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Review

Eukaryotic elongation factor 2 kinase as a drug target in cancer, and in cardiovascular and neurodegenerative diseases

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Eukaryotic elongation factor 2 kinase (eEF2K) is an unusual protein kinase that regulates the elongation stage of protein synthesis by phosphorylating and inhibiting its only known substrate, eEF2. Elongation is a highly energy-consuming process, and eEF2K activity is tightly regulated by several signaling pathways. Regulating translation elongation can modulate the cellular energy demand and may also control the expression of specific proteins. Growing evidence links eEF2K to a range of human diseases, including cardiovascular conditions (atherosclerosis, via macrophage survival) and pulmonary arterial hypertension, as well as solid tumors, where eEF2K appears to play contrasting roles depending on tumor type and stage. eEF2K is also involved in neurological disorders and may be a valuable target in treating depression and certain neurodegenerative diseases. Because eEF2K is not required for mammalian development or cell viability, inhibiting its function may not elicit serious side effects, while the fact that it is an atypical kinase and quite distinct from the vast majority of other mammalian kinases suggests the possibility to develop it into compounds that inhibit eEF2K without affecting other important protein kinases. Further research is needed to explore these possibilities and there is an urgent need to identify and characterize potent and specific small-molecule inhibitors of eEF2K. In this article we review the recent evidence concerning the role of eEF2K in human diseases as well as the progress in developing small-molecule inhibitors of this enzyme.

Keywords: mRNA translation; eEF2K; α-kinase; cardiovascular disease; hypertension; atherosclerosis; cancer; neurodegenerative disease; Alzheimer's disease; depression

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Introduction

Protein synthesis is a crucial cellular process because almost all cell functions are performed by proteins and because the levels of proteins are determined in large part by the efficiency with which their mRNAs are translated into proteins[1]. However, protein synthesis and the production of ribosomes to support this process are expensive, consuming a large proportion of the cells' energy and nutrients. Consequently, protein synthesis (mRNA translation) is subject to tight control, and numerous mechanisms have evolved to link nutrients, energy and other cues to the rates of protein production.

One such mechanism involves the phosphorylation of eukaryotic elongation factor eEF2, the protein that mediates the movement of ribosomes along mRNAs from one codon to the next during the elongation stage of translation[2]. Almost all of the energy and amino acids consumed by protein synthesis are used in elongation. When phosphorylated on Thr56 through its amino acid sequence, eEF2 cannot engage with ribosomes[3] and is essentially inactive in translation. eEF2 is phosphorylated by a highly specific protein kinase, eEF2 kinase (eEF2K). eEF2K is activated by Ca^{2+} ions via calmodulin (CaM), which immediately binds the N-terminal to its catalytic domain (Figure 1). The primary sequence of this domain does not resemble that of members of the main protein kinase superfamily, and indeed, eEF2K belongs to a small group of so-called α-kinases, which are represented by six genes in the human genome[4, 5]. Although a 3-dimensional structure for the catalytic domain of eEF2K is not yet available, such structures have been determined for two other α-kinases. This revealed a surprisingly high level of 3D similarity to members of the main kinase family, although there are also important differences[6, 7]. The excellent review articles by Drennan and Ryazanov[8] and Middelbeek *et al*[5] provide more detailed information.

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Recent discoveries have revealed roles for eEF2K in both cell physiology and disease. Given that eEF2K is the subject of a recent review[2], this article will focus on the most recent findings concerning eEF2K's roles in cancer cell biology, as well as in cardiovascular disease and in neurological functions and neuropathology. In several but not all cases, eEF2K appears to promote disease progression by protecting cells against stresses. In neurodegenerative disease, it may exacerbate disease by inhibiting protein synthesis, which is required for cognitive functions. In all cases, it is suggested that inhibition of eEF2K may be therapeutically beneficial; *eg*, by impairing cell survival in cancer and atherosclerosis and alleviating the inhibition of protein synthesis in Alzheimer's disease (AD).

Overview of the structure and control of eEF2K

In addition to the N-terminal CaM-binding domain and catalytic domain, eEF2K also contains a C-terminal region hypothesized to contain α-helical repeats and a short, extreme C-terminal region, which is highly conserved and may interact with eEF2 (Figure 1). The center of the polypeptide is predicted to be unstructured and may act as a flexible linker between the two main folded regions of the protein (Figure 1).

eEF2K undergoes autophosphorylation, with a major site being Thr-348 (of the sequence of human eEF2K[9, 10]). This position corresponds to an autophosphorylation site in the related enzyme myosin heavy chain kinase (MHCK), which, when phosphorylated, docks into a binding pocket to allow the enzyme to adopt an active conformation[11]. A similar mechanism appears to apply to eEF2K[12, 13]. This site can even be phosphorylated in *E coli,* which lacks CaM, suggesting that the activity of eEF2K in autophosphorylation is not strictly Ca/CaM dependent[13]. However, it is stimulated by Ca/CaM[13, 14]. The conformational change associated with the phosphorylation of Thr-348 enhances eEF2K activity by increasing its affinity for a (peptide) substrate and its intrinsic catalytic activity. The authors propose a two-stage 'amplifier-like' mechanism, where the first step involves the initial activation of eEF2K (off/on) and the second modulates its activity (through the stability of its active conformation, which is analogous to the volume control[10]). Further work is clearly needed to define the structural changes that underpin this regulatory mechanism. The activity of eEF2K also modulates its proteasome-mediated degradation[15, 16]; however, it appears that the ability to assume an active conformation, rather than ongoing activity, is the key determinant here[16].

In addition to its activation by CaM, eEF2K is subject to a range of other inputs. In particular, it is inactivated by signaling downstream of the mammalian target of rapamycin complex 1 (mTORC1), which is stimulated by hormones, growth factors and nutrients[2]; this can allow such anabolic stimuli to speed up the rate of elongation, and thus, protein synthesis. The first input from mTORC1 to be identified involves the 70 kDa ribosomal protein S6 kinase, p70S6K, which is activated by mTORC1 and phosphorylates eEF2K at Ser366 (human sequence numbering), inactivating it[17] (Figure 1, 2). Since then, additional links between mTORC1 and eEF2K have been delineated, including direct phosphorylation of eEF2K by mTORC1 in an *in vitro* kinase assay, a nutrient-regulated protein kinase[12, 18] (Figure 1, 2). Ser78 in eEF2K is also phosphorylated in an mTORC1-dependent manner[19] (Figure 1); this residue is adjacent to the CaM-binding motif, and its phosphorylation impairs CaM binding, and thus, the activation of eEF2K.

Because mTORC1 lies downstream of oncogenic signaling pathways, such as the phosphatidylinositol 3-kinase/Akt (protein kinase B) and Ras/Raf/MEK/ERK pathways, which are frequently dysregulated in cancers, it is likely that mTORC1 signaling is hyperactivated in a high proportion of tumors (estimated at 70%). The Ras/Raf/MEK/ERK pathway also makes direct inputs into the inactivation of eEF2K via direct phosphorylation of eEF2K by ERK (at Ser359[10]) and via $p90^{RSK}$, both of which are activated by ERK and phosphorylate

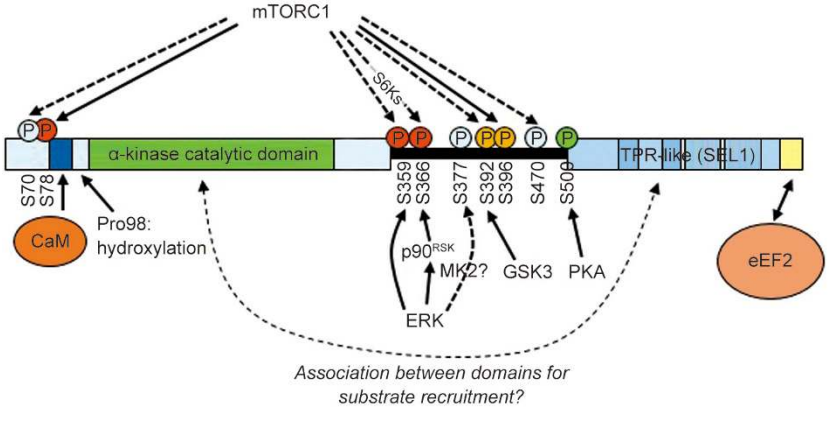


Figure 1. The structural layout of eEF2K. The drawing illustrates the major structural and functional features of eEF2K, the main known sites of phosphorylation, and the signaling pathways or kinases that regulate them. Solid lines show direct links and dashed lines show indirect links. The regions interacting with calmodulin (CaM) and eEF2 are also shown. The numbering of residues is based on human eEF2K. Green denotes active sites of phosphorylation, red denotes inhibitory sites, and grey indicates sites that are not known to affect activity. Sites in orange affect the phosphorylation of the sites at the extreme N-terminus, which can indirectly regulate activity.

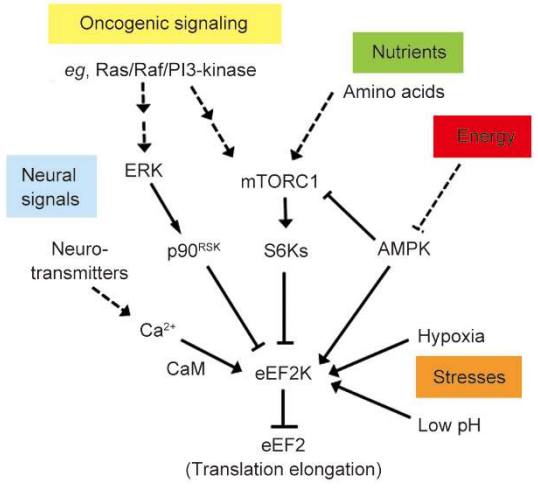


Figure 2. Regulatory connections to eEF2K. Direct links are shown by continuous lines. Dashed lines indicate indirect or multistep connections, which are associated with the indicated pathways, signals or conditions.

Ser366 (Figure 1, 2).

These pathways likely keep the activity of eEF2K at low levels in many cancer cells under nutrient-replete conditions, even though the levels of eEF2K expression are elevated, thereby allowing for high eEF2 activity and facilitating elongation. Under poor nutrient conditions, eEF2K will be activated.

eEF2 phosphorylation increases during hypoxia[20, 21], a condition where the cellular energy supply is often compromised. During hypoxia, eEF2K is activated by a mechanism that involves proline hydroxylases, which require oxygen as a co-substrate and are involved in other effects of hypoxia, such as the regulation of the transcription factor hypoxia-inducible factor 1α (HIF1α). However, the regulation of eEF2K by oxygen appears not to involve HIF1α, but rather, the direct hydroxylation of eEF2K at a highly conserved proline (Pro98; Figure 1, 2) adjacent to the CaM-binding site[22]; this residue is in a strongly conserved motif in the 'linker' between the CaM-binding motif and the catalytic domain of eEF2K. This modification limits the ability of CaM to activate eEF2K, keeping its activity low in oxygenated conditions. At low oxygen levels, where prolyl hydroxylase function is impaired, this residue is not hydroxylated, and eEF2K can be activated more strongly by calcium-bound CaM. Because there is no known way to reverse proline hydroxylation, this mechanism relies on the short half-life of the eEF2K protein to allow the replacement of hydroxylated eEF2K by unmodified enzymes. eEF2K helps to protect cells against hypoxia[20, 21], likely by reducing the rate of protein synthesis and the demand for energy. Hydroxylation of eEF2K therefore constitutes an adaptive mechanism to allow cells to cope with low oxygen levels.

A second mechanism for the regulation of eEF2K linked to CaM binding occurs at low pH values, which results in the activation of eEF2K and the phosphorylation of eEF2[23]. Recent data has shown that this effect involves several histidine residues in eEF2K, in both the CaM-binding site and its catalytic domain. Histidines are the only residues that normally undergo protonation in the shift from physiological pH (approximately 7.4) to the types of pH values that occur during physiological or pathological acidosis, which can arise in the tumor microenvironment (pH 6.4). Interestingly, eEF2K protects cancer cells against acidosis[23], again likely by slowing down protein synthesis to conserve energy.

The AMP-activated protein kinase, AMPK, is a key sensor of cellular energy levels that are activated when ATP levels fall[24]. AMPK phosphorylates a range of substrates to reduce energy demand and increase energy supply. Because protein synthesis uses a substantial amount energy, it is not surprising that AMPK can impair mTORC1 signaling, which normally promotes protein synthesis[25, 26]. The activation of AMPK leads to increased phosphorylation of eEF2[27, 28]. This likely involves the inhibition of mTORC1, a negative regulator of eEF2K, while AMPK may also phosphorylate eEF2K directly, contributing to its activation.

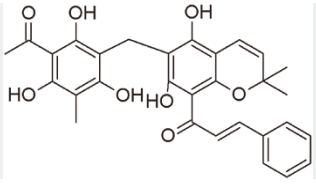
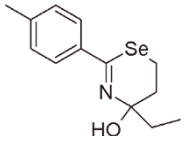
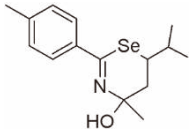
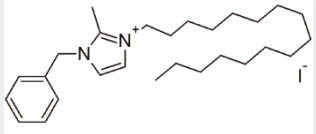
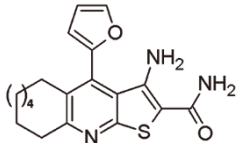
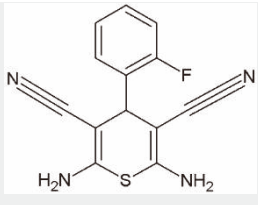
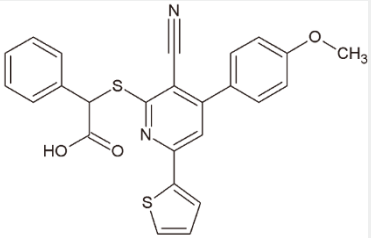
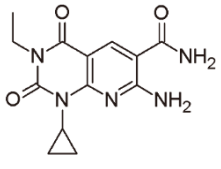
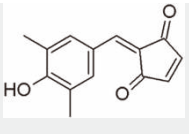
α-Kinases do not possess a direct equivalent of the activation, or 'T'-loop, which is found in members of the main protein kinase superfamily, and therefore, they do not require, or in the case of eEF2K, undergo phosphorylation within this feature of the catalytic domain (again, unlike regular protein kinases). α-Kinases do possess a different kind of loop, which approximately corresponds to the activation loop of other kinases and reflects the presence of glutamine and asparagine residues in this region, that is termed the N/D loop. One shared feature of those kinases and eEF2K is that this region plays a role in the binding and recognition of peptide or protein substrates. For example, point mutations within this region of eEF2K can alter its relative activity against eEF2, its physiological substrate, and a peptide substrate based on a myosin heavy chain (MH-1)[13].

Inhibitors of eEF2K

As an atypical α-kinase, eEF2K is not inhibited by 'classical' protein kinase inhibitors, such as staurosporine[29]. This feature makes it more challenging to identify and further develop inhibitors for eEF2K, as they cannot simply be based on known kinase inhibitors, but does mean that such compounds are less likely to exert effects on other members of the main protein kinase family. Several reports have described compounds that can inhibit eEF2K activity (Table 1).

An early report of an eEF2K inhibitor concerned rottlerin[29] but this compound inhibits various other protein kinases at concentrations lower than those needed to inhibit eEF2K[30]. Subsequently, NH125, an imidazolium derivative, was reported to be an eEF2K inhibitor[31], but subsequent studies showed it actually increases eEF2 phosphorylation within cells, probably by acting as a non-specific protein-aggregating agent[32, 33]. A thiopyran-dicarbonitrile analog was described as an eEF2K inhibitor by Devkota *et al*[34]. A-484954 was uncovered by Abbott laboratories as a specific eEF2K inhibitor, but it is only weakly effective within cells[32]. A recent study created and tested a number of pyrido[2,3-b]pyrimidine-2,4-dione derivatives and found that two such compounds inhibited eEF2K activity with submicromolar IC_{50}s *in vitro*[35]. However,

Table 1. Summary of identified eEF2K inhibitors.

Compound name	Structure*	Experimental methods	IC_{50} (μmol/L)	Ref
Rottlerin 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one, mallotoxin		Inhibitor of protein kinase Cδ. *In vitro* eEF2K kinase assay followed by autoradiography. Inhibits various other protein kinases more strongly than eEF2K.	5.3	[28, 29]
TS-2 4-ethyl-4-hydroxy-2-*p*-tolyl-5,6-dihydro-4*H*-1,3-selenazine		1,3-selenazine derivatives. *In vitro* eEF2K kinase assay followed by autoradiography.	0.36	[36]
TS-4 4-hydroxy-6-isopropyl-4-methyl-2-*p*-tolyl-5,6-dihydro-4*H*-1,3-selenazine			0.31	[36]
NH125 1-benzyl-3-cetyl-2-methylimidazolium iodide		Imidazolium histidine kinase inhibitors. *In vitro* eEF2K kinase assay followed by either a filter-based assay or immunoblotting.	0.06	[31]
Compound 34 3-amino-4-(furan-2-yl)-6,7,8,9,10,11-hexahydro-5*H*-cyclonona[b]thieno [3,2-e]pyridine-2-carboxamide		Thieno[2,3-b]pyridine analogues. *In vitro* fluorometric, coupled-enzyme assay.	0.17	[37]
2,6-diamino-4-(2-fluorophenyl)-4*H*-thiopyran-3,5-dicarbonitrile		Method 1: Fluorescence-based assay that uses the phosphorylation of a Sox-based peptide (*in vitro*).	60	[34]
2-((3-cyano-4-(4-methoxyphenyl) pyridine-2-ylthio)-2-phenylacetic) acid		Method 2: Luminescence-based assay that measures the amount of ATP remaining in the reaction (*in vitro*).	77	[34]
A-484954 7-amino-1-cyclopropyl-3-ethyl-1,2,3,4-tetrahydro-2,4-dioxopyrido[2,3-d] pyrimidine-6-carboxamide		Discovered by Abbott laboratories. *In vitro* kinase assay using myelin basic protein as substrate.	0.28	[32]
TX-1918 2-((3,5-dimethyl-4-hydroxyphenyl)-methylene)-4-cyclopentene-1,3-dione		2-hydroxyarylidene-4-cyclopentene-1,3-dione analogues. *In vitro* eEF2K kinase assay followed by autoradiography. It also inhibits Src, PKA, PKC, and EGFR-K.	0.44	[38]

* All the chemical structures in Table 1 were drawn with ChemSketch.

neither is more potent than A-484954, and each only caused partial inhibition of eEF2 phosphorylation, even at 75 μmol/L in MDA-MB-231 breast cancer cells. There is clearly an acute need to identify better eEF2K inhibitors as tool compounds to explore the roles of eEF2K in normal cell physiology and in disease. Such efforts have been hindered by the lack of a 3-dimensional structure for eEF2K but will be aided by the development of a homology model of eEF2K based on the known structures of two other members of the α-kinase family[39].

A positive role for eEF2K in cancer cell survival and tumor growth

As discussed in this section and the next, there is growing evidence of the roles of eEF2K in cancer and tumor progression, although the data at first appear conflicting (Figure 3). Several earlier studies have shown that eEF2K can protect cancer cells, especially glioma and breast cancer cells, against nutrient deprivation and some other insults[2] (Figure 3). Under such conditions, eEF2K is likely switched on by a combination of the inactivation of mTORC1 signaling and activation of AMPK. Possible mechanisms through which eEF2K may exert this effect have recently been discussed[40]. These studies, coupled with the fact that eEF2K is not essential, at least in mice under normal vivarium conditions, suggest that this enzyme may be an attractive target for cancer therapy.

Previous studies have indicated that eEF2K has a role in breast cancer cell survival[20, 41]. In the clinical setting, triple-negative breast cancers (TNBCs) are often resistant to therapy and readily metastasize. Liu *et al*[42] showed that compared to *Pten* or *P53* single mutations, the combined deletion of *Pten* and *P53* promotes the formation of claudin-low TNBC, which showed hyperactivated AKT signaling (although other data indicate that this pathway is not consistently induced in human *Pten*/*P53*-low TNBC). Such tumors also exhibited more mesenchymal features and poor clinical outcome, both of which are of interest because similar traits are observed in residual tumors after conventional therapy. The authors then employed a bias-free kinome-inhibitor screen to identify which pathways are important in these cells. Two inhibitors of eEF2K were effective, indicating that eEF2K is a promising target to tackle *Pten*/*P53*-deficient TNBC cells, especially those with high AKT activity. PI3-kinase, AKT and mTORC1 inhibitors were also identified in this screen, but they were not as effective as inhibitors of eEF2K (TX-1918 and NH125) or the c-Jun N-terminal kinase, JNK. Knocking down eEF2K suppressed cell growth two-fold under normal (nutrient-replete) conditions. By using chloroquine, an autophagy inhibitor, they found that eEF2K does not sustain tumor cell growth through autophagy. They reported eEF2K inhibitor NH125 suppressed xenograft growth of *Pten*/*P53*-deficient claudin-low TNBC in mice (BT549 cells introduced into NOD/SCID mice). The effect of TX-1918 was additive with doxorubicin treatment in mammary tumors in mice (human tumor TX-1918 cells).

Evidence that eEF2K impedes tumorigenesis

There are also data indicating that eEF2K has a negative role in cancer cell proliferation or tumor development (Figure 3). For example, the eEF2K inhibitor A-484954 has little inhibitory effect on the proliferation of lung and prostate cancer cells[32]. One recent study concerns colorectal cancer. The deletion of the *Apc* gene (adenomatous polyposis coli) is prevalent in the development of colorectal cancer. Following *Apc* deletion in

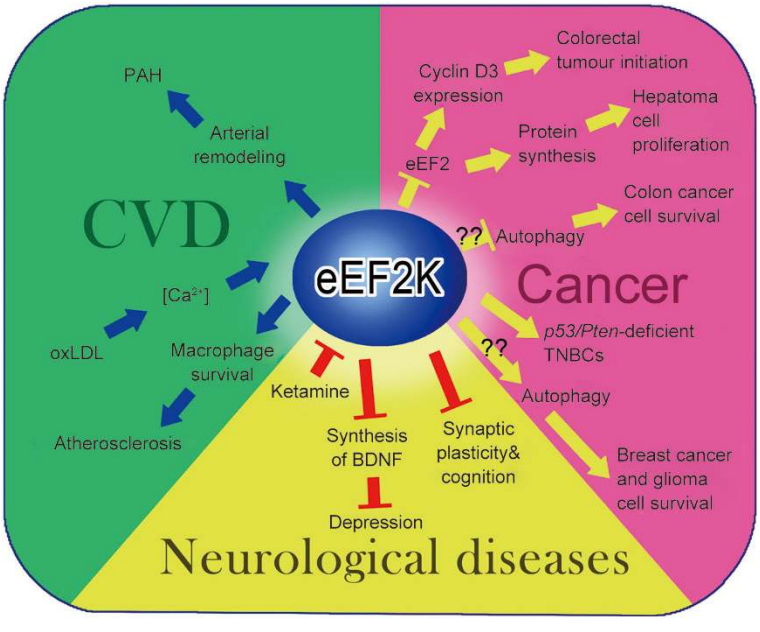


Figure 3. Links between eEF2K and the diseases discussed in this article. These include cardiovascular disease (CVD), certain cancers and Alzheimer's disease. It should be noted that eEF2K also plays a positive role in cognition in some settings, *eg*, Conditioned Taste Aversion (see text). In general, the exact connections between eEF2K and the initiation or progression of these conditions remain to be elucidated.

mice, mTORC1 signaling is more active during crypt regeneration than it is in wild-type animals (as shown by immunohistochemical staining for the phosphorylated forms of the mTORC1 targets S6 and 4E-BP1[43]). Both the stronger activation of mTORC1 and faster crypt regeneration after challenge (gamma-irradiation) in APC-deficient mice are dependent on Myc expression and can be reversed by rapamycin administration or raptor deletion, both of which impair mTORC1 signaling. Rapamycin administration can efficiently inhibit crypt regeneration and tumorigenesis driven by the loss of *Apc*. These striking data imply that components downstream of mTORC1 play an important role in these processes.

An *Apc* deficiency can increase the protein synthesis rate in the cultured intestinal crypt ([^{35}S]-labeled methionine/cysteine incorporation) and ribosome run-off rates but decrease the polysomal distribution, suggesting that elongation (but not initiation) is activated in the context of APC loss. Use of multiple knock-out and knock-in mouse models revealed that rapamycin inhibits the intestinal epithelium regeneration via mTORC1-regulated S6 kinase (S6K1)-eEF2K-eEF2 cascade. S6K1/2 knock out showed decreased intestinal regeneration, while the knock out of another effector of mTORC1 (4E-BP1/2) had no effect, and the intestinal tissue was still sensitive to rapamycin. eEF2K deletion eliminated the inhibition by rapamycin of crypt generation. This indicates that eEF2K mediates a restriction on translation, which confers sensitivity to rapamycin, raising the question as to which mRNAs' translation is critically regulated at the elongation stage. Further investigation showed that the elongation rate of cyclin D3 mRNA translation was higher in *Apc*-deficient cells than in wild-type cells and that cyclin D1 protein levels were reduced by rapamycin but not by eEF2K-knockouts. These studies indicate that blocking mTORC1 may be a useful way to treat early stage colorectal cancer, with its effects being mediated through eEF2K. These findings clearly appear to contrast with data for tumor cells *in vitro* and with more limited *in vivo* (*eg*, xenograft[44]) findings, which indicate that eEF2K aids in the survival of tumors or tumor growth, especially under conditions of nutrient starvation.

Another study supporting the conclusion that eEF2K suppresses tumorigenesis was carried out in colon cancer cells. The authors of that study discovered that eEF2K suppresses autophagy, a process that can be utilized by cancer cells to survive under stress or nutrition-deprivation conditions[45]. The authors found that the over-expression of eEF2K can significantly enhance the anti-tumor efficacy of oxaliplatin against colon cancer cells. They also found that eEF2K can negatively regulate autophagy via ATG7 and BECN1 in human colon cancer cells (HT-29 and HCT-116), which is the opposite of the pro-autophagic effect observed earlier in breast cancer cells, glioma cells and mouse embryonic fibroblasts (MEFs)[46]. They observed that LC3-II levels in cells increased and LC3 dots accumulated in cells when eEF2K was knocked down. These authors also observed that the autophagic flux increased in eEF2K knockdown cells. eEF2K negatively regulates cell viability clonogenicity, cell proliferation and size in colon cancer cells.

The same study found that the AKT inhibitor MK-2206 can significantly promote autophagy in colon cancer cells at 5 μmol/L. However, because at this concentration the phosphorylation of eEF2 is not changed, the induction of autophagy by MK-2206 cannot be completely attributed to the activation of eEF2K[45]. Moreover, knocking down eEF2K in human colon cancer cells can neither enhance the anticancer efficacy of MK-2206 nor block the autophagy response to MK-2206, suggesting that eEF2K is not required for MK-2206-induced autophagy in colon cancer cells. Interestingly, these data are the opposite of what was observed in colon cancer cells[45].

In eEF2K-depleted colon cancer cells, the AMPK-ULK1 (unc-51, such as autophagy-activating kinase 1) pathway is activated, but mTORC1 signaling is not deactivated. Xie *et al*[45] argue that higher levels of protein synthesis due to knockdown of eEF2K would deplete the ATP levels in the cell, which would further activate AMPK. To try to test the role of inhibiting translation elongation in the control of autophagy, they used the elongation inhibitor cycloheximide. This could completely block the phosphorylation of ULK1 and the LC3-II accumulation induced by eEF2K silencing, which could suggest that the removal of eEF2K activates the autophagy machinery by leading to excessive translation. However, cycloheximide has long been known to activate mTORC1 signaling[47, 48], which represses autophagy, and therefore it is hard to interpret these data. Overall, the data indicate that AMPK-ULK signaling is involved in the increased autophagy induced by eEF2K knock-down.

Further work is clearly needed to delineate the roles of eEF2K in tumor initiation or early development and in later-stage tumors, especially solid tumors. The utility of targeting eEF2K in cancer therapy may well depend on the tumor stage and type.

Wang *et al*[49] found that HLJDD (Huanglian Jiedu decoction), a traditional Chinese medicine that has been shown to have anti-cancer growth and anti-metastasis effects in clinical practice, can inhibit the proliferation of HepG2 hepatoma cells but is less potent in inhibiting the proliferation of MHCC97L cells (another type of hepatocellular carcinoma). This agent can also suppress angiogenesis (assessed using CD31 as an endothelial cell marker) and the growth of hepatocellular carcinoma xenografts (MHCC97L cells into nude mice). By using AHA (*L*-azidohomoalanine) labeling, they found that HLJDD inhibited protein synthesis in both HepG2 and MHCC97L cells at non-toxic doses. HLJDD had no effect on the phosphorylation of other translational components, such as eIF4A, eIF4E, and eIF4G. Nevertheless, by using compound C (an inhibitor of AMPK), the authors of that study found that HLJDD probably activates eEF2K via the AMPK/mTORC1 cascade. These data indicate that in hepatoma cells, the activation of eEF2K impairs tumor cell proliferation. The active components in HLJDD include geniposide, berberine, baicalin and palmatine, which have been shown to have anti-cancer effects. All of them induce the phosphorylation of eEF2 in an additive fashion. These components may also have other targets in addition to activating eEF2K.

eEF2K in cardiovascular disease

Recent studies have indicated that eEF2K has a role in cardiovascular disorders (Figure 3). Atherosclerosis is an important health challenge and a complex inflammatory disorder that involves the activation, proliferation and survival of macrophages. Oxidized low-density lipoprotein (oxLDL) blocks the apoptosis of macrophages and plays an important role in the pathogenesis of atherosclerosis[50]. eEF2K is activated by oxLDL, likely through elevated Ca^{2+}-ion concentrations, and contributes to the survival of macrophages[51]. In recent work, LDL-receptor knock-out mice were reconstituted (after irradiation) with bone marrow from mice harboring an inactivating mutation in the *eEF2K* gene and were then fed a high-fat diet to induce atherosclerosis[52]. These mice displayed lower levels of atherosclerotic plaques than did LDLR-KO mice that received bone marrow with wild-type eEF2K. This is consistent with eEF2K playing a role in macrophage survival and thus in atherogenesis. Interestingly, M1-skewed (pro-inflammatory) macrophages from the eEF2K mutant mice secreted lower levels of a range of proteins involved in inflammation (*eg*, tumor necrosis factor-α, TNF-α) and leukocyte migration.

The pathogenesis of hypertension involves vascular remodeling through the proliferation and migration of vascular smooth muscle cells. Usui *et al*[53] tested the hypothesis that eEF2K controls the proliferation and migration of SMCs. The same group found that eEF2K increased in the mesenteric artery of spontaneously hypertensive rats (SHR)[54]. They revealed that eEF2K partially mediates hypertension in SHR via vascular inflammation[55]. In cultured vascular endothelial and smooth muscle cells, eEF2K mediates TNF-α-induced vascular inflammatory responses via activation of ROS-dependent JNK and nuclear factor (NF)-κB signals[55]. Platelet-derived growth factor (PDGF-BB)-induced SMC proliferation can be inhibited by eEF2K inhibitor A-484954 (as assessed by cell number and bromodeoxyuridine incorporation). The authors then used a Boyden chamber assay to show that PDGF-BB-induced SMC migration was significantly inhibited by A-484954. The FBS-induced outgrowth of SMCs is also inhibited by this compound. Interpretation of these data is compromised by the observation that A-484954 cannot inhibit the phosphorylation of eEF2 at T56 induced by PDGF-BB, but it did interfere with the phosphorylation of p38, ERK, and HSP27.

A further potential role for eEF2K has recently been reported in pulmonary arterial hypertension (PAH)[56]. This study found that eEF2K play roles in the remodeling of pulmonary arteries during the development of PAH. Administration of A-484954 can inhibit monocrotaline-induced PAH, which the authors suggest is mediated by eEF2K through the NADPH oxidase-1/ROS/matrix metalloproteinase-2 pathway, which plays an important role in PAH.

Neurological roles of eEF2K

eEF2K is expressed in neurons, and recent evidence suggests that it may play a role in processes such as learning and memory and in depression. Because the synthesis of new proteins is crucial for long-term memory[57, 58], and Ca^{2+} ions are a key second messenger downstream of neurotransmitter receptors, there is considerable interest in the role of eEF2K in the processes that underlie synaptic plasticity, which likely provides the basis for memory. Park *et al*[59] showed that eEF2K plays an important role in the synthesis of Arc/Arg3.1, a protein involved in neurotransmitter receptor trafficking and in long-term depression, a process that reflects changes in synaptic strength. Gildish *et al*[60] used mice in which an inactivating mutation was introduced into the *eEF2K* gene. Their data showed that such animals displayed defective associative taste learning in a learning paradigm termed conditioned taste aversion. These animals also displayed abnormal brain activation, which was revealed by magnetic resonance imaging.

Given that the activation of eEF2K leads to the inhibition of protein synthesis, how could eEF2K regulate the increased synthesis of specific proteins? Early work showed that the inhibition of elongation can actually enhance the relative expression of proteins encoded by 'weak' mRNAs, which compete poorly for binding to ribosomes[61]. By making elongation the limiting step, it is argued that slower rates of elongation allow such mRNAs to bind to ribosomes and thus become translated. Presumably, the impairment of elongation would have to be transient, initially allowing this process to occur and then being relieved to allow newly recruited mRNAs to be efficiently translated. A recent study in mouse cortical neurons showed that this is indeed the case in the response to bicuculline, a γ-aminobutyric acid receptor antagonist that increases Ca^{2+}-ion concentrations[62]. This agent causes a rapid initial increase in eEF2 phosphorylation, which peaks within one min and then declines toward basal levels within 30–40 min. This decrease is driven by signaling through the classical MAP kinase (ERK) and mTORC1 pathways and is partially opposed by activation of AMPK, which can be switched on by Ca^{2+} ions. These signaling connections permit a transient rise in eEF2 phosphorylation, *ie*, a transient inhibition of elongation, which is consistent with the type of regulatory model described above.

Previous work suggested that ketamine, a fast-acting antidepressant, exerts its effects on the expression of brain-derived growth factor (BDNF), an important neuroregulatory protein, via the inhibition of eEF2K signaling, which normally negatively regulates BDNF expression[63, 64]. Ketamine enhances BDNF expression and exerts anti-depressive effects in wild-type mice but not in animals with eEF2K knock out. A study using eEF2K-deficient mice would be very important for determining whether eEF2K is a target for the development of new anti-depressant drugs. These and other aspects of the neurological roles of eEF2K are discussed in a recent review by Heise *et al*[65].

A very recent study indicates a quite different neurological role for AMPK and eEF2K in the setting of an AD model, in which amyloid β induces impairments of long-term potentiation (LTP) and depression (Figure 3). LTP is generally considered a form of synaptic plasticity associated with learning and memory formation. In this model, amyloid β causes the activation of AMPK, and this contributes to the observed impair-

ments in plasticity[66]. This raises the obvious question of which protein(s) downstream of AMPK mediate these effects. Ma *et al*[66] showed that eEF2 phosphorylation is elevated in the hippocampus (a brain area crucial for learning) of AD model mice. Most importantly in the present context, the pharmacological inhibition of eEF2K improved the deficit in LTP in the AD mice but not in controls. This suggests that eEF2K may be a relevant target for ameliorating defects associated with AD, which represents a huge and rapidly growing global health challenge. However, further work using more specific eEF2K inhibitors than the one used here (NH125) and genetic models deficient in eEF2K activity is required to establish whether eEF2K is a useful target in this context. It is also important to bear in mind the positive roles, discussed above, of eEF2K in synaptic plasticity and learning.

Conclusion

As summarized above, eEF2K appears to play a role in a range of pathologies, including cardiovascular disease, cancers, neurodegeneration and, perhaps, depression (Figure 3). The precise mechanisms by which it does this remain to be established. eEF2K may do so through its effect on protein synthesis, a process that clearly has a fundamental impact both on the proteome and the cellular 'economy' (*eg*, energy and nutrient consumption). It is also possible that eEF2K has other substrates that are involved in some of these effects, although to date none have been identified. It is important to develop specific and potent small-molecule inhibitors of eEF2K for use in cell-based studies and especially in investigations using preclinical models of disease.

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