

## Short Conceptual Overview

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# Raf kinases in signal transduction and interaction with translation machinery

**Abstract:** In recent years, a large amount of evidence has given a central role to translational control in diseases such as cancer, tissue hypertrophy and neurodegeneration. Its deregulation can directly modulate cell cycling, transformation and survival response. The aim of this review is to describe the interaction between Raf activation and the main characters of the translational machinery, such as the elongation factor 1A (eEF1A), which has been recognized in recent years as one of the most interesting putative oncogenes. A particular emphasis is given to an intriguing non-canonical role that eEF1A can play in the relationship between the Ras→Raf-1→MEK1→ERK1/2 and PI3K→Akt signaling pathways. Recently, our group has described a C-Raf kinase-mediated phosphorylation of eEF1A triggered by a survival pathway induced upon interferon alpha (IFN $\alpha$ ) treatment in the human epidermoid cancer cell line (H1355). This phosphorylation seems to be the center of the survival pathway that counteracts the well-known pro-apoptotic function of IFN $\alpha$ . Furthermore, we have identified two new phosphorylation sites on eEF1A (Ser21 and Thr88) that are substrates for Raf kinases *in vitro* and, likely, *in vivo* as well. These residues seem to have a significant functional role in the control of cellular processes, such as cell proliferation and survival. In fact, overexpression of eEF1A2 in gemcitabine-treated cancer cells caused the upregulation of phosphoAkt and an increase in cell viability, thereby suggesting that eEF1A2 could exert its oncogenic behavior by participating in the regulation of PI3K pathway.

**Keywords:** cell proliferation; Raf kinases; signal transduction; translation elongation factors.

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## Introduction

In mammals, the Raf (rapid accelerated fibrosarcoma) kinase isoforms, Raf-1/C-Raf, B-Raf and A-Raf, are encoded by three different genes. Raf-1 or C-Raf is a 74-kDa protein, ubiquitously expressed in adult tissues, that shows the highest expression in muscle, cerebellum, and fetal brain. The 94-kDa B-Raf kinase is an ubiquitous protein but is prevalently expressed in testis and neuronal tissue. The A-Raf gene product is a protein of 68-kDa and is mainly found in the urogenital tract (1).

Raf kinase isoforms in mammals show similar structural properties; they are made of three conserved regions (CR) owning specific functions. The first conserved region (CR1) contains two domains: 1) the Ras-binding domain (RBD), required for interaction with Ras and with membrane phospholipids involved in membrane recruitment and 2) the cysteine-rich domain (CRD), functioning as secondary Ras-binding site and also essential for the auto-inhibition of Raf through the interaction of CR1 with the Raf kinase domain (2). The second conserved region (CR2) contains important inhibitory phosphorylation sites that are involved in the negative regulation of Ras binding and Raf activation (3). The third conserved region (CR3) represents the C-terminal region. CR3 is the kinase domain, including the activation segment, whose phosphorylation is fundamental for kinase activation (4).

Raf kinases represent a link between Ras and the MEK-ERK kinases (extracellular signal-regulated kinases) of the TKR-Ras-MAPK pathway (TKR, tyrosine kinase receptor; MAPK, mitogen-activated protein kinase). TKRs, upon activation, recruit the guanine nucleotide exchange factor SOS and that, in turn, activates Ras proteins by exchanging guanosine-5'-diphosphate (GDP) for

guanosine-5'-triphosphate (GTP). Activated Ras•GTP binds to Raf and initiates Raf activation. Active Raf phosphorylates and activates MEK, which, in turn, phosphorylates and activates ERK. All Raf proteins have MEK1/2 kinases as substrates. Whereas the phosphorylation cascade Raf→MEK→ERK is linear, activated ERK is instead able to interact with a large number (more than 150) of cytosolic and nuclear substrates. The activation of Raf represents the most intricate step of the TKR–Ras–MAPK signaling pathway and involves several events, such as membrane recruitment, phosphorylation and protein oligomerization. Moreover, this signaling pathway is involved in the regulation of several cellular functions like cell proliferation, differentiation, migration and apoptosis (5–7).

## Ras-dependent activation of Raf kinase

Inactive Raf is present in the cytosol in a closed conformation with the N-terminal regulatory region folded over the catalytic region. The binding of the regulatory region to the kinase domain (8) is stabilized by binding to 14-3-3 protein (9–13). Raf activation is a multistep process driven by activated Ras (Ras•GTP) recruiting inactive Raf complexed with 14-3-3 and heat shock proteins, from the cytosol to the plasma membrane (14–19). Subsequently, Raf is subjected to several additional modifications before activation. Both inactive A-Raf and Raf-1 are phosphorylated on Ser259 within the CR2 region and on Ser621 in the C-terminal region and bound to the highly conserved chaperonin protein 14-3-3. Ras•GTP then promotes the transition of Raf from a closed to an open conformation by removing the interaction between the regulatory region and kinase domain (9, 12). The dephosphorylation of Ser259, dissociation of 14-3-3 from CR2 and stabilization of the open conformation are associated with Raf membrane recruitment (12, 14, 20–22). The dephosphorylation of Ser259 is catalyzed by specific phosphatases (PP2A, PP1) (14, 23–25). Ser259 can be phosphorylated by two kinases, PKA and Akt (protein kinase B, PKB). Strong evidence suggests that PKA directly phosphorylates Ser259 (10, 26), whereas Akt seems to have a direct as well as an indirect effect (27–29). Recent reports have indicated that PKA can phosphorylate additional regulatory Raf-1 residues; Ser43, whose phosphorylation interferes with binding to Ras, and Ser233, whose phosphorylation in addition to that of Ser259, enhances the binding of 14-3-3 and suppresses the catalytic activity of Raf (30). Raf membrane recruitment also facilitates its association with other proteins involved

in the activation process that stabilize the open and active conformation of Raf.

The other Raf inactivation-phosphorylation site Ser621 is conserved in all Raf isoforms and constitutes also an interaction site with 14-3-3 (31, 32). The phosphorylation of Ser621 with Ser259 seems to play different roles: 1) it is necessary for the stabilization of Raf in its closed inactive conformation; 2) its binding to 14-3-3 dimers enhances the dimerization of Raf (34–38) and allows the binding of ATP to the Raf catalytic domain (9, 13, 32, 39). However, phosphorylation of Ser621 alone appears to be required for Raf activity, increasing stability and preventing proteasomal degradation (33). The phosphorylation of Ser621 generally occurs by autophosphorylation (33, 39), although other kinases may be involved (39). The dephosphorylation of Ser621 is operated by currently unknown phosphatase(s).

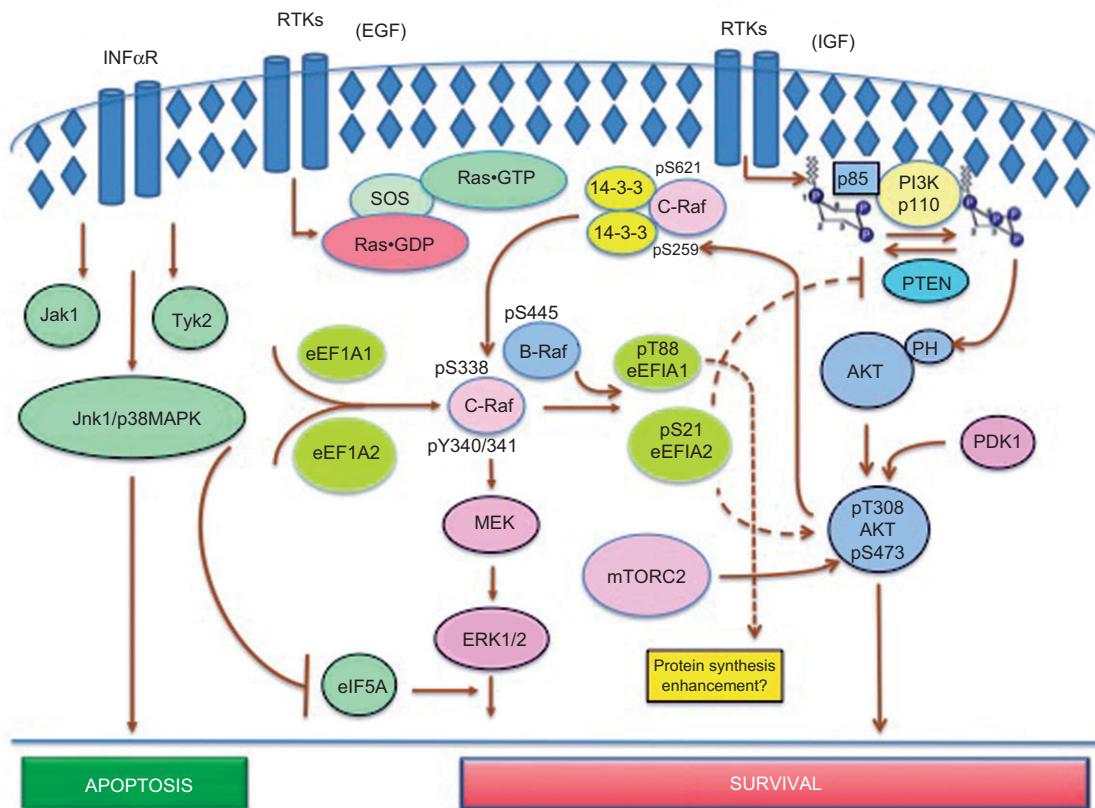
Raf activation involves instead additional phosphorylation sites, including Ser338 and Tyr340/341 in the N-region (negative charge regulatory region), upstream of CR3 as well as two putative sites in the activation segment of the kinase domain. The phosphorylation sites Ser<sub>338</sub>SY<sub>341</sub> are essential for full kinase activation and for interaction with the substrate MEK (40–42). Phosphorylation of Ser338 stimulated by growth factor is Ras-dependent and is necessary for maximal C-Raf activation (4, 40, 43, 44). A recent report indicated that Ser338 could be an autophosphorylation site induced by the dimerization of C-Raf or heterodimerization of C-Raf and B-Raf (45) or be a target for casein kinase 2 (CK2) recruited to Raf-1 and B-Raf by the scaffold kinase suppressor of Ras (KSR) (46). Moreover, Ser338 phosphorylation seems to synergize with the phosphorylation of Tyr340/341. The kinases that can be involved in the phosphorylation of Tyr341 include the Src (47, 17) and JAK family kinases (Janus Kinase) (48). Phosphorylation of Tyr340/341 stimulated by growth factor requires Ras-dependent membrane recruitment and Src family tyrosine kinases (18, 19, 40, 45), and phosphorylation of Tyr340/341 and Ser338 is required for maximal Raf activity (4, 8, 43, 44). Mutation of Tyr341 severely compromises Raf-1 kinase activity (47, 17).

Recently, the homodimerization of C-Raf and heterodimerization of C-Raf and B-Raf began to appear as important regulatory mechanisms that can strongly increase kinase activity and Raf signaling. Even though it is part of the physiological activation mechanism (36), it is not yet known when and at which step the dimerization occurs. It may also represent an alternative way for the activation of Raf that could be independent from the phosphorylation of the N-terminal (34).

## Raf→MEK→ERK signaling cascade

Once activated, Raf induces a downstream signal transduction cascade that initiates with the activation of MEK through its phosphorylation on Ser218 and Ser222. These two phosphorylations strongly increase the activity of MEK (48) (Figure 1). Increased MEK activity has been seen in a variety of leukemias; *in vitro*, constitutively activated MEK has shown both transforming ability and antiapoptotic effects, which suggests that MEK may be another important target in this pathway (49). All Raf kinases are able to bind and phosphorylate MEK *in vitro*, but their activities toward MEK are largely different. B-Raf shows the highest activity compared to Raf-1 and A-Raf (18). The fact that B-Raf shows higher basal activity might be linked to its immediate activation upon translocation to the plasma membrane due to its constitutive phosphorylation on Ser445 (corresponding to the regulated phosphorylation

site Ser338 on Raf-1) and also to the fact that the regulatory tyrosine residue of Raf-1 (Tyr341) is replaced by an aspartic acid residue (Asp448) that, because of its negative charge, mimics phosphorylation (44). Therefore, the activation of B-Raf occurs with a mechanism much simpler than that of Raf-1, and this might explain why B-Raf is a stronger activator of MEK and why B-Raf is a preferred target of mutations in human cancers (50). In addition, among all Raf kinases, B-Raf shows the strongest binding affinity toward MEK (51). In fact, MEK is normally present in the cells as a preformed complex with B-Raf and proteins, and these structures are ready to be activated. MEK-Raf-1 interaction is also regulated by scaffold proteins like KSR (46) and by phosphorylation. For instance, MEK1 can be phosphorylated on Ser298 by PAK1 (Rac effector kinase), which enhances the interaction with Raf-1 (52) and ERK2 (53), whereas the phosphorylation of MEK1 Thr292 by activated ERK prevents the phosphorylation of Ser298, thus limiting



**Figure 1** INFα and TKRs signaling pathways.

Schematic representation of the apoptotic and survival pathways mediated by INFαR and TKRs, respectively. The effect of INFαR results in a reduction of the hypusinated eIF5A1 expression levels, and eIF5A1 activity and in the upregulation of the translation factors eEF1A isoforms. The subsequent effect of EGFR is the induction of Ras→Raf→MEK→ERK1/2 signaling cascade. C-Raf and B-Raf activation/dimerization phosphorylate translation elongation factors 1A isoforms thus leading to their increase in stability and non-canonical functions. Phosphorylated eEF1A2 might regulate phosphoAkt activity, thus participating in the cross-talk between the Ras→Raf→MEK→ERK1/2 and PI3K→Akt signaling pathways.

the activation of MEK by Raf-1 (54). Thr292 is present only on MEK1, and its phosphorylation down-regulates the activity of MEK2 when MEK1-MEK2 dimerize. The observation that MEK kinase activities of Raf-1 and A-Raf are regulated in a more complicated manner with respect to that exerted by B-Raf suggests that both Raf-1 and A-Raf might exert their activity also on other not-defined substrates or that they are themselves regulators of the activity of B-Raf in the activation of the ERK pathway (55).

Downstream of MEK there are two kinases, ERK1 and ERK2, that are activated by phosphorylation on residues Thr183 and Tyr185. The phosphorylation allows the dimerization of ERK with other ERK proteins and induces the nuclear translocation and activation of a variety of nuclear targets. ERK interacts also with cytoplasmic p90RSK, whose substrates comprise several transcription factors, including cyclic AMP-response element binding protein, C-MYC, Ets, activator protein-1 and nuclear factor- $\kappa$ B (NF- $\kappa$ B), all having diverse cellular effects (56). Thus, the ERK signaling cascade couples signals from cell surface receptors to transcription factors that regulate gene expression. Depending on the stimulus and cell type, this pathway can transmit signals that modulate cell proliferation, differentiation, angiogenesis or apoptosis (57).

## Kinase-independent functions of C-RAF

Raf kinase signaling occurs also in a cascade-independent manner (58). These activities include the antagonization of pro-apoptotic factors like MST2, the mammalian sterile 20-like kinase (59), the apoptosis signal-regulating kinase 1 (ASK1) (60), the BCL-2-antagonist of cell death (BAD) (61), the positive regulation of cell migration via the Rho effector kinase Rok- $\alpha$  (62) and the activation of the transcription factor NF- $\kappa$ B (63, 64).

## Raf kinases and protein synthesis machinery

One of the TKRs that activates the Ras $\rightarrow$ Raf-1 $\rightarrow$ MEK1 $\rightarrow$ ERK1/2 signaling pathway is the epidermal growth factor receptor (EGFR). The increased expression of EGFR was found to be associated to IFN $\alpha$  therapy, in combination with chemotherapy and radiation, as a treatment for many cancers. In human epidermoid cancer cells (H1355), IFN $\alpha$  induces growth inhibition and apoptosis,

most likely through the activation of caspase cascade mediated by c-Jun N-terminal kinase (JNK-1) and/or p38 MAPK activation and the mitochondrial pathway (65). Moreover, a concomitant reduction of the hypusinated eIF5A1 expression levels and eIF5A1 activity was also observed (66). Hypusine plays a key role in the regulation of eIF5A function, as only the hypusine-containing eIF5A form is active. Intracellular hypusine content measures also the activity of eIF5A, as hypusine is contained only in this factor. The reduction of eIF5A1 expression or the inhibition of hypusine modification may cause induction or suppression of apoptosis, depending on the biological system (67). These anti-proliferative and pro-apoptotic activities are all antagonized by the epidermal growth factor (EGF). In particular, Raf-1 activity is increased by either EGF or IFN $\alpha$  and is potentiated after EGF addition.

IFN $\alpha$  treatment induces also an early up-regulation of the eukaryotic translation elongation factor 1A (eEF1A) without an increase in the levels of its transcripts. This finding suggests that the up-regulation of the intracellular content of eEF1A is post-transcriptionally modulated. In higher vertebrates, eEF1A is present in two isoforms (eEF1A1 and eEF1A2) with different expression patterns and encoded by distinct genes (68). The near-ubiquitous form, eEF1A1, is expressed in all tissues throughout development but is absent in adult muscle and heart, which express eEF1A2 instead (68, 69). eEF1A2 is also found in some other cell types, including large motor neurons, islet cells in the pancreas and enteroendocrine cells in the gut (70, 71). We showed that in H1355, treatment with IFN $\alpha$  increased both eEF1A1 and eEF1A2 expression, but to different degrees and with different kinetics. In particular, eEF1A2 was the most increased isoform, and it also showed a timing similar to the one recorded for total eEF1A. These data suggest that the eEF1A2 increase was largely responsible for the up-regulation of total eEF1A (Table 1).

siRNA downregulation of EF1A rescued the apoptosis induced by IFN $\alpha$ , which suggests a role for eEF1A in the

**Table 1** Effect of IFN $\alpha$  on the expression levels of eEF1A isoforms in H1355 cells.

eEF1A	Time of IFN $\alpha$ treatment (min)		
	0	10	30
eEF1A1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.5 $\pm$ 0.07
eEF1A2	1.0 $\pm$ 0.1	1.7 $\pm$ 0.09	2.4 $\pm$ 0.05

Human lung epidermoid cells H1355 were treated with 2500 IU/ml of IFN $\alpha$  and then analyzed by Western blot using anti-eEF1A1 and anti-eEF1A2 antibody. Films were then quantified using ImageJ 1.46r software (National Institute of Health, Bethesda, MD, USA) (72).

regulation of apoptosis. The increase of eEF1A levels mediated by  $\text{INF}\alpha$  was also associated to a phosphorylation of eEF1A mediated by Raf kinase. As further evidence, C-Raf inhibitor (BAY 43–9006) treatment induced a decrease of eEF1A phosphorylation and increased apoptosis. These data suggest the existence of an anti-apoptotic network between the translational factor 1A and the Ras-dependent signaling pathway (72). The activation of C-Raf and B-Raf by  $\text{INF}\alpha$  was presumably indirect and induced by up-regulation of EGFR. Moreover, C-Raf has been demonstrated to protect eEF1A from ubiquitin-mediated degradation (72). Interestingly, both eEF1A1 and eEF1A2 were singularly phosphorylated by B-Raf *in vitro*, whereas phosphorylation by C-Raf required the presence of both isoforms. Two new eEF1A phosphorylation sites have been identified: Thr88 and Ser21. The former was specifically mediated by B-Raf on eEF1A1, whereas the latter was present on both eEF1A isoforms and mediated by both the B- and C-Raf kinases. Thr88 phosphorylation was also identified on eEF1A1, expressed in proliferating COS 7 cells and thus suggesting that this post-translational modification is isoform-specific and probably due to structural differences between eEF1A1 and eEF1A2 (Table 2) (73). Further analyzed, phosphoThr88 seems to stabilize the elongation complex *in vivo*. In contrast, the phosphorylation of Ser21 – residue that belongs to the first GTP/GDP-binding consensus sequence (G14HVD SGKST in both eEF1A1 and eEF1A2) – could potentially prevent the binding of eEF1A to guanine nucleotides, thus allowing a switch of eEF1A activity from protein biosynthesis to different non-canonical functions (74). These findings are also supported by other evidence. In fact, expression of activated Raf-1 protein kinase in isogenic squamous carcinoma cells was associated with an increase in the steady-state mRNA

levels of eEF1A. In addition, the overexpression of eEF1A in human fibroblasts renders these cells susceptible to transformation by chemical carcinogens, which suggests eEF1A has a role in carcinogenesis (75).

In conclusion, in H1355, the upregulation of eEF1A intracellular content mediated by  $\text{INF}\alpha$  is associated with an increase in the phosphorylation of eEF1A. This effect is mediated by Raf kinases and appears to have a pro-survival function. Therefore, eEF1A is an antiapoptotic factor with activity that links the protein synthesis machinery to the growth-factor-elicited survival pathway.

## PI3K signaling pathway

A possible functional role exerted by the increased expression of eEF1A, in particular that of eEF1A2 during cell survival, could be the control of Akt function. In fact, another important pathway that is triggered by the binding of growth factors and cytokines to cell surface receptors is the PI3K signaling pathway (76). This pathway is paralleled by the Ras→Raf-1→MEK1→ERK1/2 signaling pathway and represents a major mitogenic and survival signaling pathway (Figure 1). The lipid kinase PI3K, a serine-threonine kinase known as protein kinase B (PKB), is a heterodimeric enzyme made of a regulatory subunit consisting of p85, and a catalytic subunit p110. Activation of PI3K is induced by a large number of growth factors, including insulin, insulin-like growth factors (IGF) and platelet-derived growth factors (PDGF) as well as chemokines and cytokines (76). In resting cells, PI3K has a cytoplasmic localization and is recruited by activated TKRs through the Src homology 2 domains of p85. PI3K activation is

**Table 2** Raf-mediated phosphorylation of eEF1A1 and eEF1A2.

eEF1A substrate	Raf kinase	Identified phosphopeptide	Phosphorylated residue
1 eEF1A1	B-Raf	K.pSTTTGHLIYK.C	pS21
2 eEF1A1	B-Raf	K.YYVpTIIDAPGHRDFIK.N	pT88
3 eEF1A2	B-Raf	K.pSTTTGHLIYK.C	pS21
4 eEF1A1	C-Raf DD <sup>a</sup>	–	–
5 eEF1A2	C-Raf DD	K.pSTTTGHLIYK.C	pS21
6 eEF1A1+eEF1A2	C-Raf DD	K.pSTTTGHLIYK.C <sup>b</sup>	pS21
7 eEF1A1	–	K.YYVpTIIDAPGHRDFIK.N	pT88
8 eEF1A1	–	K.p(YY)VTIIDAPGHR.D	pY85 or pY86

<sup>a</sup>C-Raf DD (constitutively active C-Raf, Bosch et al. 1997) (73).

<sup>b</sup>Phosphopeptide not specifically assigned to any two eEF1A isoforms.

1–6 Purified recombinant eEF1As and Raf kinases were used in a phosphorylation assay *in vitro*.

7–8 eEF1A1 overexpressed in COS7 cells. Phosphorylated eEF1As isolated by SDS-PAGE were then analyzed by mass spectrometry for the presence of phosphopeptides (Sanges et al. 2012) (74).

considered to be a survival signal as it protects cells from apoptosis. PI3K produces the phosphatidylinositol lipids (PI-3,4,5-P<sub>3</sub>), which can stimulate several kinases including Akt, whose activation protects cells from apoptosis (77). Akt is regulated through both localization and phosphorylation. In fact, Akt is characterized by the presence at its NH<sub>2</sub>-terminus of a pleckstrin homology (PH) domain that is crucial for its activation by growth factor stimulation. The phosphorylated product of PI3K, PI-3,4,5-P<sub>3</sub> (phosphatidylinositol 3,4,5-trisphosphate), binds to the PH domain of Akt, thus causing a conformational change and membrane localization. An Akt conformational change allows its phosphorylation on Thr308 and Ser473. These modifications are required for the activation of Akt (78); in fact, phosphatase treatment of Akt *in vitro* inactivates Akt toward its substrates. The kinase that phosphorylates Thr308 is the phosphatidylinositol dependent kinase (PDK1), whereas there are several candidates that can phosphorylate Ser473, the most likely being the mTORC2 (79). Phosphorylation of Ser473 PI3K can also be stimulated by activated Ras, which directly binds p110 (80). PTEN is a lipid phosphatase known as phosphatase and tensin homologue deleted on chromosome 10 that is negatively regulated by the PI3K signaling pathway (81). The catalytic activity of PTEN consists of the removal of the phosphate at the 3' position of PI-3,4,5-P<sub>3</sub> and its conversion to PI-4,5-P<sub>2</sub>. Following this reaction, the cellular levels of PI-3,4,5-P<sub>3</sub> are reduced and, as consequence, there is a block of PI3K activation. Thus, PTEN regulates cell growth and survival by acting as tumor suppressor.

Activated Akt controls cell proliferation, cell survival, translation, cell size and cell metabolism. Therefore, Akt is a proto-oncoprotein that, when activated, directly phosphorylates and regulates molecules involved in these processes. The constitutive activation of Raf–MAPK leads to the inhibition of Akt with a cross-talk mechanism that is still unknown. One plausible mechanism would involve a negative regulator of Akt activity that could be up-regulated by MAPK signaling. Interestingly, in the presence of cyclohexamide, an inhibitor of de novo protein synthesis, Raf was unable to inhibit Akt phosphorylation, which suggests that Raf–MAPK signaling requires protein synthesis for the inhibition of AKT (82). Therefore, to assay if the overexpression of eEF1A2 could act on the regulation of Akt activity, we assayed the phosphoAkt expression levels in Hek293 cell line after treatment of the cells with gemcitabine. It is known that gemcitabine generally synergistically potentiates the antitumor effect of several drugs by inducing the activation of the caspase-dependent apoptotic pathway, but also by suppressing the Akt survival pathway by inhibiting Akt activity (83). We found that

the expression of the eEF1A2 isoform (cloneC), normally expressed in this cell line at very low levels (control), was accompanied by a corresponding increase of phosphorylated Akt, detected with an antibody anti-phospho(Ser473)-Akt (Sigma, St. Louis, MO, USA), as compared to the same cells where no expression of eEF1A2 was induced (Table 3). This effect was also associated with an inhibition of apoptosis of approximately 70% as evaluated by cytofluorimetric analysis (Becton Dickinson; Palo Alto, CA, USA) with propidium iodide (Sigma, St. Louis, MO, USA), thus confirming the oncogenic properties of eEF1A2.

Similar behavior exerted by eEF1A2 was also described in BT549 human breast cancer cells (84). In fact, filopodia formation in eEF1A2-expressing cells was dependent on the activity of PI3K, ROCK and Akt kinases. Furthermore, eEF1A2 expression was sufficient to activate Akt in a PI3K-dependent fashion, and inactivation of eEF1A2 by short interfering RNA reduced Akt activity. In addition, eEF1A2 expression stimulated cell migration and invasion in a largely PI3K- and Akt-dependent manner. Moreover, it has been shown that eEF1A is a pAkt-interacting protein that regulates pAkt levels (85). Because eEF1A is overexpressed in breast cancer, the increased eEF1A levels might influence proliferation, survival and invasion, depending on the relative concentration of Akt isoforms. Indeed, eEF1A2 interacts specifically with Akt2. These data suggest that eEF1A2 regulates oncogenesis through Akt- and PI3K-dependent cytoskeletal remodeling (86).

## Conclusion

Molecular events causing alterations in protein synthesis and translational control have a particular role in the molecular mechanisms underlying cancer development and progression. Interestingly, several translation factors can be

**Table 3** Levels of phospho-Akt (Ser473) in gemcitabine-treated Hek293 cells.<sup>a</sup>

Gemcitabine ( $\mu\text{M}$ )	Control		Clone C	
	48 h		72 h	
0.00	0.200 $\pm$ 0.02	0.360 $\pm$ 0.04	0.750 $\pm$ 0.08	0.380 $\pm$ 0.04
0.87	0.180 $\pm$ 0.02	0.390 $\pm$ 0.04	0.250 $\pm$ 0.03	0.300 $\pm$ 0.03
1.75	0.063 $\pm$ 0.05	0.350 $\pm$ 0.04	0.210 $\pm$ 0.02	0.420 $\pm$ 0.04

Levels of phosphoAkt were evaluated after Western blot with anti-phospho Akt (Ser473) by densitometric analysis of the film with ImageJ 1.46r software and normalized with the corresponding levels of GAPDH evaluated on the same filters with anti-GAPDH antibody. Values are averages of two independent experiments.

<sup>a</sup>Lamberti A., unpublished results.

directly involved in signal transduction pathways, interact with oncogenes or probably act themselves as oncogenes. One of the molecules involved in these alterations is eEF1A, which, in addition to its canonical involvement in protein synthesis, is also associated with molecular events participating in cell transformation, tumor development and progression and apoptosis induction or inhibition. In particular, the eEF1A2 isoform seems to possess oncogenic properties as it is involved in cancer progression. Both eEF1A isoforms might play a role in the cross-talk mechanisms underlying the antagonism between the Ras→Raf-1→MEK1→ERK1/2 and the PI3K→Akt signaling pathways. Up-regulation and phosphorylation of eEF1A1 and eEF1A2 by C-Raf and/or B-Raf increase the stability of these molecules and could represent a regulatory switch for other functional roles of eEF1A, such as a direct or indirect regulator of Akt activity.

A more thorough investigation concerning specific changes in the translation apparatus for certain types of

human cancers in relation to their stage, grade, histopathology and exposure to standard anticancer therapies should be conducted. In conclusion, we think that one of the possible ways to improve tumor therapies is to clarify the specific cancer-associated changes in the translation machinery. This research could probably provide an opportunity to develop selective anti-tumor inhibitors selectively directed against translation factors.

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## References

- Sridhar SS, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther* 2005; 4: 677–85.
- Tran NH, Wu X, Frost JA. B-Raf and Raf-1 are regulated by distinct autoregulatory mechanisms. *J Biol Chem* 2005; 280: 16244–53.
- Dhillon AS, Meikle S, Yazici Z, Eulitz M, Kolch W. Regulation of Raf-1 activation and signaling by dephosphorylation. *EMBO J* 2002; 21: 64–71.
- Chong H, Lee J, Guan KL. Positive and negative regulation of Raf kinase activity and function by phosphorylation. *EMBO J* 2001; 20: 3716–27.
- Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol* 2004; 5: 875–85.
- Leicht DT, Balan V, Kaplun A, Singh-Gupta V, Kaplun L, Dobson M, Tzivion G. Raf kinases: function, regulation and role in human cancer. *Biochim Biophys Acta* 2007; 1773: 1196–212.
- Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. *Oncogene* 2007; 26: 3279–90.
- Tran NH, Frost JA. Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition. *J Biol Chem* 2003; 278: 11221–6.
- Terai K, Matsuda M. Ras binding opens c-Raf to expose the docking site for mitogen-activated protein kinase kinase. *EMBO Rep* 2005; 6: 251–5.
- Dumaz N, Marais R. Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. *J Biol Chem* 2003; 278: 29819–23.
- Cutler RE Jr, Stephens RM, Saracino MR, Morrison DK. Autoregulation of the Raf-1 serine/threonine kinase. *Proc Natl Acad Sci USA* 1998; 95: 9214–9.
- Hibino K, Shibata T, Yanagida T, Sako Y. A RasGTP-induced conformational change in C-RAF is essential for accurate molecular recognition. *Biophys J* 2009; 97: 1277–87.
- Rommel C, Radziwill G, Lovric J, Noeldeke J, Heinicke T, Jones D, Aitken A, Moelling K. Activated Ras displaces 14-3-3 protein from the amino terminus of c-Raf-1. *Oncogene* 1996; 12: 609–19.
- Kubicek M, Pacher M, Abraham D, Podar K, Eulitz M, Baccarini M. Dephosphorylation of Ser-259 regulates Raf-1 membrane association. *J Biol Chem* 2002; 277: 7913–9.
- Leevers SJ, Paterson HF, Marshall CJ. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* 1994; 369: 411–4.
- Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF. Activation of Raf as a result of recruitment to the plasma membrane. *Science* 1994; 264: 1463–7.
- Marais R, Light Y, Paterson HF, Marshall CJ. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J* 1995; 14: 3136–5.
- Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J Biol Chem* 1997; 272: 4378–83.
- Chong H, Vikis HG, Guan KL. Mechanisms of regulating the Raf kinase family. *Cell Signal* 2003; 15: 463–9.
- Light Y, Paterson H, Marais R. 14-3-3 antagonizes Ras-mediated Raf-1 recruitment to the plasma membrane to maintain signaling fidelity. *Mol Cell Biol* 2002; 22: 4984–96.
- Roy S, McPherson RA, Apolloni A, Yan J, Lane A, Clyde-Smith J, Hancock JF. 14-3-3 facilitates Ras-dependent Raf-1 activation in vitro and in vivo. *Mol Cell Biol* 1998; 18: 3947–55.
- Brummer T, Martin P, Herzog S, Misawa Y, Daly RJ, Reth M. Functional analysis of the regulatory requirements of B-Raf and the B-Raf(V600E) oncoprotein. *Oncogene* 2006; 25: 6262–76.
- Ory S, Zhou M, Conrads TP, Veenstra TD, Morrison DK. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol* 2003; 13: 1356–64.

24. Rodriguez-Viciano P, Oses-Prieto J, Burlingame A, Fried M, McCormick F. A phosphatase holoenzyme comprised of Shoc2/Sur8 and the catalytic subunit of PP1 functions as an M-Ras effector to modulate Raf activity. *Mol Cell* 2006; 22: 217–30.
25. Jaumot M, Hancock JF. Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions. *Oncogene* 2001; 20: 3949–58.
26. Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H, Kolch W. Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol Cell Biol* 2002; 22: 3237–46.
27. Guan KL, Figueroa C, Birtva TR, Zhu T, Taylor J, Barber TD, Vojtek AB. Negative regulation of the serine/threonine kinase B-Raf by Akt. *J Biol Chem* 2000; 275: 27354–9.
28. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 1999; 286: 1738–41.
29. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* 1999; 286: 1741–4.
30. Gerits N, Kostenko S, Shiryaev A, Johannessen M, Moens U. Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: comradeship and hostility. *Cell Signal* 2008; 20: 1592–607.
31. Yip-Schneider MT, Miao W, Lin A, Barnard DS, Tzivion G, Marshall MS. Regulation of the Raf-1 kinase domain by phosphorylation and 14-3-3 association. *Biochem J* 2000; 351: 151–9.
32. Tzivion G, Luo Z, Avruch J. A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* 1998; 394: 88–92.
33. Noble C, Mercer K, Hussain J, Carragher L, Giblett S, Hayward R, Patterson C, Marais R, Pritchard CA. CRAF autophosphorylation of serine 621 is required to prevent its proteasome-mediated degradation. *Mol Cell* 2008; 31: 862–72.
34. Garnett MJ, Rana S, Paterson H, Barford D, Marais R. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Mol Cell* 2005; 20: 963–9.
35. Rajakulendran T, Sahmi M, Lefrancois M, Sicheri F, Therrien M. A dimerization-dependent mechanism drives RAF catalytic activation. *Nature* 2009; 461: 542–5.
36. Rushworth LK, Hindley AD, O'Neill E, Kolch W. Regulation and role of Raf-1/B-Raf heterodimerization. *Mol Cell Biol* 2006; 26: 2262–72.
37. Weber CK, Slupsky JR, Kalmes HA, Rapp UR. Active Ras induces heterodimerization of cRaf and BRaf. *Cancer Res* 2001; 61: 3595–8.
38. Ritt DA, Monson DM, Specht SI, Morrison DK. Impact of feedback phosphorylation and Raf heterodimerization on normal and mutant B-Raf signaling. *Mol Cell Biol* 2010; 30: 806–19.
39. Dhillon AS, Yip YY, Grindlay GJ, Pakay JL, Dangers M, Hillmann M, Clark W, Pitt A, Mischak H, Kolch W. The C-terminus of Raf-1 acts as a 14-3-3-dependent activation switch. *Cell Signal* 2009; 21: 1645–51.
40. Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M. Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. *Mol Cell Biol* 1997; 17: 4509–16.
41. Xiang X, Zang M, Waelde CA, Wen R, Luo Z. Phosphorylation of 338SSYY341 regulates specific interaction between Raf-1 and MEK1. *J Biol Chem* 2002; 277: 44996–5003.
42. Edin ML, Juliano RL. Raf-1 serine 338 phosphorylation plays a key role in adhesion-dependent activation of extracellular signal-regulated kinase by epidermal growth factor. *Mol Cell Biol* 2005; 25: 4466–75.
43. Goetz CA, O'Neil JJ, Farrar MA. Membrane localization, oligomerization, and phosphorylation are required for optimal raf activation. *J Biol Chem* 2003; 278: 51184–9.
44. Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J* 1999; 18: 2137–48.
45. Zang M, Gong J, Luo L, Zhou J, Xiang X, Huang W, Huang Q, Luo X, Olbrot M, Peng Y, Chen C, Luo Z. Characterization of Ser338 phosphorylation for Raf-1 activation. *J Biol Chem* 2008; 283: 31429–37.
46. Ritt DA, Zhou M, Conrads TP, Veenstra TD, Copeland TD, Morrison DK. CK2 is a component of the KSR1 scaffold complex that contributes to Raf kinase activation. *Curr Biol* 2007; 17: 179–84.
47. Fabian JR, Daar IO, Morrison DK. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol Cell Biol* 1993; 13: 7170–9.
48. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; 22: 153–83.
49. Hoyle PE, Moyer PW, Steelman LS, Blalock WL, Franklin RA, Pearce M, Cherwinski H, Bosch E, McMahon M, McCubrey JA. Differential abilities of the Raf family of protein kinases to abrogate cytokine dependency and prevent apoptosis in murine hematopoietic cells by a MEK1-dependent mechanism. *Leukemia* 2000; 14: 642–56.
50. Mercer KE, Pritchard CA. Raf proteins and cancer: B-Raf is identified as a mutational target. *Biochim Biophys Acta* 2003; 1653: 25–40.
51. Papin C, Denouel A, Calothy G, Eychene A. Identification of signalling proteins interacting with B-Raf in the yeast two-hybrid system. *Oncogene* 1996; 12: 2213–21.
52. Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE, Cobb MH. Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *EMBO J* 1997; 16: 6426–38.
53. Eblen ST, Slack JK, Weber MJ, Catling AD. Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol Cell Biol* 2002; 22: 6023–33.
54. Eblen ST, Slack-Davis JK, Tarcsafalvi A, Parsons JT, Weber MJ, Catling AD. Mitogenactivated protein kinase feedback phosphorylation regulates MEK1 complex formation and activation during cellular adhesion. *Mol Cell Biol* 2004; 24: 2308–17.
55. Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, Kolch W. Raf family kinases: old dogs have learned new tricks. *Genes Cancer* 2011; 2: 232–60.
56. Shelton JG, Steelman LS, Lee JT, Knapp SL, Blalock WL, Moyer PW, Franklin RA, Pohnert SC, Mirza AM, McMahon M, McCubrey JA. Effects of the RAF/MEK/ERK and PI3K/AKT signal transduction pathways on the abrogation of cytokine-dependence and prevention of apoptosis in hematopoietic cells. *Oncogene* 2003; 22: 2478–92.
57. Kholodenko BN, Hancock JF, Kolch W. Signalling ballet in space and time. *Nat Rev Mol Cell Biol* 2010; 11: 414–26.



58. Baccarini M. Second nature: biological functions of the Raf-1 'kinase'. *FEBS Lett* 2005; 579: 3271–7.
59. O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. *Science* 2004; 306: 2267–70.
60. Chen J, Fujii K, Zhang L, Roberts T, Fu H. Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. *Proc Natl Acad Sci USA* 2001; 98: 7783–8.
61. Wang HG, Rapp UR, Reed JC. Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* 1996; 87: 629–38.
62. Ehrenreiter K, Piazzolla D, Velamoor V, Sobczak I, Small JV, Takeda J, Leung T, Baccarini M. Raf-1 regulates Rho signaling and cell migration. *J Cell Biol* 2005; 168: 955–64.
63. Li S, Sedivy JM. Raf-1 protein kinase activates the NF- $\kappa$ B transcription factor by dissociating the cytoplasmic NF- $\kappa$ B-I $\kappa$ B complex. *Proc Natl Acad Sci USA* 1993; 90: 9247–51.
64. Baumann B, Weber CK, Troppmair J, Whiteside S, Israel A, Rapp UR, Wirth T. Raf induces NF- $\kappa$ B by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. *Proc Natl Acad Sci USA* 2000; 97: 4615–20.
65. Caraglia M, Vitale G, Marra M, Budillon A, Tagliaferri P, Abbruzzese A. Alpha-interferon and its effects on signalling pathways within cells. *Curr Protein Pept Sci* 2004; 5: 475–85.
66. Caraglia M, Passetto A, Beninati S, Leardi A, Nicolini L, Improta S, Pinto A, Bianco AR, Tagliaferri P, Abbruzzese A. Interferon  $\alpha$ 2 recombinant and epidermal growth factor modulate proliferation and hypusine synthesis in human epidermoid cancer KB cells. *Biochem J* 1997; 324: 737–41.
67. Caraglia M, Park MH, Wolff EC, Marra M, Abbruzzese A. eIF5A isoforms and cancer: two brothers for two functions? *Amino Acids* 2013; 44: 103–9.
68. Lund A, Knudsen SM, Vissing H, Clark B, Tommerup N. Assignment of human elongation factor 1 $\alpha$  genes: EEF1A maps to chromosome 6q14 and EEF1A2 to 20q13.3. *Genomics* 1996; 36: 359–61.
69. Lee S, Francoeur AM, Liu S, Wang E. Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1  $\alpha$  gene family. *J Biol Chem* 1992; 267: 24064–8.
70. Chambers DM, Peters J, Abbott CM. The lethal mutation of the mouse wasted (*wst*) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1A, encoded by the *Eef1a2* gene. *Proc Natl Acad Sci USA* 1998; 95: 4463–8.
71. Newbery HJ, Loh DH, O'Donoghue JE, Tomlinson VA, Chau YY, Boyd JA, Bergmann JH, Brownstein D, Abbott CM. Translation elongation factor eEF1A2 is essential for post-weaning survival in mice. *J Biol Chem* 2007; 282: 28951–9.
72. Lamberti A, Longo O, Marra M, Tagliaferri P, Bismuto E, Fiengo A, Viscomi C, Budillon A, Rapp UR, Wang E, Venuta S, Abbruzzese A, Arcari P, Caraglia M. C-Raf antagonizes apoptosis induced by IFN- $\alpha$  in human lung cancer cells by phosphorylation and increase of the intracellular content of elongation factor 1A. *Cell Death Differ* 2007; 14: 952–62.
73. Bosch E, Cherwinski H, Peterson D, McMahon M. Mutations of critical amino acids affect the biological and biochemical properties of oncogenic A-Raf and Raf-1. *Oncogene* 1997; 15: 1021–33.
74. Sanges C, Scheuermann C, Zahedi RP, Sickmann A, Lamberti A, Migliaccio N, Baljuls A, Marra M, Zappavigna S, Reinders J, Rapp U, Abbruzzese A, Caraglia M, Arcari P. Raf kinases mediate the phosphorylation of eukaryotic translation elongation factor 1A and regulate its stability in eukaryotic cells. *Cell Death Dis* 2012; 3: e276. DOI: 10.1038/cddis.2012.16.
75. Patel S, Wang FH, Whiteside TL, Kasid U. Constitutive modulation of Raf-1 protein kinase is associated with differential gene expression of several known and unknown genes. *Mol Med* 1997; 3: 674–85.
76. Wymann MP, Marone R. Phosphoinositide 3-kinase in disease: timing, location, and scaffolding. *Curr Opin Cell Biol* 2005; 17: 141–9.
77. Datta S, Brunet A, Greenberg M. Cellular survival: a play in three Acts. *Genes Dev* 1999; 13: 2905–27.
78. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* 1996; 15: 6541–51.
79. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005; 307: 1098–101.
80. Rodriguez-Viciana P, Warne PH, Dhand R Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 1994; 370: 527–32.
81. Stiles BL. Phosphatase and tensin homologue deleted on chromosome 10: extending its PTENacles. *Int J Biochem Cell B* 2009; 41: 757–61.
82. Menges CW, McCance DJ. Constitutive activation of the Raf-MAPK pathway causes negative feedback inhibition of Ras-PI3K-AKT and cellular arrest through the EphA2 receptor. *Oncogene* 2008; 27: 2934–40.
83. Bartholomeusz C, Yamasaki F, Saso H, Kurisu K, Horto-bagyi GN, Ueno NT. Gemcitabine overcomes erlotinib resistance in EGFR-overexpressing cancer cells through downregulation of Akt. *J Cancer* 2011; 2: 435–42.
84. Amiri A, Noei F, Jeganathan S, Kulkarni G, Pinke DE, Lee JM. eEF1A2 activates Akt and stimulates Akt-dependent actin remodeling, invasion and migration. *Oncogene* 2007; 26: 3027–40.
85. Pecorari L, Marin O, Silvestri C, Candini O, Rossi E, Guerzoni C, Cattalani S, Mariani SA, Corradini F, Ferrari-Amorotti G, Cortesi L, Bussolari R, Raschellà G, Federico MR, Calabretta B. Elongation Factor 1 alpha interacts with phospho-Akt in breast cancer cells and regulates their proliferation, survival and motility. *Mol Cancer* 2009; 8: 58. DOI: 10.1186/1476-4598-8-58.
86. Lau J, Castelli LA, Lin ECK, Macaulay S. Identification of elongation factor 1A as a potential associated binding partner for Akt2. *Mol Cell Biochem* 2006; 286: 17–22.