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Raf kinases mediate the phosphorylation of eukaryotic translation elongation factor 1A and regulate its stability in eukaryotic cells

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We identified eukaryotic translation elongation factor 1A (eEF1A) Raf-mediated phosphorylation sites and defined their role in the regulation of eEF1A half-life and of apoptosis of human cancer cells. Mass spectrometry identified *in vitro* S21 and T88 as phosphorylation sites mediated by B-Raf but not C-Raf on eEF1A1 whereas S21 was phosphorylated on eEF1A2 by both B- and C-Raf. Interestingly, S21 belongs to the first eEF1A GTP/GDP-binding consensus sequence. Phosphorylation of S21 was strongly enhanced when both eEF1A isoforms were preincubated prior the assay with C-Raf, suggesting that the eEF1A isoforms can heterodimerize thus increasing the accessibility of S21 to the phosphate. Overexpression of eEF1A1 in COS 7 cells confirmed the phosphorylation of T88 also *in vivo*. Compared with wt, in COS 7 cells overexpressed phosphodeficient (A) and phospho-mimicking (D) mutants of eEF1A1 (S21A/D and T88A/D) and of eEF1A2 (S21A/D), resulted less stable and more rapidly proteasome degraded. Transfection of S21 A/D eEF1A mutants in H1355 cells increased apoptosis in comparison with the wt isoforms. It indicates that the blockage of S21 interferes with or even supports C-Raf induced apoptosis rather than cell survival. Raf-mediated regulation of this site could be a crucial mechanism involved in the functional switching of eEF1A between its role in protein biosynthesis and its participation in other cellular processes.

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Eukaryotic translation elongation factor 1A (eEF1A) is one of the most abundant proteins in cells.[1,2] In high vertebrates a gene family encodes for two distinct isoforms, eEF1A1 and eEF1A2.[3] The coding region of the *eEF1A* gene is highly conserved throughout eukaryotes. In humans, the almost identical (92% sequence identity) amino-acid sequences of the eEF1A isoforms differ in length only for one additional C-terminal amino-acid residue present in the second isoform. eEF1A belongs to the family of GTP-binding proteins and promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of the ribosome during the elongation cycle in protein biosynthesis. Moreover, the GTPase activity of eEF1A is also used to enhance the accuracy of codon recognition.[4,5] The functions of eEF1A in the elongation cycle have been extensively investigated in eubacteria, for example, *Escherichia coli* (EF-Tu) as well as in archaea, for example, *Sulfolobus sulfataricus*[6,7] and eukaryotes, for example, *Saccharomyces cerevisiae*. These studies showed the high conservation of the elongation factor between the three kingdoms as well as among a wide range of different species confirming its importance for life.

In higher vertebrates eEF1A1 is expressed during development but is absent in the adult muscle and heart[8,9] that, on the other hand, express high levels of eEF1A2 as well as other cell types including large motor neurons, islet cells in the pancreas and neuroendocrine cells in the gut.[10] Despite sharing 92% sequence identity, paralogous human eEF1A1 and eEF1A2 have likely different functional profiles. eEF1A1 has been shown to be involved in additional non-canonical functions, including actin-binding and bundling, apoptosis, nuclear transport, proteasomal-mediated degradation of damaged proteins, heat shock and transformation.[11] It is not

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✠He passed away on 26th October 2011.
Keywords: EF-1A; Raf kinases; signal transduction; apoptosis; ubiquitin; mass spectrometry
Abbreviations: eEF1A, eukaryotic translation elongation factor 1A; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GST, glutathione S transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NTA, nitrilotriacetic acid; PKC, protein kinase C
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yet clear how many of these non-canonical functions are shared by the two isoforms but, in humans, eEF1A2 has been shown to have oncogenic properties when overexpressed inappropriately. It has been involved in the ovarian, breast, pancreatic, liver and lung cancer.[12] Regarding this oncogenic potential, the underlying cancerogenic mechanism needs to be further investigated, including the identification of further potential oncogenic mutations that have not been identified yet.[13]

It is known that different signalling pathways, such as the phosphatidylinositol 3-kinases/Protein Kinase B/mammalian target of rapamycin and the Ras-mitogen-activated protein kinase signalling cascades,[14] are involved in the control of the translation apparatus. Not much is clear about the direct regulation of eEF1A even though several groups have been studying phosphorylation and regulation of this elongation factor.[15] A summary of so far eEF1A-identified phosphorylation sites[16–22] is reported in Table 1 of Supplementary Information. Remarkably, eEF1A results to be a substrate for phosphorylation by the classical protein kinase C (PKC) isoforms *in vitro*. More precisely, PKC δ phosphorylates eEF1A at Threonine 431 (based on murine sequence)[22] and increases its activities in translation elongation whereas a nuclear PKC isoform (PKCβ1) could phosphorylate eEF1A2 on S53.[21] Moreover, recent findings showed an *in vivo* interaction between eEF1A and C-Raf kinase during a survival response mediated by epidermal growth factor (EGF)-dependent Ras/extracellular signal-regulated kinase (ERK) pathway during the treatment of human lung cancer cells with alpha interferon (IFNα).[23,24] In detail, this interaction induced eEF1A phosphorylation and increased its expression by protecting the factor from proteasome-dependent degradation. In this study, we have defined the Raf kinase-mediated phosphorylation sites in both eEF1A isoforms *in vitro* and their possible involvement in the regulation of apoptosis in lung cancer cells *in vivo*.

Results

Assays of Raf activity on eEF1A isoforms purified from *E. coli*. On the basis of our previous results,[23] we analyzed the ability of Raf kinases to phosphorylate recombinant eEF1A1-His and eEF1A2-His purified from *E. coli in vitro* according to the procedures described in Supplementary Information. The kinase assays were performed as reported in Materials and Methods using recombinant B-Raf (wt B-Raf or constitutively active mutant B-Raf V600E)[25] as well as C-Raf DD (constitutively active C-Raf DD)[26] purified from baculovirus-infected Sf9 cells as described previously.[27] Inactive C-Raf K75D and B-Raf K75D were used as negative controls. The results, reported in Figure 1, showed the presence of a radioactive band with a size corresponding to that of eEF1A1-His (50.5 kDa), thus indicating that wt B-Raf and the constitutively active B-Raf V600E were able to phosphorylate both eEF1A1-His and eEF1A2-His *in vitro* (Figure 1a, lanes 1–4 and Figure 1d, lanes 1–2). To verify the reproducibility of the results, the kinase assay was performed using a different concentration of B-Raf obtaining similar results (Figure 1g, lanes 1–2 and Figure 1k, lanes 1–2, respectively). The correspondence of the ^{32}P-signal with eEF1A-His was confirmed by probing the membranes with

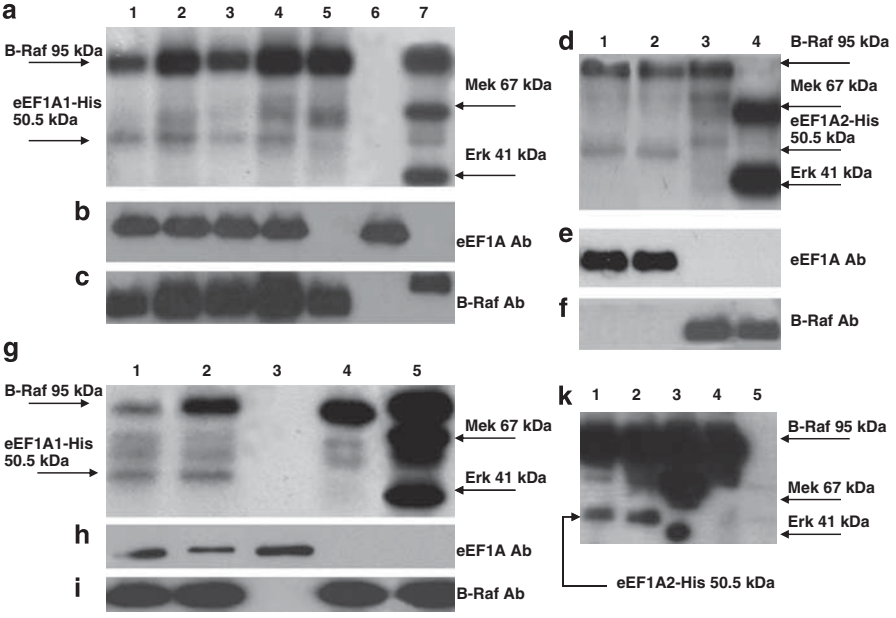

Figure 1 B-Raf kinase assay of eEF1A1-His and eEF1A2-His. (**a**) Autoradiography of the assay on eEF1A1-His in the presence of B-Raf wt (10 ng/μl) and B-Raf V600E (10 ng/μl), respectively. Lanes: 1, B-Raf wt (diluted 1 : 5) + eEF1A1-His; 2, B-Raf wt + eEF1A1-His; 3, B-Raf V600E (diluted 1 : 5) + eEF1A1-His; 4, B-Raf V600E + eEF1A1-His; 5, B-Raf wt; 6, eEF1A1-His and 7, B-Raf wt + Mek + ERK. (**b** and **c**) Immunoblotting against antibody (Ab) anti-eEF1A and anti-B-Raf, respectively. (**d**) Autoradiography of the assay on eEF1A2-His in the presence of B-Raf wt (10 ng/μl). Lanes: 1, B-Raf wt (diluted 1 : 5) + eEF1A2-His; 2, B-Raf wt + eEF1A2-His; 3, B-Raf wt; 4 and B-Raf wt + Mek + ERK. (**e** and **f**) Immunoblotting against Ab anti-eEF1A and anti-B-Raf, respectively. (**g**) Autoradiography of the assay on eEF1A1-His in the presence of B-Raf wt (20 ng/μl). Lanes: 1, B-Raf wt (diluted 1 : 5) + eEF1A1-His; 2, B-Raf wt + eEF1A1-His; 3, eEF1A1-His; 4, B-Raf wt and 5, B-Raf wt + Mek + ERK. (**h** and **i**) Immunoblotting against Ab anti-eEF1A and anti-B-Raf, respectively. (**k**) Autoradiography of the assay on eEF1A2-His in the presence of B-Raf wt (20 ng/μl). Lanes: 1, B-Raf wt (diluted 1 : 5) + eEF1A2-His; 2, B-Raf wt + eEF1A2-His; 3, B-Raf wt + Mek + ERK; 4, B-Raf wt and 5, eEF1A2-His

anti-eEF1A antibody (Figures 1b, e and h). In addition, the radioactive band was also analyzed for trypsin digestion (Supplementary Information). C-Raf DD instead did not promote any phosphorylation on eEF1A1-His and eEF1A2-His (Figure 2a, lanes 2 and 3, respectively). Therefore, based on the hypothesis that the presence of both eEF1A isoforms could enhance C-Raf activity *in vitro*, equal amounts of eEF1A1-His and eEF1A2-His were mixed, preincubated at 37 °C in kinase buffer for 10 min and then, in case of a C-Raf DD-mediated phosphoryation, radioactively marked by ^{32}P-labelled ATP. A phosphorylation signal corresponding to the size of eEF1A-His was observed in the sample containing both proteins (Figure 2a, lane 4). This data could be confirmed by the fact that the relative densitometric evaluation of C-Raff DD autophosphorylation showed a significant reduction in samples containing both isoforms and in the control (Figure 2b, lanes 4 and 5). The correspondence of the ^{32}P-band and eEF1A-His was confirmed by probing the same membrane with anti-eEF1A antibody (Figure 2c).

Identification of phosphorylation sites on eEF1A1 and eEF1A2 mediated by Raf kinases *in vitro*. To identify the amino-acid residues of eEF1A1-His and eEF1A2-His phosphorylated by Raf, recombinant eEF1A-His isoforms expressed in *E. coli* were incubated with either B-Raf or C-Raf DD in the presence of unlabelled ATP and analyzed by mass spectrometry. As reported in Table 1, analysis of the phosphopeptides showed that B-Raf phosphorylated eEF1A1-His and eEF1A2-His, thus confirming the above reported results of radioactive kinase assays. C-Raf DD did not show any phosphorylation activity on eEF1A1-His, as also observed for radioactive kinase assays whereas an eEF1A2-His phosphopeptide containing phosphorylated S21 was detected. The latter result, apparently in contrast to the radioactive kinase assays (Figure 2) can be explained by a low C-Raf DD activity on eEF1A2-His that is not detectable using conventional methods (e.g., *in vitro* kinase assay). Thus, this finding is because of the specificity of mass spectrometry. Table 1 and Supplementary Information report the mass spectrometry identified phosphopeptides.

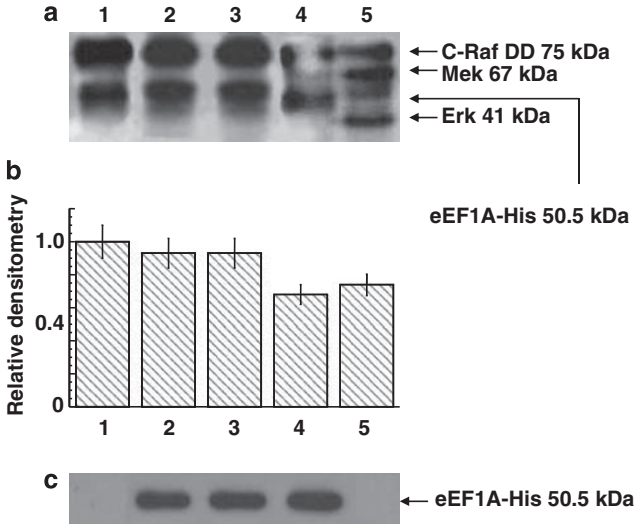

Figure 2 C-Raf DD kinase assay of eEF1A1-His and eEF1A2-His. (**a**) Autoradiography. Lanes: 1, C-Raf DD; 2, C-Raf DD + eEF1A1-His; 3, C-Raf DD + eEF1A2-His; 4, C-Raf DD + eEF1A1-His + eEF1A2-His and 5, positive control C-Raf DD + Mek + ERK. (**b**) Relative densitometric evaluation of C-Raf DD signal intensity. (**c**) Western blot with anti-eEF1A antibody. Each measurement and western blot was carried out in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments

Identification of phosphorylation sites of eEF1A1 expressed in COS 7 cells. To assess if the identified eEF1A phosphorylation sites were present in functional and active proliferating cells, glutathione S-transferase (GST)-eEF1A1 and eEF1A2-HIS were expressed in COS 7 cells and, 24 h after transfection, the recombinant proteins were purified according to the procedure described in Supplementary Information. Mass spectrometry analysis of phosphopeptides obtained after tryptic digestion of the proteins extracted from the SDS-PAGE, identified two phosphorylation sites on GST-eEF1A1 (Table 2). One of these sites (T88) was identical to that identified on recombinant eEF1A1-His purified from *E. Coli* after *in vitro* kinase assay in the presence of B-Raf (Table 1). No phosphopeptides were instead identified on eEF1A2-HIS.

Expression and stability of eEF1A1 and eEF1A2 and their mutants in COS 7. To study the relevance of eEF1A phosphorylation sites found *in vitro* and *in vivo*, S21 and T88 on eEF1A1 and S21 on eEF1A2 were mutated using site-directed mutagenesis and expressed in COS 7 cells.

Table 2 Phosphorylation sites identified on eEF1A1 expressed in COS 7 cells

Substrates	Phosphopeptide sequence	Modified amino-acid residue
GST-eEF1A1	K.YYVpTIIDAPGHRDFIK.N	T88
GST-eEF1A1	K.p(YY)VTIIDAPGHR.D	Y85 or Y86

Table 1 Phosphorylation sites identified on eEF1A1 and eEF1A2 expressed in *Escherichia coli*

Kinases	Substrates	Phosphopeptide sequence	Modified amino-acid residue
B-Raf	eEF1A1-His	K.pSTTTGHLIYK.C	S21
B-Raf	eEF1A1-His	K.YYVpTIIDAPGHRDFIK.N	T88
B-Raf	eEF1A2-His	K.pSTTTGHLIYK.C	S21
C-Raf DD	eEF1A1-His	–	–
C-Raf DD	eEF1A2-His	K.pSTTTGHLIYK.C	S21
C-Raf DD	eEF1A1-His+eEF1A2-His	K.pSTTTGHLIYK.C[a]	S21

[a]Phosphopeptide not ascribable specifically to any of two eEF1A isoforms

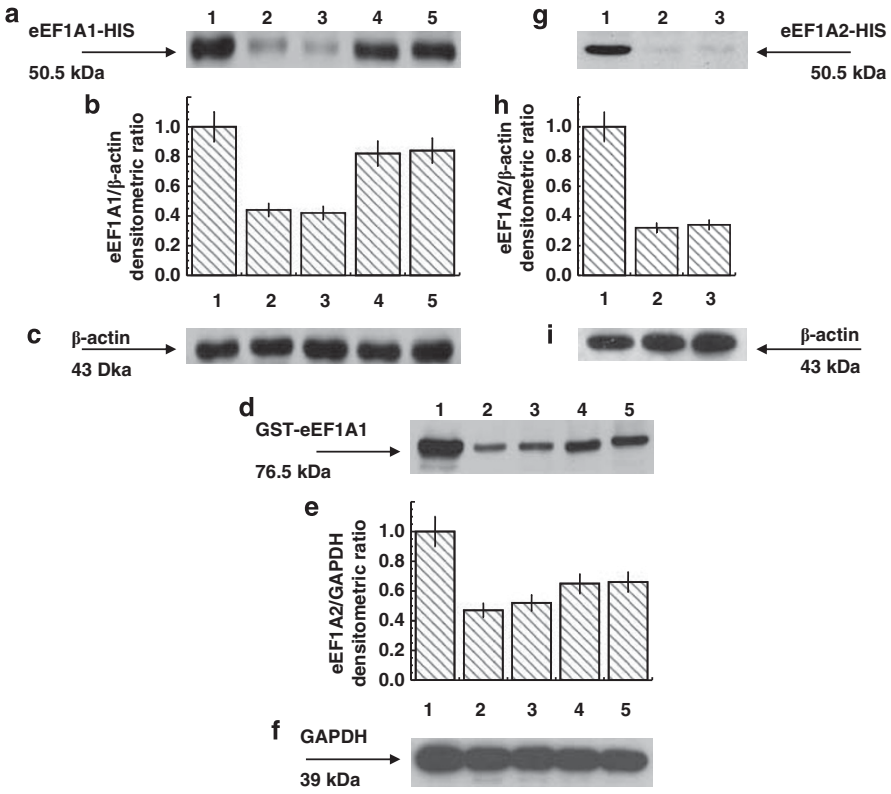

Figure 3 Expression levels of GST-eEF1A1, eEF1A1-HIS and eEF1A2-HIS and corresponding mutants in COS 7 cells. (**a**, **g** and **d**) Western blots of cell lysates with antibody anti-His and anti-GST, respectively. (**a**) Lanes: 1, eEF1A1-HIS wt; 2, eEF1A1-HIS S21A; 3, eEF1A1-HIS S21D; 4, eEF1A1-HIS T88A; 5, eEF1A1-HIS T88D. (**d**) Lanes: 1, GST-eEF1A1 wt; 2, GST-eEF1A1 S21A; 3, GST-eEF1A1 S21D; 4, GST-eEF1A1 T88A; 5, GST-eEF1A1 T88D. (**g**) Lanes: 1. eEF1A2-HIS wt; 2, eEF1A2-HIS S21A and 3, eEF1A2-HIS S21D. (**b**, **e** and **h**) Relative band intensity evaluation. (**c**, **i** and **f**) Western blots of cell lysates with antibody anti-β-actin and anti-GAPDH, respectively. Each measurement and western blot was carried out in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments

To mimic the negative charge of the phosphate group, serine/threonine was replaced with aspartic acid (S21D and T88D), whereas the phosphorylation-deficient site was realized by substituting serine/threonine with alanine (S21A and T88A). To compare the expression levels of these proteins, wt GST-eEF1A1, wt eEF1A1-HIS, wt eEF1A2-HIS and their corresponding mutants were expressed for 48 h and analyzed by immunoblotting. As reported in Figure 3, western blot analysis with anti-His antibody or anti-GST antibody of cell lysates showed that compared with wt, the modified proteins were expressed at lower levels. In particular, compared with wt eEF1A1-HIS, the densitometric evaluation of the band intensities from cell lysates (Figure 3a) with respect to that of β-actin (Figure 3c) showed that the expression level of S21A and S21D mutants was lower than that of the T88A and T88D mutants (Figure 3b). Similar results were also obtained by analyzing the expression level of eEF1A1 and its mutant fused to GST. In fact, western blots of the cell lysates (Figure 3d) with anti-GST antibody confirmed the lower expression of the GST-eEF1A1 mutants with respect to GST-eEF1A1 wt (Figure 3e). Compared with wt eEF1A2-HIS, also the expression of its corresponding S21 mutants with anti-His antibody (Figure 3g) appeared to be reduced as shown by the densitometric evaluation of the band intensity (Figure 3h) from cell lysates with respect to that of β-actin (Figure 3i). These findings suggested that compared with wt GST-eEF1A1, eEF1A1-HIS and eEF1A2-HIS the respective mutant proteins appeared less stable *in vivo*.

S21 has a crucial role in the ubiquitination status and subsequent proteasomal degradation of eEF1A isoforms *in vivo*. As reported above, mutation of RAF-mediated phosphorylation sites on eEF1A interfere with eEF1A protein expression levels. This indicates that Raf might regulate the stability of both eEF1A isoforms in an ubiquitin dependent and thus proteasome-dependent manner. To investigate this possibility, COS 7 cells were co-transfected transiently with eEF1A1-HIS or eEF1A2-HIS, respectively, and HA-ubiquitin for 36 h. In the following 12 h, cells were treated with the proteasomal inhibitor MG-132. Overexpressed eEF1A-HIS isoforms were precipitated using Ni-nitrilotriacetic acid (NTA) and immunoblotted against HA-ubiquitin using an anti-HA antibody. As shown in Figure 4 ubiquitination levels of both isolated phosphorylation resistant mutants of eEF1A-HIS (S21A and T88A) were increased in comparison with the ubiquitination levels of isolated phosphorylation-mimicking mutants (S21D and T88D) in MG132 non-treated cells (Figure 4a). Remarkably, in MG132-treated cells ubiquitination levels of isolated eEF1A1-HIS wild-type and corresponding mutants were comparable (Figure 4b). Similar results were obtained when eEF1A2-HIS wt and corresponding mutants (S21A and S21D) were co-expressed together with HA-ubiquitin in COS

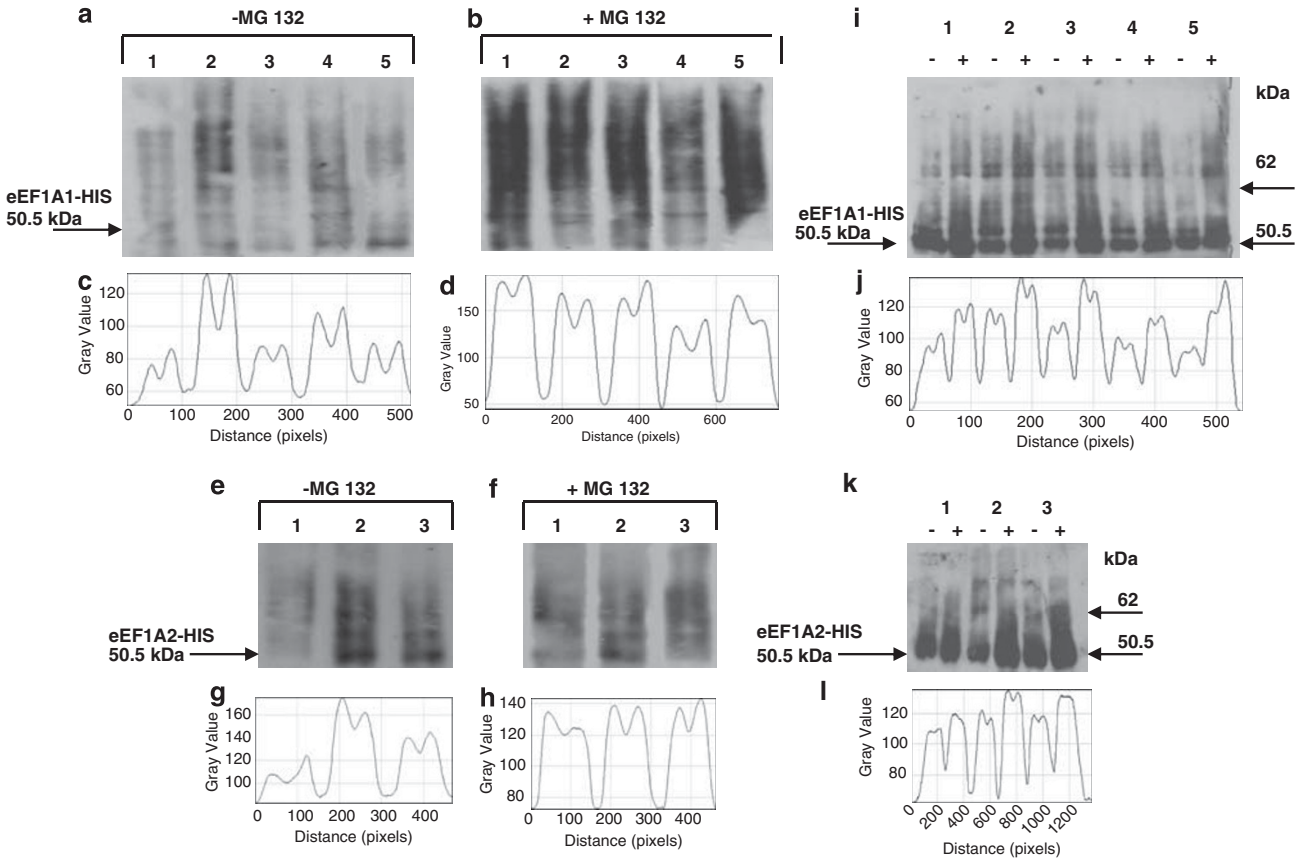

Figure 4 Ubiquitination assay of eEF1A1-HIS and eEF1A2-HIS and their mutants. After cotransfection of COS 7 cells with wt eEF1A-HISs and corresponding mutants with HA-ubiquitin wt and treatment of cells in the absence (−) or in presence (+) of MG132, cell extracts were analyzed after pull-down for eEF1A1-HIS (**a**, **b**, **i**) and eEF1A2-HIS (**e**, **f**, **k**), respectively, by immunoblotting with anti-HA (**a**, **b**, **e**, **f**) and anti-His (**i**, **k**) antibodies. (**a**, **b**, **i**) Lanes: 1, eEF1A1-HIS wt; 2, eEF1A1-HIS S21A; 3, eEF1A1-HIS S21D; 4, eEF1A1-HIS T88A and 5, eEF1A1-HIS T88D. (**e**, **f**, **k**) Lanes: 1, eEF1A2-HIS wt; 2, eEF1A2-HIS S21A and 3, eEF1A2-HIS S21D. Band intensities were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA) (**c**, **d**, **g**, **h**, **j** and **l**)

7 cells (Figure 4e). In detail, the ubiquitination level of eEF1A2-HIS S21A was increased in comparison with the ubiquitination level of eEF1A2-HIS S21D in the absence of MG132, whereas in the presence of MG132 ubiquitination levels of eEF1A2-HIS wt, eEF1A2-HIS S21A and eEF1A2-HIS S21D were quite similar (Figure 4f). Similar results were also obtained when in a similar independent experiment. Immunoblotting analysis with anti-His antibody of the cell extracts after His-pull-down showed that compared with intact eEF1A1 and eEF1A2, the polyubiquitination level was slightly higher for the mutants (Figures 4i and k, respectively).

S21 positively regulates apoptosis in H1355 cells. In order to assay the biological effects of the eEF1A isoforms and their mutants, H1355 cells were transfected with wt eEF1A-HIS and the corresponding S21A and S21D constructs. As reported in Figure 5, compared with wt, transfections of H1355 cells with eEF1A-HIS mutants showed in general an increase in the apoptosis evaluated after 72 h by FACS analysis following double labelling of the cells with annexin V and propidium iodide. In particular, eEF1A1-HIS S21A and S21D mutants induced mainly an increase of early apoptosis whereas eEF1A2-HIS S21A and S21D mutants induced an increase of late apoptosis. Details regarding the FACS analysis of the samples are reported in Supplementary Information.

Discussion

In this work, the role of eEF1A post-translational modifications induced by its interaction with Raf kinases has been examined. The work was based on our previous finding that following treatment of a human epidermoid cancer cell line (H1355) with INFα, revealed a phosphorylation of eEF1A mediated by C-Raf kinase, following activation of a survival pathway induced by the EGF receptor.[23] Therefore, the aim was the identification of the specific amino-acid residues of eEF1A isoforms phosphorylated by Raf using a proteomic approach. Here, we describe for the first time that both eEF1A1 and eEF1A2 were phosphorylated *in vitro* by B-Raf and C-Raf on S21. This residue belongs to the first consensus sequence (G,A)XXXXGK(T,S) of the GTP/GDP-binding site of GTP-binding proteins[28] that are highly conserved in both human variants (G_{14}HVDSGKST in both eEF1A1 and eEF1A2).

According to the 3D model of EF1A•GDP•EF1B from yeast, the S21 (S21 in human eEF1A1 and eEF1A2) side

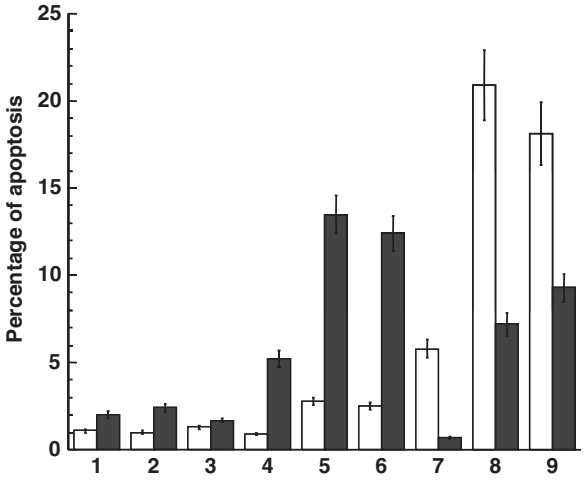

Figure 5 Effects of transfection with eEF1A-HIS isoforms and their S21 mutants on apoptosis in H1355 cells. FACS analysis after double labelling with annexin V and propidium iodide of H1355 cells transfected with eEF1A-HIS constructs for 72 h as described in Materials and Methods and Supplementary Information. White bars: percentage of annexin V-FITC and PI positive (late apoptosis). Black bars: percentage of annexin V-FITC positive (early apoptosis). Samples: 1, control cells; 2, cells treated with lipophectamine; 3, cells transfected with void vector; 4, eEF1A1-HIS wt, 5, eEF1A1-HIS S21A; 6, eEF1A1-HIS S21D; 7, eEF1A2-HIS wt, 8, eEF1A2-HIS S21A and 9, eEF1A2-HIS S21D. Error bars indicate the maximum deviation from the mean value of two independent experiments

chain is linked directly to the oxygen of both phosphates of the GDP nucleotide.[29] In addition, also T22, D156, S192, G193 and W194 (all in domain I) are important for the binding of GTP/GDP ligands.[30] In fact, mutations of the amino-acid residues involved in the binding of guanine nucleotide such as D156N of yeast EF1A resulted in a marked reduction in translational fidelity.[31] Therefore, the binding of the guanine nucleotides on eEF1A could be potentially prevented by phosphorylation of the S21 residue. This phosphorylation could cause a conformational change that directs eEF1A to interact with other partners leading to different functions far from protein biosynthesis, such as actin bundling, essential for regulation of actin cytoskeleton and cell morphology during several cellular processes.[32] These observations are indirectly confirmed by the finding that GST-eEF1A1 expressed in normal proliferating cells (COS 7), did not show any modifications of S21. In fact, in a contest of normal cell growth, most of eEF1A molecules present in the cells are involved in its canonical function during protein biosynthesis.[33]

We also found that eEF1A1 is specifically phosphorylated on T88 by B-Raf *in vitro*. This phosphorylation on T88 was also identified using mass spectrometry on eEF1A1 expressed in proliferating COS 7 cells. However, we cannot affirm that T88 *in vivo* phosphorylation is due to a specific B-Raf activity on eEF1A1. The fact that phosphorylated T88 was found only on eEF1A1 suggests that this post-translational modification is due to structural differences between eEF1A1 and eEF1A2. Thus, a participation of phosphoT88 in that residue corresponding specific cellular events with respect to those involving the phosphorylation of S21 is conceivable.

We also identified in proliferating COS 7 cells a peptide containing not clearly defined phosphorylation at the level of Y85 or Y86. Large-scale proteomics studies[20] already revealed that, besides Y86, other tyrosine residues present in both human eEF1A variants are phosphorylated. Albeit both isoforms share a sequence homology of 98%, only eEF1A1, as we also confirmed by our results, is phosphorylated on Y86. Moreover, it has been recently reported that eEF1A1 and eEF1A2 interact with SH2 and SH3 domains of different signalling molecules and the binding interaction sites have been localized at the level of tyrosine residues (Y86 and Y167) of the G-domain of eEF1A.[34]

To study the relevance of these identified phosphorylated residues, namely S21 and T88, we analyzed the biochemical function of mutated eEF1As by transient expression in COS 7 cells, even though no phosphorylation for S21 was observed *in vivo*. One possible explanation for the lack of S21 phosphorylation in COS 7 cells might be ascribed to the fact that this post-translational modification could impair the involvement of eEF1A in protein synthesis (a fundamental process in active proliferating cells). Instead, this modification might occur in tumor cells following the activation of a signal transduction pathway inducing tumorigenesis. Considering the fact that Raf kinases have an important role in tumorigenesis and inter alia cellular proliferation, a Raf-mediated phosphorylation of eEF1A isoforms *in vivo* is expectable and further confirmed by the *in vitro* data. Furthermore, S21 is part of the GDP/GTP-binding pocket and thus has an important structural role for the function of eEF1A. At the same time it is also a possible phosphorylation residue (as evidenced by the *in vitro* data shown here) affecting the eEF1A expression levels in COS 7 cells. Compared with the corresponding wt eEF1A, overexpression of eEF1A1 and eEF1A2 S21A/D mutants in COS 7 cells showed a marked reduction of protein levels as determined by western blot of cell lysates. This reduction was most likely due to an increase of their proteasomal degradation as indicated by western blot analysis of pull-down samples from cells co-transfected with eEF1A constructs and HA-ubiquitin in the absence or presence of the proteasomal inhibitor MG132. Protein levels of the ubiquitinated phospho-deficient mutant (S21A) were higher in comparison with protein levels of the ubiquitinated phospho-mimicking mutant S21D (Figure 4). Consequently, we assume that S21 is a crucial site for the half-life of eEF1A and its phosphorylation could be a regulatory switch for other functional roles of eEF1A. Moreover, the finding that C-Raf required the presence of both eEF1A isoforms for its phosphorylation activity *in vivo,* suggested the formation of a potential eEF1A heterodimer. Remarkably, EF1A dimerization has been already described for Tetrahymena EF1A. In that case, EF1A, that is also an activator of a PI 4-kinase, bound actin and increased actin bundling.[35] EF1A bundles filamentous actin through dimer formation whereas EF1A monomers do not.[36]

In order to support the potential interaction between eEF1A isoforms *in vivo,* recombinant GST-eEF1A1 wt and mutants were cotransfected with eEF1A2-HIS wt in COS 7 cells and then analyzed by western blot after GST pull-down. As shown in Figure 6, the expressed proteins were detected with both anti-GST and anti-His antibodies. In particular, the expression level of GST-eEF1A1 and S21D and T88D mutant was quite similar to that observed in the experiment reported in Figure 3.

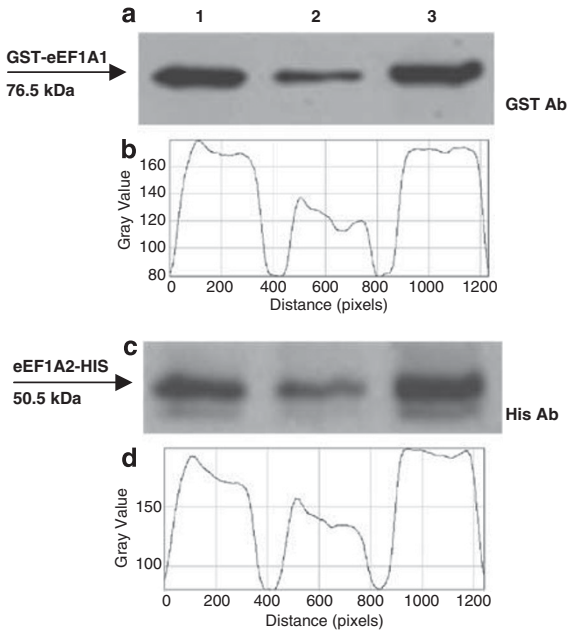

Figure 6 Cotransfection of GST-eEF1A1 and eEF1A2-HIS in Cos 7 cells. GST-eEF1A1 wt and mutants were cotransfected with eEF1A2-HIS wt in COS 7 cells. After 24 h, the cells were harvested, lysed and analyzed after GST pull-down with antibody anti-GST (GST Ab, **a**) and anti-His (His Ab, **c**). Lanes: 1, cells transfected with GST-eEF1A1 wt and eEF1A2-HIS wt; 2, cells transfected with GST-eEF1A1 S21D and eEF1A2-HIS wt; 3. Cells transfected with GST-eEF1A1 T88D and eEF1A2-HIS wt. Band intensities were evaluated with Image J software (**b** and **d**)

Also similar was the level of the signal as detected with anti-His antibody. These results confirmed *in vivo* the eEF1A1•eEF1A2 interaction observed *in vitro*. The heterodimerization hypothesis was also investigated generating a docking model of the two isoforms interaction (r-1.pdb) at GRAMM-X Protein–Protein Docking Web Server v.1.2.0[37,38] using 3D models of eEF1A1 (eEF1A1.pdb) and eEF1A2. (eEF1A2.pdb). The pdb files for human eEF1A1 and eEF1A2 were generated at Swiss-Model Server[39] using as template the structure of yeast eEF1A (PDB ID: 1F60, chain A).[29] As illustrated in Figure 7, the docking model obtained supports the formation of a possible heterodimer between eEF1A1 and eEF1A2. In particular, this model shows that the M-domain of one isoform is in contact to the G-domain of the other and *vice versa*. The heterodimer formation somehow could induce a conformational change in one or in both eEF1A isoforms that allows the phosphorylation of S21. However, because of the high sequence similarity between eEF1A isoforms, the formation of eEF1A1 or eEF1A2 homodimer cannot be excluded.

The effects of the eEF1A isoforms in mediating cell proliferation or apoptosis might depend on their respective intracellular abundance.[40] Therefore, we analyzed the biological effects of eEF1A wt and mutants during their transient overexpression in H1355 cells. The data obtained showed that eEF1A-mutated recombinant proteins, lacking the amino-acid residues important for the phosphorylation (T/S exchanged against A/D by site-directed mutagenesis) and thus unable to be regulated by C-Raf, induced apoptosis rather than cell survival. This result is in agreement with our previous

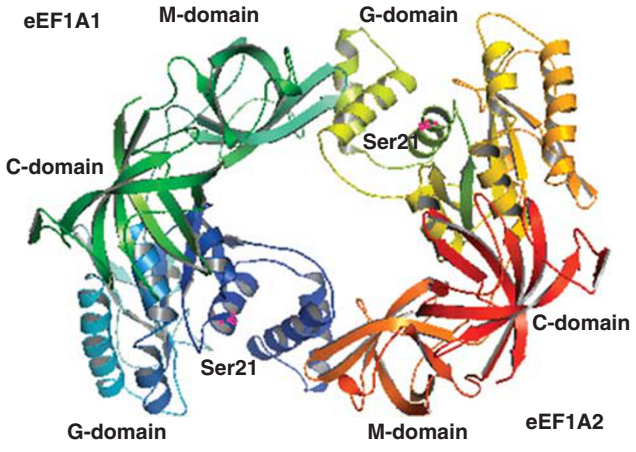

Figure 7 Imitation of a 3D model of an eEF1A1•eEF1A2 heterodimer. The heterodimer representation was obtained from the molecular docking pdb file (r-1.pdb) using PyMol software (DeLano Scientific LLC, San Carlos, CA, USA). In both eEF1A isoforms, the position of S21 is highlighted

work showing that the upregulation of the intracellular content of eEF1A is associated to an increase in the phosphorylation of serine and threonine residues of the protein mediated by C-Raf and that this effect appeared to have a pro-survival function.[23] Interestingly, eEF1A1 S21 mutants induced an increase of early apoptosis whereas eEF1A2 S21 mutants induced an increase of late apoptosis. The latter aspect may be also in agreement with the antiapoptotic role proposed for eEF1A2[41] as the lower stability of the eEF1A2 mutants probably abolishes its protective function and causes apoptotic events.

Conclusions

In conclusion, we have identified eEF1A amino-acid residues (S21 and T88) that are phosphorylated by Raf kinase *in vitro* and eEF1A amino-acid residues (T88 and Y85/86) that are phosphorylated by a not identified kinase *in vivo.* Mutations of S21 and T88 affect the stability of the elongation factor *in vivo*. These residues have most likely an important functional role in the control of cellular process, such as cell proliferation and apoptosis.

Materials and Methods

Expression and purification of recombinant eEF1A1-His and eEF1A2-His from *E. coli*. eEF1A1 and eEF1A2 cDNAs were cloned in pET22b(+) expression vectors (Novagen, Darmstadt, Germany) in frame to the His-Tag sequence and overexpressed in *E. coli* strains (Rosetta, BL21C43) according to the procedure reported in Supplementary Information. Activity of the purified proteins was tested *in vitro* according to the procedure already described[42] and summarized in Supplementary Information.

Expression and purification of recombinant eEF1A1 and eEF1A2 from COS 7 cells. eEF1A1 and eEF1A2 cDNAs were cloned in mammalian expression vectors (pEBG and pCDNA 3.1, respectively) and expressed in COS 7 cells. Because of the lack of an isoform-specific antibody raised against the two isoforms, eEF1A1 was fused to GST (GST–eEF1A1) whereas eEF1A2 was provided with a His-Tag (eEF1A2-HIS). Vectors were transfected with jet polyethylenimine solution (Polyplus tranfection) according to the manufacturer's protocol. The procedure for the purification of both proteins is reported in Supplementary Information.

***In vitro* kinase assay.** The Raf kinase assay was performed *in vitro* on recombinant eEF1A-His purified from *E. coli*. Depending on the kinase and the substrate, recombinant proteins were mixed in a 1 : 3/1 : 5 or 2 : 3/2 : 5 ratio ensuring substrate excess. The reaction mixture contained 300 or 600 ng of purified kinase, purified substrate 600–1000 ng, 1 × kinase buffer (10 × kinase buffer: 25 mM glycerol-phosphate, 50 mM TrisHCl, pH 7.5, 2 mM DTT, 10 mM MgCl2 and 25 mM ATP) to a final volume of 30 μl. For the radioactive labelling assay 5–10 μCi [γ-^{32}P]ATP (Hartmann Analytics, Braunschweig, Germany), corresponding to 0.5–1.0 μl, were added. The mixture was incubated at 37 °C for 1 h on a thermomixer at 600 r.p.m. The reaction was stopped by addition of 4 × Laemmli loading buffer (2.4 ml 1 M Tris pH 6.8, 0.8 g SDS, 4 ml 100% glycerol, 0.01% bromophenol blue, 1 ml β-mercaptoethanol and 2.8 ml water),[43] and analyzed by immunoblotting with phosphospecific antibodies or, in case of radioactive labelling, the nitrocellulose membrane was exposed to an X-ray film at −80 °C overnight (ON). The protein concentration was determined according to Lowry.[44] Analysis of trypsin digestion of the radio-labelled band extracted from gels was performed according to van der Geer and Hunter.[45]

Vector construction. Full-length eEF1A1 and eEF1A2 cDNAs were cloned in PET-22b(+) using pAN7[46] and eEF1A2 pcDNA 3.1. (gift of Charlotte R Knudsen, Aarhus)[47] vectors as templates. The following primers were used: EF1 (forward) 5′-CCCTAAAAGCCCATATGGGAAAGG-3′ and EF2 (reverse) 5′-CTTCCACCAC GGATCCAGAGTGGG-3′. A2 (forward) 5′-TGGAATTCACTCATATGGGCAAG-3′; A2 (reverse) 5′-CTCGAGTCAGTGATGGTGATG-3′.

Full-length eEF1A1 cDNA was cloned in pcDNA 3.1 using the pAN7 vector as a template. The following primers were used: EFB (forward) 5′-GCCAAAATGGG ATCCGAAAAGACT-3′ corresponding to the region containing M_1GKEKT and EFA (reverse) 3′-ATTCATTTACCGGTCTGAGCTTTCTG-5′ overlapping with the Q_{456}KAQKAK (stop codon) region of eEF1A.

For the pEBG vector containing full-lenght EF1A1 fused to GST the following primers were used: Pebg (forward) 5′ AAAAAAGGATCCATGGGAAAGGAAAAG ACTCATATCAACATTGTC-3′ and PEBG (reverse) 5′-AAAAAATCTAGATCATTTA GCCTTCTGAGCTTTCTGGGCAGACTT-3′.

The eEF1A mutants were generated by site-direct mutagenesis using the corresponding vectors as templates. The following self-complementary oligonucleotides were used: eEF1A1 S21A (forward): 5′-GTA GAT TCG GGC AAG GCG ACC ACT ACT GGC CAT-3′, eEF1A1 S21A (reverse): 5′-ATG GCC AGT AGT GGT CGC CTT GCC CGA ATC TAC-3′, eEF1A1 S21D (forward): 5′-GTA GAT TCG GGC AAG GAT ACC ACT ACT GGC CAT-3′, eEF1A1 S21D (reverse): 5′-ATG GCC AGT AGT GGT ATC CTT GCC CGA ATC TAC-3′, eEF1A1 T88A (forward): 5′-AGC AAG TAC TAT GTG GCG ATC ATT GAT GCC CCA-3′, eEF1A1 T88A (reverse): 5′-TGG GGC ATC AAT GAT CGC CAC ATA GTA CTT GCT-3′, eEF1A1 T88D (forward): 5′-AGC AAG TAC TAT GTG GAT ATC ATT GAT GCC CCA-3′, eEF1A1 T88D (reverse): 5′-TGG GGC ATC AAT GAT ATC CAC ATA GTA CTT GCT-3′, eEF1A2 S21A (forward): 5′-GTG GAC TCC GGA AAG GCG ACC ACC ACG GGC CAC-3′, eEF1A2 S21A (reverse): 5′-GTG GCC CGT GGT GGT CGC CTT TCC GGA GTC CAC-3′, eEF1A2 S21D (forward): 5′-GTG GAC TCC GGA AAG GAT ACC ACC ACG GGC CAC-3′, eEF1A2 S21D (reverse): 5′-GTG GCC CGT GGT GGT ATC CTT TCC GGA GTC CAC-3′.

Following amplification, performed with Pfu polymerase (Stratagene, Santa Clara, CA, USA), the PCR products were treated with Dpn I endonuclease (Fermentas, Milan, Italy, 1 h at 37 °C plus inactivation for 30 min at 80 °C) specifically digesting the methylated parental DNA strand. The duration of the elongation cycle was calculated based on the activity of the Pfu polymerase (2 min/1000 bp).

Pull-down and western blot analysis. Pull-down of GST-eEF1A1 or eEF1A2-HIS was performed using GST-sepharose (Amersham, Milan, Italy) or Ni-NTA agarose (Qiagen, Milan, Italy), respectively. Cell extracts (500 μg) were incubated with equal amounts of pre-equilibrated resin (150 μl slurry/1 mg protein extract) for 2 h at room temperature (RT) or ON at 4 °C, respectively. After the incubation, the resin was washed two times (centrifugation for 2 min at 2000 r.p.m. and 4 °C) in 1 ml of 1 × phosphate buffered saline (PBS), in the case of GST-sepharose, or in 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, in the case of Ni-NTA to reduce nonspecific bound proteins, 0.05% Tween 20, pH 8.0, resuspended in 30 μl of 4 × Laemmli loading buffer, heated to 95 °C for15 min and subjected to western blot analysis. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Milan, Italy). All films were scanned using Adobe photoshop software (San Jose, CA, USA).

Antibodies used were as follows: mouse monoclonal antibody anti-EF1A (Upstate, Billerica, MA, USA), rabbit polyclonal antibody anti-GST (Santa Cruz, Santa Cruz, CA, USA), mouse monoclonal antibody anti-actin (Santa Cruz), mouse monoclonal antibody anti-His (Qiagen) and rabbit polyclonal anti-HA antibody (Abcam, Cambridge, MA, USA).

Mass spectrometry measurements. Mass spectrometry analysis of eEF1A-His samples overexpressed in *E. coli* was performed after separation of the recombinant proteins by SDS-PAGE (NuPAGE (Darmstadt, Germany) Novex 4–12% Bis-Tris gels; MOPS buffer system). Gels were subjected to silver staining, and the respective bands were excised and washed according to Shevchenko *et al*.[48]

Mass spectrometry analysis of GST-eEF1A and eEF1A2-HIS overexpressed in COS 7 cells was performed after separation of the proteins on 4–12% BisTris SDS Gels (Invitrogen, Karlsruhe, Germany). Afterwards protein band was visualized according to Blum *et al*.[49] with slight modifications, excised, washed and in-gel digested as described previously.[50,51]

Detailed procedures for mass spectrometry measurements are reported in Supplementary Information.

Cell culture and transfection. COS 7 cells (African green monkey kidney) were grown in Dulbecco's modified Eagle medium (Gibco, Monza, Italy) with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco) and antibiotics (penicillin–streptomycin, Gibco). The human lung epidermoid carcinoma cell line (H1355), obtained from American Type Tissue Collection (Rockville, MD, USA), was grown in an RPMI 1640 medium supplemented with heat-inactivated FBS, 100 U/ml penicillin, 100 μ/ml streptomycin and 1% L-glutamine at 37 °C. The cells were grown in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

Cells were detached from confluent 100-mm dishes. About 2×10^5 cells were incubated in electroporation buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM glucose) and 15 μg/μl of DNA construct in 20 mM HEPES. Cells were electroporated at 250 V and at 975 mF for 6 s and incubated at 37 °C for 24 h. Following incubation the cells were processed for western blotting analysis and apoptosis detection as described below.

Immunoprecipitation. H1355 cells were scraped, washed twice in cold PBS and resuspended in 20–40 μl of lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0,25% sodium deoxycholate, 150 mM NaCl, 1 μg/ml aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4 and 1 mM NaF) for 30 min on ice and centrifuged at 14 000 × *g* for 20 min at 4 °C. Total protein extracts (500 μg) were subjected to immunoprecipitation with 2 μg of anti-eEF-1A for 16 h at 4 °C. Immune complexes were bound to 20 μl of protein A-agarose for 2 h at 4 °C. The protein A-agarose/immune complex was washed four times with cold PBS and resuspended in 20 μl of SDS loading buffer, heated for 5 min at 95 °C and subjected to western blotting analysis. Cytosolic proteins (2 μg) were separated by SDS-PAGE, electrotransferred to nitrocellulose and probed with anti-eEF-1A and anti-His antibodies. Blots were developed using enhanced chemiluminescence detection reagents (SuperSignal West Pico, Pierce) and exposed to X-ray films. Bands were quantified by using Adobe photoshop software.

Ubiqutination assay. COS 7 cells were grown and co-transfected, as above mentioned, either with eEF1A1-HIS or with eEF1A2-HIS constructs and HA-ubiquitin for 36 h and subsequently incubated in the presence or absence of MG132 (Calbiochem, Milan, Italy) for 12 h. Immunoblotting analysis of Ni-NTA isolated proteins has been performed as above reported, probing the corresponding membrane with anti-HA antibody.

Flow cytometric analysis of apoptosis. Apoptotic cell death was analysed by Annexin-V–fluorescein isothiocyanate (FITC) staining. Annexin-V–FITC binds to phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis. Labelling of apoptotic cells was performed using an Annexin-V kit (MedSystems Diagnostics, Vienna, Austria). Briefly, after transfection, H1355 cells were incubated in complete RPMI 1640 for 72 h. Subsequently, all cells (flowing and attached) were incubated with Annexin-V–FITC in a binding buffer (provided by the manufacturer) for 10 min at RT, washed and resuspended in the same buffer as described by the manufacturer. Apoptosis was evaluated measuring the % of mean fluorescence intensity (% MFI) using FL1-H (Log scale) by the FACSCalibur software (Applied Biosystems, Monza, Italy). For each sample, 20 000 events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)