interactome of tumors to identify new targets. We propose that development of therapeutic approaches to essential targets, such as eIF4F, should also be vigorously supported since they sidestep the problem of expression heterogeneity encountered with many of the current molecular targeted therapies. Better models are also now required to understand how to best target these in a heterogenous setting within a population of cancer cells, while minimizing impact on normal cells and tissues.

Conflict of interest statement

Nothing declared.

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