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## Cellular Interaction of Human Eukaryotic Elongation Factor 1A Isoforms

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### Abstract

Besides its canonical role in protein synthesis, the eukaryotic translation elongation factor 1A (eEF1A) is also involved in many other cellular processes such as cell survival and apoptosis. We showed that eEF1A phosphorylation by C-Raf *in vitro* occurred only in the presence of eEF1A1 and eEF1A2, thus suggesting that both isoforms interacted in cancer cells (heterodimer formation). This hypothesis was recently investigated in COS-7 cells where fluorescent recombinant eEF1A isoforms colocalized at the level of cytoplasm with a FRET signal more intense at plasma membrane level. Here, we addressed our attention in highlighting and confirming this interaction in a different cell line, HEK 293, normally expressing eEF1A1 but lacking the eEF1A2 isoform. To this end, His-tagged eEF1A2 was expressed in HEK 293 cells and found to colocalize with endogenous eEF1A1 in the cytoplasm, also at the level of cellular membranes. Moreover, FRET analysis showed, in this case, the appearance of a stronger signal mainly at the level of the plasma membrane. These results confirmed what was previously observed in COS-7 cells and strongly reinforced the interaction among eEF1A isoforms. Moreover, the formation of eEF1A heterodimer in cancer cells could also be important for cytoskeleton rearrangements rather than for phosphorylation, most likely occurring during cell survival and apoptosis.

**Keywords:** eukaryotic translation elongation factor 1A (eEF1A), confocal microscopy, FRET, pull-down assay, immunoblotting

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## 1. Introduction

Eukaryotic elongation factor 1A (eEF1A) belongs to the family of GTP-binding proteins and it is the second most abundant protein in the cellular environment. It catalyzes the first step of the elongation cycle by promoting the GTP-dependent binding of aminoacyl-tRNA to the A-site of the ribosome [1–3]. eEF1A exists as two isoforms eEF1A1 and eEF1A2 [4], and in humans, they share almost identical amino acid sequences (92% sequence identity). eEF1A1 is ubiquitously present except in skeletal and cardiac muscle, while eEF1A2 expression is restricted in the brain, skeleton muscle, heart, and other cell types including large motor neurons, islet cells in the pancreas, and neuroendocrine cells in the gut [5], and it is currently found in all vertebrates [6]. Besides their role in polypeptide synthesis, paralogous human eEF1A1 and eEF1A2 act as “moonlighting” proteins [7] owing to several noncanonical functions such as cytoskeleton remodeling by binding and bundling filamentous actin [8, 9], apoptosis, nuclear transport, proteasome-mediated degradation of damaged proteins, heat shock, and transformation [10–12]. Overexpression of eEF1A1 or eEF1A2 in Hela cells led to increased cell growth [7], whereas the disruption of eEF1A1 resulted in actin cytoskeleton defects under basal conditions and in response to palmitate, thus suggesting that eEF1A1 mediates lipotoxic cell death, secondary to oxidative and ER stress, by regulating cytoskeletal changes critical for this process [13]. These findings highlighted that eEF1A1 was involved in both cell proliferation and apoptosis, though the relationship between eEF1A1 and apoptosis is still unclear. By contrast, eEF1A2 seems to play antiapoptotic properties in ovarian, breast, pancreatic, liver, and lung cancer [14]; however, this oncogenic potential deserves further investigation [15].

The possible interaction between eEF1A molecules was first characterized in *Tetrahymena* as eEF1A dimer was able to bundle actin filament [16]. Subsequently, the identification of dimeric eEF1A was also reported in both chicken and human B cell lines [17]. Recent investigations indicated that, compared to eEF1A2, eEF1A1 showed a higher property of self-association [18]. Moreover, under oxidant condition, eEF1A1 was able to form intermolecular disulfide bonds [19]. Recent findings showed that C-Raf kinase interacts *in vivo* with eEF1A during a survival response mediated by epidermal growth factor (EGF) following the treatment of human lung cancer cells with  $\alpha$ -interferon (IFN $\alpha$ ) [20]. Moreover, phosphorylation of eEF1A *in vitro* by C-Raf on S21 required the presence of both eEF1A isoforms, thus suggesting that the existence of an eEF1A1/eEF1A2 complex and the S21 phosphorylation represented a regulatory mechanism responsible for the switch from eEF1A canonical to noncanonic functions [21]. On the basis of these findings, we recently showed the possible direct interaction between the eEF1A isoforms by using fluorescence resonance energy transfer (FRET) [22]. Compared to our previous work, here we settled for a different experimental approach mainly based on pull-down, confocal microscopy, and FRET analysis based on IgG-FITC (donor)- and IgG-TRITC (acceptor)-conjugated antibodies in HEK 293 cells transfected with recombinant His-tagged eEF1A2 isoform.

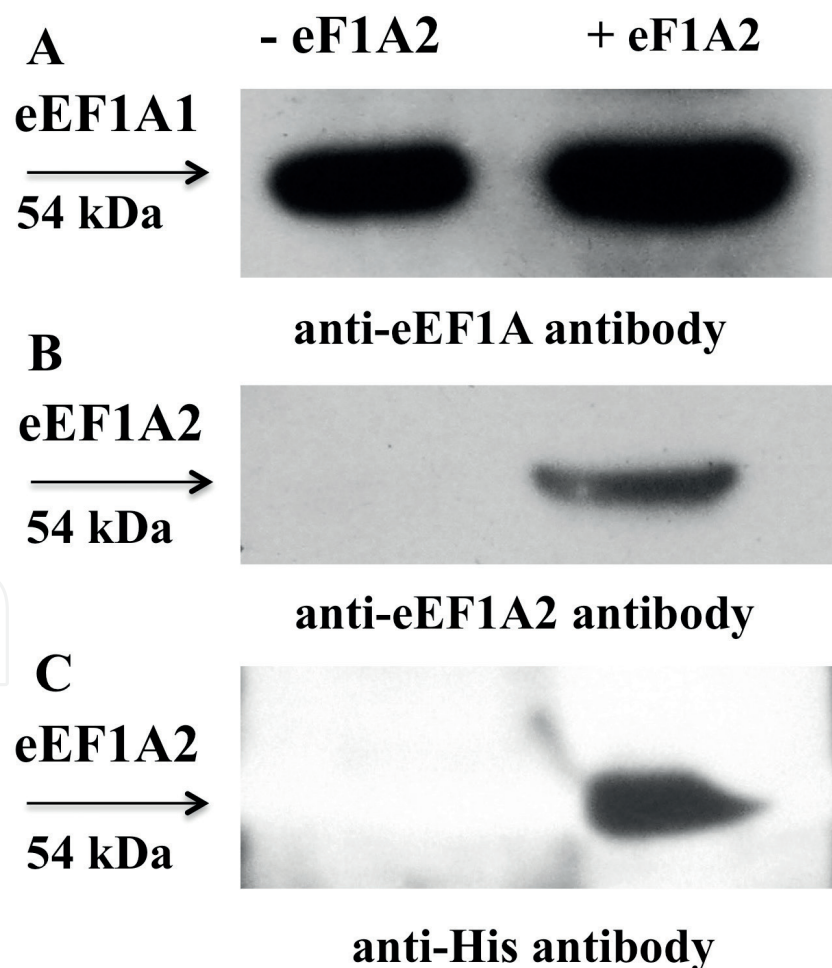
## 2. Expression and interaction of eEF1A1 and eEF1A2 in HEK 293 cells

To assess the possible physiological interaction between eEF1A isoforms in a natural cellular environment, such as the cytoplasm of intact cells, human embryonic kidney 293 (HEK 293)

cell line was used as an experimental system. This choice was derived from the finding that HEK 293 cells normally express substantial levels of eEF1A1 isoform, whereas the eEF1A2 isoform is absent.

## 2.1. Expression in HEK 293 of eEF1A1 and eEF1A2

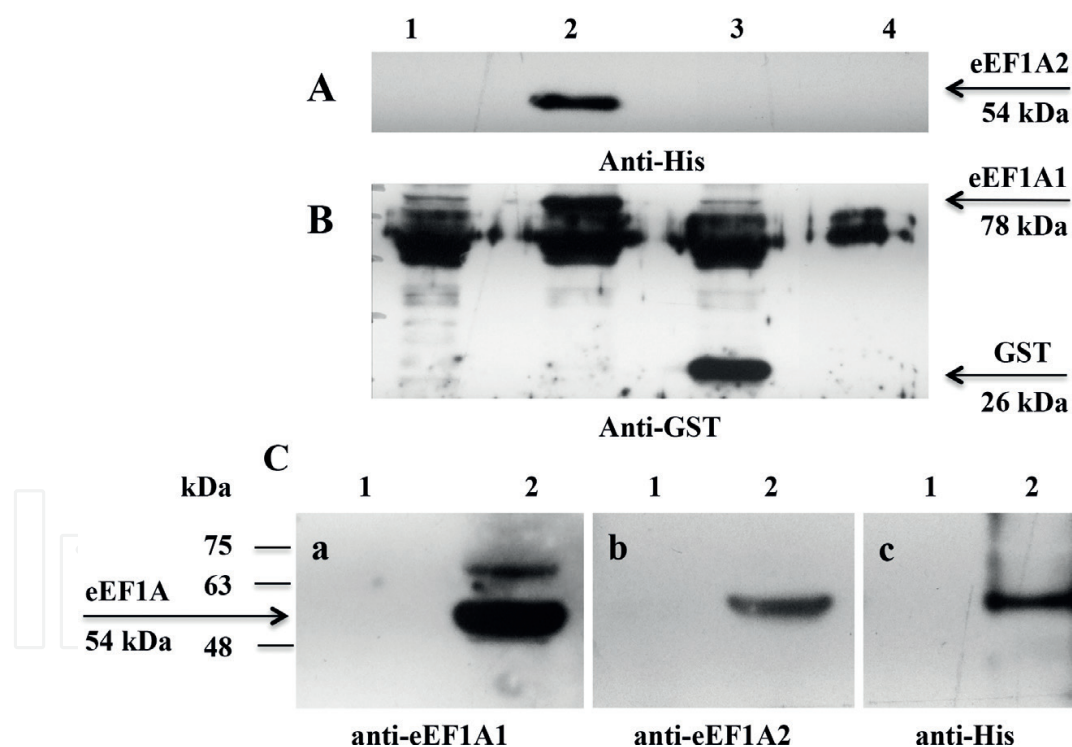
First, the efficiency of pcDNA3.1-eEF1A2(His)<sub>6</sub> (gift from C. R. Knudsen, Aarhus, Denmark [23]) to transfect HEK 293 cells was evaluated. As reported in **Figure 1A**, compared to non-transfected HEK 293, cells transfected with recombinant eEF1A2 isoform showed an increase in the expression of the 54 kDa bands corresponding to the molecular weight of eEF1A. Subsequently, the expression level of eEF1A2 using a specific anti-eEF1A2 antibody (prepared as already reported [22]) was analyzed. As shown in **Figure 1B**, eEF1A2 isoform was revealed only in HEK 293 cells transfected with pcDNA3.1-eEF1A2(His)<sub>6</sub> and confirmed with the anti-His antibody (Merck, Germany) (**Figure 1C**).



**Figure 1.** Expression of eEF1A isoforms in HEK 293 cells. HEK 293 cells were transfected with pcDNA3.1-eEF1A2(His)<sub>6</sub>, and after 24 h from transfection, cell extracts were analyzed by Western blot using commercial mouse anti-eEF1A antibody (A), anti-eEF1A2 antibody (B), and rabbit anti-His antibody (C). Lanes: -eEF1A2, non-transfected HEK 293 cells; +eEF1A2, HEK 293 cells transfected with pcDNA3.1-eEF1A2(His)<sub>6</sub>.

## 2.2. Both eEF1A1 and eEF1A2 immuno-interact after pull-down

The possible interaction between eEF1A isoforms was analyzed by pull-down experiment. To this purpose, GST-eEF1A1 (kindly supplied by C. Sanges, Wurzburg, Germany [21]) and pcDNA3.1-eEF1A2(His)<sub>6</sub> constructs were co-transfected in HEK 293 cells and, after 24 h from transfection, cell extracts were analyzed by Western blot following GST-agarose and Ni-NTA-agarose pull-down. As shown in **Figure 2**, compared to controls, GST pull-down of co-transfected cells showed the presence of a band of 54 kDa corresponding to the size of eEF1A2(His)<sub>6</sub> (**Figure 2A**, lane 2), whereas Ni-NTA pull-down showed the presence of a band of about 78 kDa corresponding to the size of the construct GST-eEF1A1 (**Figure 2B**, lane 2). **Figure 2B** (lane 3) also shows the presence of a band of about 26 kDa corresponding to the GST protein. This finding suggested that GST by itself somehow interacted with Ni-NTA matrix; thus, the result shown in line 2 could be partly due to an interaction of the GST moiety present in GST-eEF1A1 with Ni-NTA and not with eEF1A2. Therefore, to further confirm the interaction between eEF1A isoforms, a different approach was undertaken after transfection of HEK 293 cells with pcDNA3.1-eEF1A2(His)<sub>6</sub>. In fact, as reported in **Figure 2C**, compared to cells transfected with pcDNA3.1 empty vector, cells transfected with eEF1A2(His)<sub>6</sub> showed, after Ni-NTA



**Figure 2.** Co-transfection of GST-eEF1A1 and eEF1A2-His in HEK 293 cells. GST-eEF1A1 and pcDNA3.1-eEF1A2(His)<sub>6</sub> were cotransfected in HEK 293 7 cells. After 24 h, the cells were harvested, lysed, and analyzed after GST pull-down with antibody anti-His (A) and after Ni-NTA pull-down with anti-GST antibody (B). (A) Lanes: 1, non-transfected cells; 2, cells transfected with GST-eEF1A1 and pcDNA3.1-eEF1A2(His)<sub>6</sub>; 3, cells transfected with GST and pcDNA3.1-eEF1A2(His)<sub>6</sub>; 4, GST-agarose alone. (B) Lanes: 1, non-transfected cells; 2, cells transfected with GST-eEF1A1 and pcDNA3.1-eEF1A2(His)<sub>6</sub>; 3, cells transfected with GST and pcDNA3.1-eEF1A2(His)<sub>6</sub>; 4, Ni-NTA alone. (C) pcDNA3.1-eEF1A2(His)<sub>6</sub> was co-transfected in HEK 293 7 cells. After 24 h, the cells were harvested, lysed, and analyzed after Ni-NTA pull-down with anti-eEF1A1, anti-eEF1A2, and anti-His antibody. (a-c) Lanes: 1, cells transfected with empty vector; 2, cells transfected with pcDNA3.1-eEF1A2(His)<sub>6</sub>.



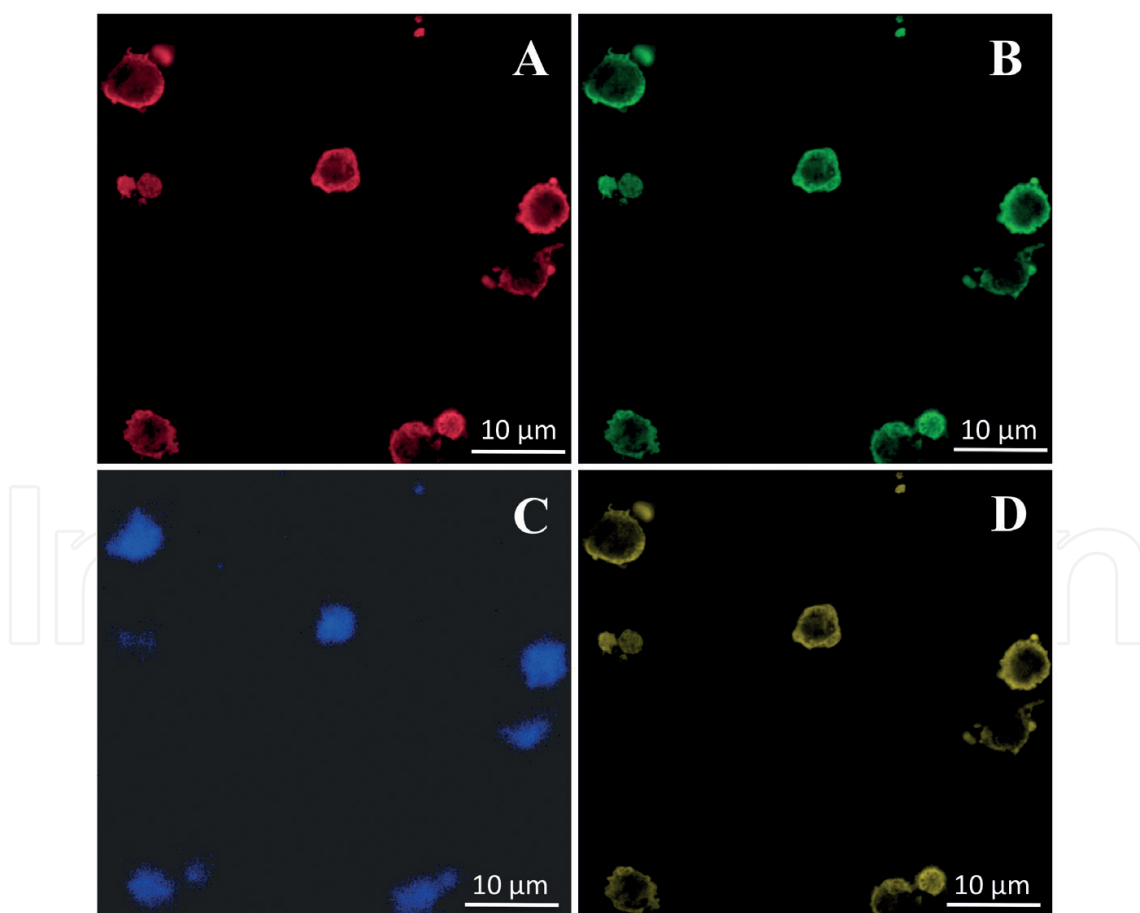
pull-down of cell extracts, the presence of a band of 54 kDa that was recognized by the specific anti-eEF1A1 (prepared as already reported [22]) (**Figure 2C-a**, lane 2) and anti-eEF1A2 (**Figure 2C-b**, lane 2) antibodies, the latter confirmed also with anti-His antibody (**Figure 2C-c**, lane 2).

### 2.3. Both eEF1A1 and eEF1A2 colocalize in HEK 293 cells

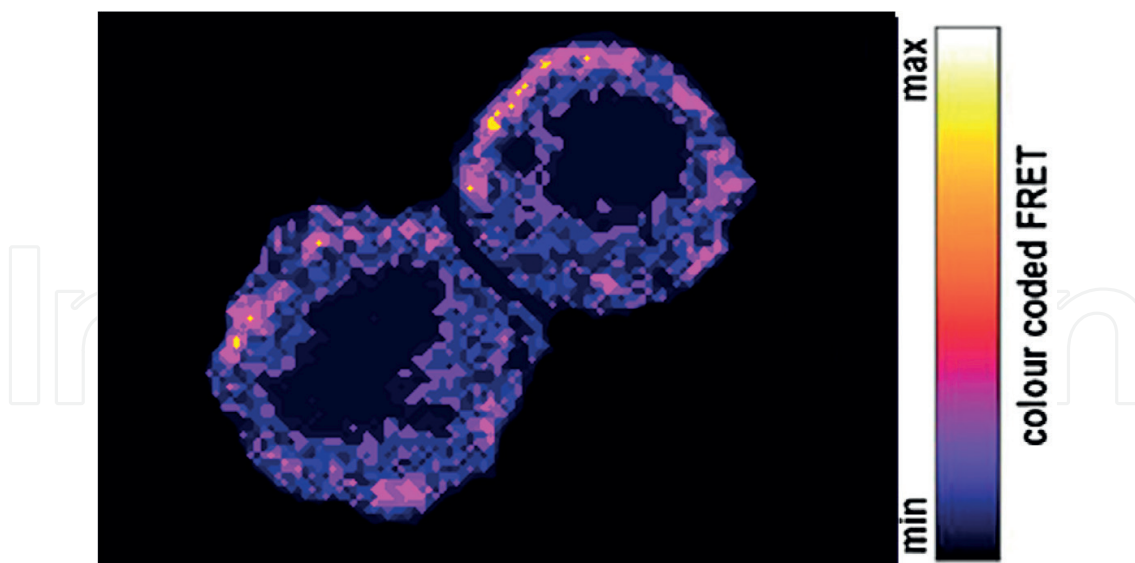
The intracellular colocalization of eEF1A1 and eEF1A2 was first analyzed by confocal microscopy. As shown in **Figure 3**, HEK 293 cells after 48 h from transfection with pcDNA3.1-eEF1A2(His)<sub>6</sub> construct revealed that both endogenous eEF1A (**Figure 3A**) and transfected eEF1A2(His)<sub>6</sub> (**Figure 3B**) shared a cytoplasmic localization. The superimposition of the two panels (merged image, **Figure 3D**) showed that both eEF1A isoforms exhibited a cytoplasmic colocalization with specific signals more intense at the level of the plasma membrane.

### 2.4. FRET analysis showed that both eEF1A1 and eEF1A2 interact in HEK 293 cells

The interaction between endogenous eEF1A and transfected eEF1A2(His)<sub>6</sub> was further investigated by sensitized emission FRET method. FRET effect was performed by confocal microscope



**Figure 3.** Colocalization of eEF1A1 and eEF1A2(His)<sub>6</sub> in HEK 293 cells. HEK 293 cells were transfected with pcDNA3.1-eEF1A2(His)<sub>6</sub>, and after 48 h from transfection, cells were analyzed by confocal microscopy. (A) eEF1A1, (B) eEF1A2, (C) nuclear staining, and (D) merged images.



**Figure 4.** FRET analysis of the interaction between eEF1A1 and eEF1A2. Representative pseudocolor images of cells labeled with rabbit anti-eEF1A1 and mouse anti-His primary antibodies followed by FITC and TRITC secondary-labeled antibodies.

that allowed discriminate proteins that colocalize in the same cellular compartment from those that are instead involved in specific molecular interactions. FRET effects were calculated using ImageJ plug-in software [24]. **Figure 4** shows the representation of the FRET effects where the blue color is indicated at low signal, whereas yellow-white color designated a high signal. The images clearly showed the interaction between eEF1A1 and transfected eEF1A2(His)<sub>6</sub> within the cytoplasm with specific signals more intense especially at the level of the plasma membrane.

### 3. Discussion

FRET is a powerful technique suitable for studying *in situ* interactions between biological molecules in cellular environments [25]. FRET can be assessed from the transfer of energy from one fluorescent molecule (donor) to another fluorescent molecule (acceptor). This process occurs optimally only if the two molecules are properly oriented and reasonably at a narrow distance (usually 1–10 nm) [26]. By this technique, the interaction between eEF1A1 and eEF1A2 in order to reinforce our hypothesis on the formation of an eEF1A1-eEF1A2 heterodimer [21] was highlighted. In a different approach, we afforded this aspect by expressing chimeric eEF1As fused to CFP and YFP as donor and acceptor (CFP-eEF1A1 and YFP-eEF1A2) in COS-7 cells, respectively [22]. However, some criticisms emerged that could have affected the FRET results such as (1) the possible interaction of the expressed chimeric proteins with endogenous enzymes, (2) self-association between eEF1A molecules (i.e., homodimer formation), and (3) the overexpression in COS-7 cells of both constructs that could have generated an artificial FRET signal mainly at the level of plasma membrane. Therefore, to overcome these concerns and to confirm that both eEF1A isoforms interact in the cellular environment, we used a different approach based on the use of IgG-FITC (donor)- and IgG-TRITC (acceptor)-conjugated antibodies. To this end, HEK 293 cell line, lacking the expression of eEF1A2 isoform, was transfected with

pcDNA3.1-eEF1A2(His)<sub>6</sub> and the interaction of the recombinant eEF1A2(His)<sub>6</sub> with endogenous eEF1A1 was assessed by pull-down, confocal microscopy and FRET analysis. The results obtained showed that the endogenous eEF1A1 and the expressed eEF1A2 interacted in HEK 293 cells at the level of both cytoplasm and plasma membrane. Moreover, the FRET image highlighted a more intense signal at the level of the plasma membrane. These data confirmed those reported in our previous work [22], thus strongly confirming the association in the cells of eEF1A isoforms.

The homodimer association of eEF1As has recently emerged from the crystallization of rabbit eEF1A2 [27] or as proposed in *Tetrahymena*, in order to explain actin bundling essential for the regulation of actin cytoskeleton and cell morphology during several cellular processes [16]. The possible association between eEF1A isoforms was instead proposed by Sanges et al. [21] in studying the control of eEF1A function in cancer cells *via* phosphorylation and by Lee et al. [28] in studying the interaction of eEF1A2 with the tumor suppressor protein p16<sup>INK4a</sup>. Since eEF1A1 and eEF1A2 display a very high amino acid sequence identity (above 97%), the overall structures appear quite similar, as can be predicted by bioinformatic analysis at the GRAMM-X docking Web Server v.1.2.0 [29, 30], using rabbit eEF1A2 (PDB 4C0S chain A) as template [27]. These considerations suggest that both eEF1A1 and eEF1A2 complexes are present in the cells either as homodimer or as heterodimer. These complexes are most likely associated with regulatory noncanonical functions of eEF1As.

## 4. Conclusions

Because eEF1A dimers are involved in actin bundling [31, 32], it emerges that the fraction of eEF1A as dimer is mostly involved in the actin cytoskeleton rearrangement. Therefore, the cellular distribution of eEF1A molecules between monomeric and dimeric form regulates the functional role of eEF1A in translation or in actin bundling. Because actin chains and translational system coexist in the cells and maybe also functionally dependent [33, 34], the transition “monomer-dimer-monomer” of eEF1A should be relatively easy depending on the cell conditions [35]. This interconversion may be regulated by the reversible posttranslational modifications of eEF1A [36] and its interactions with the protein partners such as Raf kinases [20, 21]. Therefore, it is possible that in cells coexpressing both isoforms, like cancer cells, eEF1A heterodimer formation could also be important for cytoskeleton rearrangements rather than for some phosphorylation catalysis most likely occurring during cell survival and apoptosis [20, 21].

## 5. Materials and methods

### 5.1. Cell culture and transfection

HEK 293 cells, obtained from the American Type Tissue Collection (Rockville, MD, USA), were grown at 37°C in a 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle medium (DMEM) (Gibco, Monza, Italy) supplemented with 10% heat-inactivated FBS (GIBCO), 100 U/ml penicillin, 100 mg/ml streptomycin, and 1% L-glutamine.



For Western blot analysis, cells ( $300 \times 10^3$ /well) were transfected with GST-eEF1A1 (1  $\mu$ g), pcDNA3.1-eEF1A2(His)<sub>6</sub> (1  $\mu$ g) and pcDNA3.1 (3  $\mu$ g) as control using Lipofectamine 2000 or K2. Twenty-four hours after transfection, cells were collected and the corresponding extract analyzed with mouse monoclonal anti-eEF1A antibody.

For confocal microscopy and FRET analysis, cells ( $10 \times 10^3$ ) were layered on 10-mm glass coverslips, grown at confluence and then transfected with pcDNA3.1-eEF1A2(His)<sub>6</sub> (1  $\mu$ g) or with pcDNA3.1 (1  $\mu$ g) as controls. Cells were analyzed after 24 h of incubation.

## 5.2. Cytosolic extracts, pull-down assay, and Western blot

After growth, HEK 293 were scraped, washed twice in PBS, resuspended for 30 min on ice in 20–40  $\mu$ l of lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mg/ml aprotinin, leupeptin, pepstatin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF), and then centrifuged at  $14,000 \times g$  for 20 min at 4°C.

Pull-down assay for GST-eEF1A1 or eEF1A2(His)<sub>6</sub> was carried out using GST-sepharose (Amersham, Milan, Italy) or Ni-NTA agarose (Qiagen, Milan, Italy), respectively. In detail, 500  $\mu$ g of cell extracts was incubated with pre-equilibrated resin (about 150  $\mu$ l slurry/1 mg protein extract) for 2 h at room temperature (RT) or ON at 4°C, respectively. Subsequently, for GST pull-down, the resin was washed two times (centrifugation for 2 min at 2000 r.p.m. 4°C) with 1 ml of 1× phosphate-buffered saline (PBS), whereas for Ni-NTA pull-down, the resin was washed two times with 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, and 20 mM imidazole, to reduce nonspecific bound proteins, 0.05% Tween 20, pH 8.0. Successively, the samples were resuspended in 30  $\mu$ l of 4× Laemmli loading buffer, heated to 95°C for 15 min and subjected to Western blot analysis.

Protein concentration was determined by a modified Bradford method, using the Bio-Rad protein assay and compared with bovine serum albumin (BSA) standard curve. 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).

## 5.3. Confocal laser scanning microscopy

Human embryonic kidney cells (HEK 293 Cell line) were treated for 20 min with glutaraldehyde 2.5% in PBS, washed three times with PBS, permeabilized for 10 min with 0.1% Triton-X100 and finally washed in PBS. Cells were then blocked for 20 min with 1% BSA in PBS, and after apposite washes, cells were incubated with rabbit anti-eEF1A1 antibody (GenScript, Piscataway, NJ, USA) and mouse anti-His polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:300 in 1% BSA for 1 h. After washing three times with PBS, cells were incubated for 1 h with the appropriate secondary antibodies conjugated to fluorochromes and diluted 1:1000 in 1% BSA. Incubation with TOPRO 3-Iodide (Invitrogen Molecular Probes Eugene, OR, USA) diluted 1/1000 in BSA 1% was done for staining of the nucleus. After this, cells were washed properly with PBS and then observed with a Nikon Confocal Microscope C1 equipped with an EZ-C1 Software for data acquisition by using 60× oil immersion objective.

#### 5.4. FRET analysis

HEK 293 cells ( $7 \times 10^3$  cells/cm<sup>2</sup>) were grown for 24 h on glass coverslips under standard conditions (37°C, 5% CO<sub>2</sub>). Cells were then rinsed with PBS, fixed for 10 min with formaldehyde (3.7% in PBS), permeabilized for 10 min with Triton X-100 (0.1% in PBS), and blocked for 20 min in bovine serum albumin (BSA) (1% in PBS). Subsequently, each sample was incubated for 1 h with 5 µg/ml of mouse anti-His and 5 µg/ml of human anti-eEF1A1 antibodies. Following PBS washes, cells were treated for 1 h with goat anti-mouse IgG FITC-conjugated antibody (donor) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1 µg/ml) and with goat anti-rabbit IgG-TRITC-conjugated antibody (acceptor) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (10 µg/ml). Finally, after 3× washes in PBS, confocal images were collected using a Nikon Confocal Microscope C1 furnished with EZ-C1 software. FRET analysis was carried out as already reported [24]. “FRET” images give the calculated amount of FRET for each pixel in the merged images. The ImageJ plug-in color codes the relative FRET efficiency, which is reported by the displayed color bar, on the right of the images.

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#### Conflicts of interest

The authors declare that there is no conflict of interest.

#### Disclosure statement

Nothing to declare.

#### Author contributions

NM, IR, and NMM were involved in WB analysis and cell extract preparation; GS and CS were involved in cell transfection; ER was in charge of tissue culture; VQ and FP performed confocal and FRET analysis; PA and AL were involved in the reading and approval of the manuscript.

## Abbreviations

CFP	cyan fluorescent protein
DMEM	Dulbecco's modified Eagle medium
eEF1A	eukaryotic elongation translation factor 1A
EGF	epidermal growth factor
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
GST	glutathione S-transferase
Ni-NTA	nickel-nitrilotriacetic acid
PBS	phosphate-buffered saline
TRITC	tetramethylrhodamine
YFP	yellow fluorescent protein

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