Structural cues for understanding eEF1A2 moonlighting

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Abstract: Spontaneous mutations in the *EEF1A2* gene cause epilepsy and severe neurological disabilities in children. The crystal structure of eEF1A2 protein purified from rabbit skeletal muscle reveals a post-translationally modified dimer that informs about sites of interaction with numerous binding partners, including itself, and maps these mutations onto the dimer and tetramer interfaces. The spatial locations of the side chain carboxylates of Glu301 and Glu374, to which phosphatidylethanolamine is uniquely attached via an amide bond, define the anchoring points of eEF1A2 to cellular membranes and interorganellar membrane contact sites. Additional bioinformatic and molecular modelling results provide novel structural insight into the demonstrated binding of eEF1A2 to SH3 domains, the common MAPK docking groove, filamentous actin, and phosphatidylinositol-4 kinase III β . In this new light, the role of eEF1A2 as an ancient, multifaceted, and articulated protein at the crossroads of autophagy, oncogenesis and viral replication appears very distant from the “canonical” one of delivering aminoacylated-tRNAs to the ribosome that has dominated the scene and much of the thinking for many decades.

The eukaryotic translation elongation factors eEF1A1 and eEF1A2 are 92% identical and 98% similar (Figure S1 in the Supporting Information) but their mutually exclusive presence in most normal cells is tissue- and development-specific.^[1] Recent whole exome sequencing of parent-child trios has led to the discovery of an increasingly growing number of *de novo* heterozygous missense mutations in *EEF1A2* genes from children with neurological deficits ranging from a mild epileptic phenotype to intractable epilepsy and severe intellectual disabilities that are typically associated to subtle facial dysmorphic features.^[2] Remarkably, a 15.8-kb deletion that removes the promoter and first exon of *EEF1A2*, thereby abolishing

eEF1A2 protein activity altogether, was found to be solely responsible for the autosomal recessive ‘wasted’ (*wst*) phenotype discovered in Jackson Laboratory’s mice in 1982. This genetic alteration, which gives rise to pathological changes similar to those of ataxia telangiectasia in humans, is characterized by a high degree of chromosomal damage and motor neuron degeneration that starts at day 21 after birth and causes death by day 28.^[3] The study of *EEF1A1* and *EEF1A2* gene expression profiles during development in wild-type (+/+), heterozygous (+/*wst*), and homozygous (*wst/wst*) mice revealed that eEF1A2 protein is present only in brain, heart and muscle of (+/+) and (+/*wst*) animals, with the onset of its expression coinciding with a concomitant decrease in detectable eEF1A1 protein. In (*wst/wst*) tissues, eEF1A2 was absent, as expected, but the scheduled decline of eEF1A1 during postnatal development took place just as in their (+/+) counterparts, strongly suggesting that the developmental genetic switch in charge of silencing *EEF1A1* is still functional in these animals.^[4] In parallel, prior to postnatal day 7, eEF1A1 is the major gene product in the brain of adult (+/+) mice but only eEF1A2 is found in terminally differentiated neurons by postnatal day 14; in contrast, eEF1A1 continues to be present in non-neuronal cells. This postdevelopmental takeover by eEF1A2 protein and the concomitant decline in *EEF1A1* expression observed in brain neurons and skeletal muscle cells also happens in cardiac myocytes and motor neurons in the spinal cord^[5] for reasons that are not fully understood.

Taken together, these clinical and experimental findings support a critical role for eEF1A2 in neurodevelopment but neither the mechanism(s) underlying the varying severity of symptoms nor the reason(s) why mutations invariably occur at positions encoding amino acids that are evolutionarily highly conserved in the protein sequence (and therefore subjected to strong selective pressure) is presently known.

Significantly, minor specific sites of normal *EEF1A2* expression have also been found in several specialized human body compartments^[2a, 6] and transcripts encoding *EEF1A2* are uniquely overexpressed in plasmacytomas (tumors of mature plasma cells) and subsets of human multiple myeloma (MM) cell lines.^[7] *EEF1A2* expression is also elevated during the progression and dissemination of several solid tumors including breast, ovarian, pancreatic and lung cancers –in cases of ductal breast carcinoma, accompanied by *EEF1A1* underexpression^[8]–, and this increase has been repeatedly associated to poor prognosis. On the contrary, high *EEF1A1* expression in resected colon adenocarcinomas has been correlated to significant overall survival.^[9]

Furthermore, eEF1A2 is known to promote not only the malignant transformation of a variety of mammalian cells^[10] but also formation of filopodia and lamellipodia –long cellular

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processes composed of bundles of parallel actin filaments—that are important for normal cell migration and cytoskeletal reorganization. These effects have been related to the ability of eEF1A2 to bind and activate phosphatidylinositol-4 kinase III β (PI4KB),^[11] the enzyme (EC 2.7.1.67) responsible for catalyzing the phosphorylation of the hydroxyl at the D4 carbon of the inositol ring in phosphatidylinositol (PI) to yield PI 4-phosphate (PI4P), the major precursor in the synthesis of other phosphoinositides, including phosphatidylinositol-4,5 bisphosphate (PIP₂). Importantly, PI4KB—unlike other lipid kinase family members^[12]—is not an integral membrane protein and therefore needs to be recruited from the periphery to membranes in the Golgi complex (GC) and the trans-Golgi network (TGN), where it is primarily localized. Upon activation, PI4KB plays crucial roles in cytokinesis and regulation of lipid and protein transport from the GC to the plasma membrane (PM).^[12–13]

Remarkably, both eEF1A1/2 and PI4KB are also essential host factors for genome replication of *Picornaviridae*^[14] such as enteroviruses (e.g. poliovirus, coxsackieviruses, and human rhinoviruses), Kobuviruses (e.g. Aichi virus), and coronaviruses.^[15] This is so because all single-stranded positive sense RNA [(+)RNA] viruses subvert cellular membranous structures (e.g. single-membrane tubules, double-membrane vesicles [DMVs], or more complex multilamellar bodies) from their host cells to use them as suitable microenvironments for viral RNA synthesis. These so-called replication organelles (RO) or viral replication complexes (VRC) stem from the TGN and are in physical contact with lipid droplets, which suggests that lipid shuttling facilitates RO proliferation and genome replication.^[16] This explains why PI4KB and PIP₂ localize to VRC.^[17]

From this broad-scope but necessarily succinct summary, it becomes apparent that the ancient, extremely abundant, and highly homologous eEF1A1 and eEF1A2 proteins from phylogenetically distant eukaryotes—from yeast to plants and animals—have evolved, in common with their prokaryotic EF1A (aka EF-Tu) counterparts,^[18] the capacity to execute diverse functions besides delivering aminoacyl-tRNAs (aa-tRNAs) to the ribosome. Ample experimental evidence strongly supports the current view that a protein's ability to moonlight is dependent on particular post-translational modification (PTM) patterns, which provide not only multitasking abilities and different subcellular localizations but also several potential means of pharmacological intervention.^[19] Apart from some of the more common reversible PTMs such as (i) lysine acetylation, (ii) lysine mono-, di- and trimethylation, (iii) serine, threonine, and tyrosine phosphorylation, (iv) arginine mono-ADP-ribosylation (MARylation), (v) α -N-terminal methylation^[20] (following methionine removal), and (vi) methyl esterification at the C-terminus,^[21] both eEF1A1 and eEF1A2 undergo a rather unique PTM consisting of the covalent attachment to two glutamate residues (301 and 374)—via an amide bond—of phosphatidylethanolamine (PE),^[22] a key player in membrane architecture with a well-defined role as “a nonprotein molecular chaperone”.^[23]

To shed structural light on the effect of eEF1A2's PTMs, some of which occur in, or near, short linear motifs (SLiMs) present in surface-exposed and highly conserved amino acid stretches, we purified eEF1A2 from a traditional natural

source, rabbit skeletal muscle, and obtained crystals that were employed to solve its 3D-structure by x-ray crystallography. The concurrent use of mass spectrometry (MS), bioinformatics, molecular modelling and simulation methods allowed us to gather novel structural information that accounts for eEF1A1/2 moonlighting functions both in the cytosol and at particular membrane locations.

Results and discussion

Overview of the structure

The crystal structure of the eEF1A2:GDP complex was solved at 2.7 Å resolution (Supporting Table S1) and displays two copies in the asymmetric unit arranged as a head-to-tail compact dimer comprising residues 5–454 in chain A and residues 4–461 in chain B (Figure 1). This dimeric arrangement, first observed in PDB entry 4C0S—reportedly containing phosphorylated Ser163 and Thr239—, was originally discussed mostly in the context of nucleotide exchange and dissociation from the 80S ribosome.^[24] In contrast, the rabbit skeletal muscle eEF1A2 structure that we have solved reveals (i) the conserved covalent linkage of ethanolamine phosphoglycerol (EPG) to Glu301 and Glu374, which provides a sound foundation to account for the interaction of eEF1A2 with cellular membranes via the phosphatidylethanolamine precursor;^[22] (ii) the phosphorylation of Ser300 that is known to prevent aa-tRNA binding;^[25] (iii) the first experimental evidence of *N*^ω-carboxymethylation of Arg266, which might be an indication of MARylation; (iv) the structuring as an α -helix of the previously unresolved C-terminal region, which has been conclusively demonstrated to be the exclusive actin-binding site in eEF1A1 and eEF1A2;^[26] and (v) the characterization of two alternate locations for Ser53, a residue in the GTP-binding domain that is targeted by *Legionella pneumophila* glucosyltransferases Lgt1 and Lgt2 (when eEF1A is loaded with GTP and aa-tRNA)^[27] to inhibit protein synthesis and is also known to undergo a well-established Ca²⁺-activated, PKC β 1-dependent^[28] phosphorylation. The importance of a low pH (5.6) in crystallization is in consonance with (i) the dual protonation on N δ and N ϵ of His95 and His367 because both nitrogens are found at hydrogen bonding distance from the carboxylate oxygens of Asp91 and Glu68 (in the case of His95) and Asp362 and Glu45' (in the case of His367); and (ii) the protonation of the Asp252 carboxylate for which we calculate a pK_a of 6.0–6.6.^[29]

Description of eEF1A2 chains A and B

Each eEF1A2 chain folds into three well-characterized structural domains (Figure 1 and SI Figures S2 and S3), namely, domain I (residues 4–234), where GDP/GTP binding takes place, and domains II (241–328) and III (337–454 in chain A and 337–461 in chain B), both of which are β -barrels. A best-fit superposition of individual domains reveals a high degree of structural similarity between chains A and B (domain I: rmsd = 0.39 Å; domain II: rmsd = 0.27 Å; domain III: rmsd = 0.24 Å). Nevertheless, the overall root-mean-square deviation (rmsd) for all the C α atoms of 1.16 Å highlights slightly different inter-domain arrangements, which can be attributed to the distinct crystal environment surrounding each chain. These differences are most prominent in the C-terminal region of domain

III where residues 456–462 could be unambiguously traced in chain B and shown to fold into a lysine-rich α -helix (SI Figure S4). The preceding stretch remains loosely bound to the protein core by virtue of hydrophobic contacts and several β -sheet-like hydrogen bonds to β 4 of domain II. The mutually interacting residues and atoms involved are Lys444:Glu336, OG(Thr452):O(Thr286), OG(Ser454):OE1/OE2(Glu288), OG(Ser454):OD1(Asn314) plus the helix dipole, and ⁴⁵⁵AqkA⁴⁵⁸:Val277. As a result of these interactions, the orientation of the C-terminus in this eEF1A2:GDP complex is distinct from that observed in the bovine mitochondrial EF-Tu:GDP complex (PDB code 1D2E),^[30] the only other EF1A/eEF1A crystal structure displaying a C-terminal region (SI Figure S5).

Figure 1. Three-dimensional structure of the eEF1A2 dimer as observed in the crystallographic unit cell. Chains A and B are represented as cartoons and colored in pink and yellow, respectively. The C_{α} of the post-translationally modified residues identified in this work are displayed as yellow spheres and labeled. The GDP molecules are shown as green spheres.

The loop comprising residues 50–56, which is more poorly and distinctly structured in chain A compared to chain B, turned out to be quite recalcitrant to modelling in previous independent work (PDB entry 4C0S)^[24] (see SI Figure S6 for a comparison of the major structural differences between both

PDB entries). We initially encountered the same difficulties because this loop faces not only the GDP/GTP binding site but also the 214–225 hairpin of chain B from a symmetry-related copy of another dimer. In our case, with the aid of the RCD+ loop server^[31] and molecular dynamics (MD) simulations in the unit cell (see Movie S1), we were able to (i) build this loop connecting helices H2 and H3 into the electron density map for both chains, (ii) probe the effect of phosphorylation at Ser53 (see below), and (iii) directly observe the motions of Phe54, Tyr56 and Trp196 that appear to be involved in nucleotide binding and exit (see Movie S2).

A GDP molecule is bound to each eEF1A2 monomer but no electron density corresponding to a coordinated Mg^{2+} ion is discernible near the phosphates. The guanine ring is sandwiched between the C_{β} of Trp22 and the alkyl side chain of Lys154, with N1 and N2 donating two hydrogen bonds to the carboxylate of Asp156 and O6 accepting two longer hydrogen bonds from the hydroxyl of Ser194 and the backbone NH of Trp196. The α -phosphate O1A is hydrogen bonded by the OG and NH atoms of Thr22 whereas the terminal β -phosphate is stabilized by the NH groups of Asp17, Gly19, Lys20 and Ser21, as well as the amino group of Lys20 and the hydroxyl group of Ser21. The ribose hydroxyls are solvent-exposed and face the 50–56 loop from another monomer in the crystal lattice so that four different nucleotide environments are discernible in the crystal lattice, of which only three would be compatible with Ser53 phosphorylation or glucosylation. The absence of clear electron density for Mg^{2+} in our structure supports the independence from this divalent cation of GDP binding to eEF1A2 and the disengagement between the presence of Mg^{2+} and the structural rearrangements involved in nucleotide exchange, as opposed to prokaryotic EF1A – previously known as EF-Tu – in which the residue positionally and functionally equivalent to Ser21 is a Thr.^[32]

Besides the differences within the nucleotide-binding sites, other distinct structural features can be noted between chains A and B that mainly involve small rearrangements in several loops and in the intrinsically disordered region (IDR) encompassing 122–127. This IDR appears folded into an α -helix in chain B due to the strong hydrogen bonds established between the negatively charged Glu122 and Glu124 carboxylates with the positively charged side chains of Lys154 (whose aliphatic side chain extends over the guanine ring of the bound GDP molecule) and Arg166. In chain A, this region displays dual occupancies due to the proximity of the same amino acids from a symmetry-related copy of the same chain. It becomes apparent, therefore, that replacement of any of these two glutamic acids with lysine (as observed in children encoding the p.(E122K) and p.(E124K) mutations in their *EEF1A2* genes) will have highly detrimental structural consequences. Nonetheless, it is noteworthy that the clinical neurological manifestations emerging from these two amino acid changes in eEF1A2 are amongst the least severe.^[2a, 33] Other minor loop displacements ranging from 1.5 to 2 Å throughout the structure lead to subtle shifts of secondary structure elements. This can be observed, for example, in the loop connecting β 3 to β 4 (residues 380–386) in domain III, which shows a reorientation of 1.8 Å and causes a maximum lateral displacement of 1.6 Å of the β 4 strand.

eEF1A2 dimer stabilization

The presence in the crystal structure of two intimately associated eEF1A2 monomers, which are overall identical except for some minor loop rearrangements most likely due to distinct crystal lattice contacts, bears the questions of the significance of the dimer and the nature of the stabilizing forces. Dimer formation entails the burial of $\sim 2,200$ Å² of surface area per monomer, $\sim 50\%$ of which is nonpolar. A global 0.514 Voro-

MQA score,^[34a] which combines statistical potentials with use of Voronoi tessellation of atomic balls (threshold = 0.4), supports the high quality of our structural model whereas visualization of the local residue-based VoroMQA scores along the protein chain^[34b] allows the protein-protein interaction interface to be directly mapped onto the amino acid sequence (Figure 2A). Remarkably, none of these interfacial residues undergoes any known PTM but an abundance of amino acids that are replaced due to spontaneous mutations in humans and give

rise to inborn neurodevelopmental disorders^[2] is clearly apparent.

Figure 3. Amino acid modifications in eEF1A2 observed by X-ray crystallographic analysis. A) The eEF1A2 backbone is represented as a red cartoon and the C α of each modified residue is displayed as a labelled yellow sphere. The associated electron density (2F_o-F_c map contoured at 1 σ) for each modified residue is depicted in the boxes where the letter in brackets refers to the protein chain. N ϵ -CM-Arg: N ϵ -carboxymethylated arginine. B) Side-by-side arrangement of two A monomers in the crystal lattice. P-Ser: phosphorylated serine; EPG-Glu: ethanolamine phosphoglycerol covalently bonded to glutamate. The fact that most PTMs are more clearly observable (i.e. have lower B factors) in one of the monomers present in the asymmetric unit reflects, once again, the different crystal environments around each protein chain. In particular, the electron density is much poorer for the EPG bonded to Glu301 in chain B (exposed to solvent channels and not modelled in the final structure deposited with the Protein Data Bank) whereas in chain A this modification points towards the same modified residue in chain A' of a symmetry-related copy of another dimer.

High interface-specific local scores are patent at Gly70, Ile71, Asp252, and Arg423 (known mutation sites), and also at Tyr86 and Lys100, vicinal to Asp91 and Phe98 (mutation sites, too),

in agreement with the pattern of interchain hydrogen bonds and hydrophobic contacts (SI Table S2). Analysis of the MD trajectories *in crystallo* for the fully reconstituted protein provided binding energy estimates (Figure 2C) that show the prominent importance of Ile71 and Asp252 for dimer stabilization. Taken together, all of these findings provide a strong indication that the crystallographically observed eEF1A2 dimer, which may originate from the PM, the SR, the GC and/or the mitochondria of skeletal muscle cells (see below), is indeed biologically relevant. This view is also supported by the very low dissociation constant (9.4 pM at 25 °C) estimated by the PROtein binDing enerGY prediction (PRODIGY) server.^[36]

Post-translational modifications in eEF1A2

The extra electron density close to the side chains of some eEF1A2 residues strongly suggested the presence of several PTMs, the existence of which was assessed by crystallographic refinement (Figure 3) and independently studied by MS and MS/MS (SI Figure S7). Herein, we have focused on two PTMs: (i) attachment of EPG to Glu301 and Glu374, and (ii) phosphorylation of Ser300. In addition, crystallographic evidence was found that the guanidinium group of Arg266 is –at least– N ϵ -carboxymethylated (Figure 3A), which could be the result of either a nonenzymatic reaction or MArylation.

N ϵ -carboxymethylation of Arg266

In the immediate vicinity of Lys79 and Tyr86, and also close to the unsolved N-terminal region of eEF1A2 discussed above, is the Arg266 side chain, where we found extra electron density next to the N ϵ atom that is fully compatible with a carboxymethyl moiety (Figure 3A). Arginine is known to undergo several PTMs, namely methylation, citrullination, phosphorylation, and MArylation^[37] but also nonenzymatic carbonylation and formation of advanced glycation end products (AGEs)^[38] that include N ϵ -carboxymethyl-arginine.^[38] To the best of our knowledge, this is the first time that such modification, which has been recently described as the dominant AGE in glycosylated collagens and mouse tissues,^[39] is reported for eEF1A1/2. One important consequence is that the strong ionic interactions established between Arg266 and the carboxylates of Asp110 in domain I and Glu268 in domain III of monomeric eEF1A1(GTP) (fixed in place by Arg240 and Arg423, respectively) are also lost in the eEF1A2(GDP) dimer. Remarkably, Arg266 is highly conserved in eukaryotic eEF1A1/2 but is absent in its mitochondrial counterpart where the positionally equivalent residue is a threonine (SI Figure S1).

GDP vs. GTP conformations of eEF1A2

The three domains of eEF1A1/2 are expected, on the basis of structural knowledge gained from prokaryotic EF1A, to reorient depending on whether GDP or GTP is bound, and this inherent interdomain flexibility can account for their diverse interaction sites beyond aa-tRNA binding for amino acid delivery to the ribosome.^[40] The large structural rearrangement that is known to occur in EF1A (SI Figure S8) when it switches from the “inactive” GDP-bound (e.g. PDB id. 1TUI) to the “active” GTP-bound conformation (e.g. PDB entries 1EFT and 1TTT) was recapitulated in a homology model of human eEF1A1/2 (PDB

Figure 4. Theoretical model of a full-length eEF1A2 dimer inserted into the inner leaflet of a cellular membrane. A) Each eEF1A2 monomer is coloured differently except for the five N-terminal amino acids, which are coloured in blue. The monolayer is made up of phosphatidylcholine exclusively for illustrative purposes. Note the parallel arrangement of the fatty acid chains (green sticks) covalently bonded to the EPG moieties attached to Glu301 of both monomers. The C-terminal α -helices appear very close to the PI head groups (D4 atoms are shown as pink spheres to highlight the position of the phosphate moiety in PI4P). The locations of the other EPG-modified glutamic acids (Glu374 and Glu374' in the other subunit) are also shown to highlight their orthogonal orientation relative to Glu301 and Glu301'. The largely solvent-exposed GDP molecules within the nucleotide-binding sites are displayed as grey spheres. B) Detail of the region surrounding pSer300 and EPG-Glu301 in one subunit. Note the buttressing interactions provided by Arg240 and Arg273 as well as the spatial relation between this PTM and those taking place at the N-terminus. Bet2 and Me_K3 stand for *N,N,N*-trimethylated Gly2 (betaine) and *N,N*-dimethylated Lys3, respectively. The latter is proposed to contribute to eEF1A2 dimerization by virtue of an electrostatic interaction with the carboxylate of Asp306 (cf. Figure 2C). PE stands for phosphatidylethanolamine.

id. 1SYW)^[41] using simulation methods and the intermediate structure present in the eEF1A:eEF1B α complex from *Saccharomyces cerevisiae* (PDB id. 1F60).^[42] Incidentally, this latter crystallographic structure was recently refined by alternative interpretation of the original data –and deposited in the PDB as entry 5O8W– to account for a glutamine residue covalently linked to the side chain of Glu45.^[43] A highly similar GTP conformation of rabbit eEF1A1 –trapped in a post-hydrolysis GDP state in the presence of bound didemnin B^[44]– was recently found in the mammalian ribosomal complex solved by cryo-electron microscopy (PDB id. 5LZS).^[45] Nucleotide exchange upon eEF1B α binding takes place by means of switches I (loop 67–78) and II (90–96),^[46] and involves domains I and II. It is therefore remarkable that the mobile switch I (the “effector region”)^[47] presents two very different conformations (rmsd = 3.75 Å) in chains A and B of the eEF1A2 structure reported here (SI Figure S9), especially in light of the fact that the GDP dissociation rate constant is reported to be ~7 times lower for eEF1A2 than for eEF1A1.^[48] A short animation showing the conformational changes involved in the transition of eEF1A2 from the GDP to the GTP conformation (calculated using the morphing utility implemented in the iMODS server)^[49] can be found as Movie S3 in the Supporting Information.

In our hands, attempts to trap rabbit skeletal muscle eEF1A2 in the GTP conformation by incubating with non-hydrolysable analogues of GTP such as 5'-guanylyl imidodiphosphate (GDPNP) and following published procedures successfully employed for EF1A,^[50] proved futile. We take this as a strong indication that a considerably stabilized dimer, possibly due to the PTMs identified in this work, is isolated during the purification and crystallization procedures, thus excluding the monomeric GTP form of the protein. In fact, the recent crystallographic determination of a classical, open GDP-bound conformation in the complex of *E.coli* EF1A with GDPNP supports the emerging view that the identity of the bound guanine nu-

cleotide does not lock this GTPase in a fixed conformation unless it is a constituent part of a higher-order complex,^[51] as found in the ribosome,^[45] for example. Additional, albeit indirect, pieces of evidence supporting this interpretation are that (i) the eEF1A monomer-dimer exchange is not affected by pH changes,^[52] and (ii) the presence of GTP, GDP, or GDPNP has virtually no effect on the F-actin bundling activity of *Tetrahymena* eEF1A, whose dimeric form is reverted to the monomer by Ca²⁺/calmodulin.^[53]

Model structure of a full-length eEF1A2 dimer

Unfortunately, no electron density or MS evidence could be found informing about the recently described high-stoichiometry methylation of the N-terminal site and adjacent lysine in yeast and human eEF1A^[20] which, following methionine removal, renders a trimethylated Gly2 (and thereby a positively charged betaine) at the new N-terminus and a dimethylated Lys at position 3 (Figure 4B). The missing N- and C-terminal residues in both chains, as well as incomplete side chains in one or both monomers due to lack of electron density or high B-factors, were built in for completeness with a view to running C α -restrained MD simulations, *in crystallo*, of the eEF1A2 dimer and two different dimers of dimers (obtained by applying the symmetry operators to the dimer present in the unit cell) in the presence of explicit solvent and counterions. By means of these computational methods, we could sample the side-chain conformational preferences and calculate reliable molecular electrostatic potential (MEP) maps for dimers and tetramers (SI Figure S10). We also model-built the covalently bonded PE molecules by attaching the fatty acids myristate^[22b] and palmitate to the EPG moieties. The resulting eEF1A2 models revealed the parallelism of the PE molecules attached to Glu301 in chains A and B and the fact that they point in the same direction as the heavily methylated N-termini and the Lys-rich C-terminal α -helices of both monomers (Figure 4).

Figure 5. The eEF1A2 tetramer. A) Higher-order association of two eEF1A2 dimers in the crystal lattice gives rise to a tetramer and additional interfacial contact sites (boxed), which include Glu122 and Glu124 (amino acids replaced by lysine due to *EEF1A2* mutations in children affected with neurodevelopmental disorders). B) In the tetramer, each monomer is coloured differently and the myristoyl and palmitoyl chains (green sticks) of the PE molecules covalently attached to Glu374 have been model-built in their full length for enhanced clarity. Note the spatial convergence and parallel orientation of these fatty acid chains, which are proposed to get inserted into membranes. The EPG moieties attached to Glu301 in each monomer are also displayed and labelled. Bound GDP molecules are displayed as sticks with C atoms coloured in grey. C) BAR domain of human Sorting Nexin 9 (Spx9) (PDB ID: 3DYT). D) Ribbon and line representation of the eEF1A2 tetramer (grey) in which local contact sites between the associated eEF1A2 dimers^[34a] are coloured in red (image provided by the VoroMQA server).^[34b]

This overall arrangement immediately suggested to us the feasibility of PE insertion into the inner leaflet of a model membrane for eEF1A2 anchoring, aided by electrostatic attraction between the positively charged basic residues at both N- and C-termini and the negatively charged phospholipid/phosphoinositide heads. In Figure 4 we chose to model a PI4P patch on the cytosolic side of the cell membrane because of the prominent roles that this phosphoinositide plays in the eEF1A2-mediated (i) recruitment of effector proteins at membrane-cytosol interfaces, (ii) assembly of Golgi-derived clathrin structures, and (iii) receptor signaling on PM lipid rafts.^[54] This well-defined structural layout raises the captivating possibility of distinct preferences for particular lipid compositions in membrane microdomains as well as precise localization of eEF1A2 in distinct subcellular compartments.^[55] Intriguingly, the other EPG-modified residues (Glu374 and Glu374') are found to stick out in a virtually perpendicular orientation relative to Glu301 and Glu301' (Figure 4A, right) although this view changes quite dramatically when a higher-order tetrameric ensemble is considered (Figure 5). In a tetramer generated using the crystal symmetry, the parallel PE chains also point in the same direction towards a hypothetical membrane, but additionally the two pairs face opposite sides of the tetramer, which may indicate simultaneous binding to a double-membrane structure. Furthermore, the crescent shape of the top twin monomers is suggestive of some lipid-dependent curvature preferences that are reminiscent of those associated to Bin/Amphiphysin/Rvs (BAR) domains of proteins

implicated in membrane trafficking regulation and cytoskeletal rearrangements (Figure 5C). The presence of EPG-Glu374 in domain III in this type of eEF1A2 tetramer may be particularly relevant because (i) this region has been shown to mediate formation of spine- and filopodia-like protrusions in embryonic neurons,^[56] and (ii) the equivalent residue in *Trypanosoma brucei* eEF1A (Glu362) is the single site of EPG attachment.^[57]

Identification of a bipartite SLiM in eEF1A2 for binding to SH3 domains and to the common MAPK docking groove

SRC Homology 3 (SH3) domains bind proline-rich motifs (PRMs) in eukaryotes and eEF1A2 has been shown to interact with those present in phospholipase C γ 1 (PLC γ 1), the non-receptor Src family kinase (SFK) members Crk, Fgr, and Fyn,^[40b] and the adapter protein SORBS2.^[40a] Because both the Eukaryotic Linear Motif (ELM) resource^[58] and the LMDI-Pred server^[59] assigned a high probability, as a putative SH3-binding region, to the first half of the arginine-containing and not fully extended ²³⁵ILPPTRPDKPLRLPL²⁵⁰ PRM in eEF1A2, we used Dali^[60] to perform a best-fit structural superposition of all peptide-bound SH3 domains present in the Protein Data Bank (Figure 6A). We found very high structural similarity between the ²³⁵ILPPTRP²⁴¹ stretch in eEF1A2 and the ¹⁸⁶PPVPPQRP¹⁹³ peptide from the signal transducing adaptor protein SLP-76 bound to the PLC γ 1 SH3 domain. In this complex (PDB ID: 1YWO), selectivity arises from (i) the shape complementarity of a continuous hydrophobic strip of

carbon atoms, (ii) three hydrogen bonds involving the main chain carbonyl oxygens of Pro187, Val188, and Pro190, and (iii) the Pro193-assisted lodging of the guanidinium moiety of the “compass” Arg192 inside a pocket where it forms salt bridges with the carboxylates of Asp808 and Glu809 (Figure 6B, left). This means that the only truly sequence-specific determinant for binding to the PLC γ 1 SH3 domain –conformational selection notwithstanding– is this side-chain electrostatic complementarity, which therefore arises as a crucial factor for proper peptide orientation and also as an important discriminant factor for SH3 domain selectivity. This recognition pattern can be achieved almost identically by the first half of the eEF1A2 PRM, which is endowed with the same polarity and register.

Since the side chains of Tyr800, Asp808, Glu809, Trp828, Asn844, and Tyr845 of PLC γ 1 SH3 domain are directly involved in the sequence-dependent recognition of ²³⁵ILPPTRP²⁴¹ in eEF1A2 and this amino acid sequence is highly conserved in eEF1A proteins over evolutionary time (Figure 6C), we performed a search to identify potential SH3-containing interactors involved in cytoskeleton organization, actin binding, and signal transduction. We found that our identified binding motif in PLC γ 1 SH3, with putative affinity for the eEF1A2 PRM, is indeed present not only in other known binders such as Crk, Fgr, Fyn, and SORBS2 (Figure 6A), but also in (i) regulators of actin cytoskeleton dynamics such as Abl interactor 2 (Abi-2), (ii) Src substrate cortactin, (iii) numerous adapter proteins such as intersectins, Grb2, Nck1 and Nck2, and (iv) several guanine nucleotide exchange factors (GEFs), e.g. β PIX (aka Rho GEF7), the GEF for Rac1 (SI Figure S11).

Interestingly, eEF1A and SORBS2 have been shown to co-localize at the PM.^[40a] Furthermore, the *N*-terminal Met-Gly-Cys sequence in the so-called SH4 domain of Fyn –and other SFKs that are known to interact with eEF1A2– is responsible for anchoring to the cytoplasmic face of the PM upon removal of methionine and fatty acid acylation, which happens to be dual in Fyn, i.e. myristoylation of Gly via an amide bond and palmitoylation of Cys via a thioester bond. These two PTMs are necessary for properly orienting the peptide chain with respect to the lipid bilayer and precede the subsequent trimethylation at Lys7 and/or Lys9.^[61] It is therefore remarkable that both types of interactors, Fyn and eEF1A2, undergo similar PTMs for membrane trafficking from the GC to the PM.

The second half of the highly conserved eEF1A2 PRM, ²⁴³DKPLRLPL²⁵⁰, encompasses a ‘classical motif’ (Figure 6C) that is used to anchor peptides to a ‘common docking’ groove in mitogen-activated protein kinases (MAPKs). This surface region is separate from the active site and contributes to increasing specificity in the selection of binding partners that contain promiscuous active sites, particularly regulatory kinases and phosphatases, as well as scaffolding proteins. This putative docking motif in eEF1A2 (also present in eEF1A1) fits the consensus $\Psi_{1-3}\chi_{3-7}\Phi_A\chi\Phi_B$, where Ψ , Φ , and χ denote positively charged, hydrophobic, or any intervening residues,

respectively.^[62] Consequently, the identified binding mode would be selective, in principle, for c-Jun amino-terminal kinase 1 (JNK1), JNK2 and JNK3 (Figure 6D) due to the electrostatic complementarity between Lys244 and Glu329. Additional but less discriminatory stabilization arises from (i) the burial of the hydrophobic side chains of Pro245, Leu248 and Leu250, and (ii) hydrogen bonds between the CO and NH backbone atoms of Arg247 and Leu248 and the guanidinium and CO groups of Arg127 and Ser161, respectively (Figure 6E).

It is noteworthy that the stretchable eEF1A2 PRM is properly exposed for binding to SH3 domains and MAPK docking grooves neither in the presently characterized GDP-bound dimer (Figure 6D) nor in the more compact GTP-bound monomer (Movie S4). Given that this PRM connects domain I to domain II, the evidence that we have gathered strongly supports the view that other distinct –and possibly transient– states exist for eEF1A2 beyond the classical “on-off” conformational equilibrium at domain I.

Actin binding

Actin is the major component of the cytoskeleton and one of the most highly conserved proteins from yeast to human. The percentage of eEF1A that is associated with actin has been estimated at 70–90%, depending on cell type, and the C-terminal ~50 residues of domain III have been identified as the major (or single) actin-binding site in dimers of *Tetrahymena* eEF1A, *Dictyostelium* eEF1A, rabbit liver eEF1A1,^[26] and rabbit skeletal muscle eEF1A2.^[63] Consistently with the protonation of interfacial residues described above, the affinity of eEF1A for filamentous actin (F-actin) weakens as the pH increases over the physiological range –presumably due, at least in part, to dimer dissociation– whereas its affinity for aa-tRNA as a monomer remains constant between pH 6.5 and 7.0.

The cleft between subdomains I and III of actin, which falls near the hinge for domain motions, is a common binding site for many unrelated actin-binding proteins (ABP)^[64] that regulate the transition between monomeric G-actin and F-actin, which is structurally and functionally asymmetric. Most ABP contain signaling domains and other protein-protein interaction modules, and eEF1A proteins are no exceptions. Among the numerous actin-binding domains, the prototypical and versatile WASP-homology domain 2 (WH2) does not show any significant sequence conservation but displays a remarkable periodicity of hydrophobic and positively charged amino acids. Because we observed residues 451–461 of one eEF1A2 monomer clearly structured as an α -helix, we reasoned that the C-terminal region could be broadly considered a *bona fide* single WH2 motif with the ability to undergo a disorder-to-order transition upon binding to actin.^[65]

Figure 6. A bipartite SLiM in eEF1A2 for binding to SH3 domains and to the common MAPK docking groove. A) Structure-based multiple sequence alignment (MSA) of selected SH3 domains that are known to bind eEF1A2 (an extended MSA can be found in SI Figure S11). The crucial affinity-determining amino acids are coloured in red. B) Theoretical models of the ²³⁵ILPPTRP²⁴¹ amino acid stretch in eEF1A2 in the same overall conformation as the ¹⁶⁶PPVPPQRP¹⁹³ peptide from signal transducing adaptor protein SLP-76 (in complex with the SH3 domain of rat PLCγ1) bound to the SH3 domains of (from left to right) human PLCγ1, Crk and Fyn (PDB IDs shown on the right column), as representative examples of SH3 domains containing Asp-Glu, Glu-Asp and Asp-Asp pairs. C) Phylogenetic tree of selected eEF1A proteins from different species and multiple sequence alignment of their PRMs showing the high degree of conservation over evolutionary time of the xPxR Px motif, in which the underlined Arg is proposed to play a prominent role in SH3-type discrimination, and the KPLRPL, in which the underlined Lys putatively provides the MAPK subtype specificity. D) Schematic cartoon representation of an eEF1A2 monomer (grey) displays the PRM linking domains 1 and 2 as sticks coloured in magenta. E) Multiple sequence alignment of human JNK1, JNK2, and JNK3 MAPK domains that contain the identified binding determinants (red, labels in F) and hydrophobic pockets (bold) for eEF1A2 binding. F) Theoretical model of human JNK1 (enveloped in a semi-transparent surface, PDB ID 1UKH) in complex with an N- and C-capped peptide derived from the ²⁴³DKPLRLPL²⁵⁰ stretch in eEF1A2 adopting the same backbone conformation as found in JNK-interacting protein-1 (JIP1). An AMP-PNP molecule (sticks) and two Mg²⁺ ions (green spheres) bound at the ATP-binding site (taken from PDB ID 1JNK after best-fit superposition) are shown for reference. The enlarged area on the right shows the anchoring residues as sticks and the hydrogen bonds as dashed yellow lines. The two aquamarine spheres represent bridging water molecules found in the crystal structures.

Figure 7. Proposed mode of interaction between F-actin and the C-terminal region of eEF1A2. A) The molecular electrostatic potential calculated for two contiguous actin monomers in two strands making up a short fibre is colour-coded (from -5.0 kT/e , red, to 5.0 kT/e , blue) and displayed on the van de Waals surface to show the favourable orientation of the dipole moment of the C-terminal α -helix of eEF1A2 (orange) and the electrostatic complementarity with the charged amino group of Lys453 and remaining lysine residues (side chains as sticks). B) Detail of the modelled complex formed between the C-terminal α -helix (brown) of eEF1A2 and a reduced F-actin representation (two short intertwined filaments, blue). Seven snapshots from the 250-300 ns post-equilibration period of the MD simulation are displayed to show the conservation of the α -helical structure and the convergent arrangement of the C-terminus, which includes the carboxymethyl ester, around the actin protomer-protomer interface. Note how the side chains of Lys453 and Gln456 (sticks), in the mid-helix region, project into the cavity where the C-terminal carboxylate of actin Phe375 (sticks) is located. C) Overlaid snapshots of full-length eEF1A2 dimer (grey surface) taken every 5 ns from the last 50-100 ns of the MD simulation in solution showing the relative conformational freedom of both C-termini (orange and brown cartoons), which conserve their α -helical structure only transiently and populate different regions of phase space. D) Theoretical model showing two separate actin fibres (each filament in a shade of blue; PDB ID: 3G37) crosslinked by two eEF1A2 dimers (with monomers coloured in red and cream), each bound to a different actin filament over one half-turn of the F-actin helix. Note the axial periodicity of actin monomers and the peripheral and winding distribution around the actin fibres of additional eEF1A2 C-terminal α -helices from other dimers (not shown for simplicity) that would stabilize the observed bundles.

To test this hypothesis, we model-built the sequence encompassing residues 445 to 463 of eEF1A2 as an α -helix and structurally aligned it onto a superimposed set of similar β -actin-bound α -helices from an assortment of ABP trying to find the ideal register. We found an optimal alignment when the protonated amino group in the extended side chain of Lys453 donates direct hydrogen bonds to the C-terminal carboxylate of actin Phe375 (Figure 7A). The eEF1A2 helix remained

structured during 250 ns of the ensuing MD simulations (Figure 7B) whereas the last few amino acids sampled conformational space over time until they converged into a solution that placed the amino group of the terminal Lys463 close to the carboxylate of Asp57' from the vicinal actin protomer.

We also explored the dynamic behavior of the full-length eEF1A2 dimer in solution when both C-termini (residues

Figure 8. Theoretical model of the (eEF1A2)₂:(PI4KB)₄ complex. A) Optimized structure-based sequence alignment of select stretches of domain I of eEF1A2 chain B (orange) onto their counterparts in human Ras-related protein Rab-11A (green) in complex with PI4KB (PDB ID: 4D0L). B) Overall macromolecular mimicry between domain I of eEF1A2 and Rab-11A that is optimal for the G nucleotide-binding site and excludes (i) the insertion corresponding to α -helix H2 in eEF1A2 and (ii) the distinct folding of the stretch giving rise to H3 in eEF1A2. C) Model of a putative membrane-anchored eEF1A2 dimer (red and orange) in complex with two PI4KB dimers (green). D) Best-fit superposition of PI4KB-bound chains A (red) and B (orange) from eEF1A2. *Circled areas:* Note the alternate and mutually exclusive locations of the side chains of Ser53 and Phe54, which are proposed to be involved in entry and exit of the guanine nucleotide, as well as the alternate conformations of the loop encompassing Phe123, a residue found in between Glu122 and Glu124, two amino acids whose replacement by lysine due to spontaneous mutations is related to human genetic disorders. The GDP molecule has been replaced with the Mg²⁺-bound 5'-guanosine-diphosphate-monothiophosphate (gray sticks) found in the PI4KB:Rab11:FIP3 complex (PDB ID: 4D0M) that is displayed in A). Key residues in eEF1A2 and PI4KB are displayed as sticks and only a few are labeled for enhanced clarity.

445–463) are initially structured as α -helices necessarily detached from domain II of each monomer (Figure 7C). Our MD simulation results support the propensity of both C-termini to remain structured as α -helices, at least transiently, and additionally show that they can become IDRs and change their orientation relative to domains II and III, as observed in the crystal structures (SI Figure S5).

Finally, we carried out multiple best-fit rmsd superpositions of this region onto the C-terminal helices docked into F-actin from rabbit skeletal muscle to envision how an eEF1A2 dimer inter-

acting with two actin subunits from two separate fibers can promote and/or stabilize actin bundles. Since each fiber is made up of two right-handed intertwined strands of F-actin and a whole helical repeat contains 13 actin monomers,^[66] this is the expected axial periodicity for eEF1A2 dimers crosslinking two parallel actin fibers because the first binding cleft of each repeat is found in an identical relative orientation (Figure 7D). The winding distribution of additional clefts in F-actin allows multiple C-terminal α -helices from other eEF1A1/2 dimers to bind and thus stabilize the observed bundles.

Figure 9. Loop stabilization by Ser53 phosphorylation. A) Overlay of representative snapshots from the MD simulations showing the direct hydrogen bonds between the GDP ribosyl hydroxyls and a phosphorylated Ser53 (pSer53). B) Overlay of representative snapshots from the same MD simulations showing the alternative loop arrangement at another interface due to the swapping of positions between pSer53 and Phe54.

Interaction of eEF1A2 with PI4KB

A direct physical and functional interaction between eEF1A2 and PI4KB has been established, with a calculated stoichiometry of 1:1, although up to a 2-fold molar excess of recombinant eEF1A2 was required to maximally increase *in vitro* PI4KB activity.^[11] Because PI4KB plays an essential role in maintaining the structural integrity of the GC by virtue of a demonstrated direct interaction with the GTP-bound form of the small GTPase Rab11 and crystal structures of the PI4KB:Rab11 complex in the absence and presence of the Rab11 effector protein FIP3 are publicly available,^[67] we wondered whether it should be possible to build a model of the eEF1A2:PI4KB complex that could account for the known experimental findings. This was indeed achieved when domain I of eEF1A2 was structurally superimposed onto Rab11 and a feasible binding interface over the nucleotide-binding pocket was revealed (Figure 8). The resulting membrane-attached complex (Figure 8C) has a stoichiometry of 2:4 and shows how an eEF1A2 dimer that is post-translationally modified through covalent amide bond formation between the carboxylate of Glu301 and PE can provide a suitable interface for binding (and thereafter activate) two PI4KB dimers. In fact, this structural model nicely accounts for the finding that both full-length eEF1A2 and a $\Delta^{321-464}$ variant promote PI4KB-mediated PIP₂ accumulation at the PM and production of filopodia whereas eEF1A2 Δ^{1-320} and eEF1A2 $\Delta^{163-464}$ do not.^[11] Furthermore, the

location of Ser53 in our model complex allows us to envisage a much stronger interaction when this residue is phosphorylated (Figure 9) by mammalian PKC β 1,^[28] a Ca²⁺-dependent Ser/Thr-kinase, and provides a rationale to the observation that a Ca²⁺-dependent phosphorylation in eEF1A is necessary for PI4KB activation.^[68]

Differences between eEF1A2 and eEF1A1

The reciprocal and temporally regulated patterns of expression of *EEF1A1* and *EEF1A2* in developing skeletal myocytes, neurons and other specialized cells, together with the fact that *EEF1A2* is overexpressed in certain tumors, pose several intriguing questions. First, which functions carried out by eEF1A2 are exclusive and why cannot they be performed by eEF1A1? Second, where in the sequence are these differences encoded and what is their dependence on particular PTMs? Among the six cysteines present in eEF1A1, the only one that is not conserved in eEF1A2 is found at position 234 (SI Figure S1). This solvent-accessible Cys234 is known to be essential for eEF1A1 oligomerization (through disulfide bond formation with Cys31) under oxidative stress^[69] and also a target site (together with other proteins localized within the ER/Golgi complex) of S-nitrosylation in human cells;^[70] this is clearly not possible for Thr234 in eEF1A2. In contrast, Cys411 –spatially close to the mutated p.(P333L) position in children suffering from neurodevelopmental disorders– is known to be glutathionylated in both eEF1A1 and eEF1A2,^[71] and a protein described as eEF1A1 has been reported to form both intra- and intermolecular disulfide bonds in a mammalian neuronal cell line (HT22) following exposure to various oxidative insults.^[72] On the other hand, intense H₂O₂-induced thiol oxidation has been observed in yeast eEF1A1^[73] and a mixed disulfide between eEF1A1 and thioredoxin has been described in *D. discoideum*.^[74]

Another interesting albeit subtle difference, in the light of our models, is that the methylcarboxylated C-terminus of eEF1A1, which lacks the penultimate glycine residue present in eEF1A2 (SI Figure S1), is likely to make distinct subsite contacts which could account for the finding that the resulting actin bundles are macroscopically different.^[63]

Concluding remarks and outlook

The proteins encoded in the two cell type-specific paralogs *EEF1A1* and *EEF1A2* undertake the common, best-known task of delivering aa-tRNA to the ribosome for translation, but important differences in other functions have continually emerged over the years. Thus, eEF1A1 supports viral RNA replication in infected cells^[14, 16] whereas eEF1A2 promotes not only normal neuron and myocyte development but also malignancy and invasiveness in several tumors. Extant experimental evidence indicates that some of the moonlighting functions of these two proteins are performed by both free and membrane-anchored dimers. The present results provide structural insight into these findings by revealing that (i) the *EEF1A2* mutations responsible for neurological disabilities in children^[2] are located at dimerization hotspots and/or affect amino acids present at crucial locations involved in the binding of other macromolecular partners, e.g. PI4KB, and (ii) PE attachment to Glu301 and Glu374 establishes well-defined geometries for insertion into lipid monolayers

that, to the best of our knowledge, have not been previously described. Inspiringly, this little known biochemical reaction –despite being evolutionarily conserved between *Trypanosoma brucei* and *Homo sapiens*– is reminiscent of the reversible conjugation to PE that takes place on the C-terminal glycine (upon arginine removal) of microtubule-associated protein light chain 3 (LC3) –or Atg8 in yeast– and is necessary for stable association of this essential autophagy protein with the autophagosome membrane.^[75] In turn, the phagophore, a crescent-shaped double-membrane structure (aka the isolation membrane) that sequesters the cargo before forming the autophagosome vesicle is akin to the DMV that is generated during (+)RNA replication. We therefore think that formal –yet largely unaddressed and worth of study– similarities exist between LC3 and eEF1A2 in terms of PE-dependent membrane shaping and residence, as well as recruitment of specific proteins to specialized cellular locations. In this respect, it is striking that both eEF1A1 and eEF1A2 appear as high-confidence candidate interactors with Atg proteins in the Human Autophagy Interaction Network (AIN).

Considering that eEF1A has been found anchored at the ER membrane^[76] and localized close to nuclear and plasma membranes,^[77] it is tempting to speculate that the presence of PE-bound eEF1A would promote insertion of the lipid chain into particular membrane domains, thus facilitating interactions with other membrane components and the underlying cytoskeleton.^[55] We note that this form of membrane attachment for an eEF1A dimer would place both N- and C-termini pointing to the phospholipid/phosphoinositide heads (Figure 4) even though the C-terminal helices could still engage in alternate binding to other proteins, e.g. actin, upon small torsional changes in the stretch connecting it to the β -barrel of domain III (Figure 7B). Our modelling and simulation of the full-length protein facilitates understanding of several “non-canonical” eEF1A functions that are known to take place close to the PM, e.g. (i) co-localization with F-actin in lamella and filopodia,^[78] (ii) interaction of the PRM with SH3 domain-containing proteins including the adaptor SORBS2, which is involved in the assembly of signaling complexes;^[40a] (iii) binding of phosphotyrosine-containing sites of both eEF1A1 and eEF1A2 to SH2 domains of several membrane-anchored signaling molecules, such as Crk, Fgr, Fyn, Grb2, RasGAP, Shc and Shp2;^[40b] and most importantly in the current context, (iv) activation by eEF1A2 of PI4KB, an enzyme that supplies the essential PI4P for determining and maintaining the identity of several cell organelles despite the extensive membrane flux.^[79]

The mutually exclusive presence of eEF1A1 and eEF1A2 in most normal mammalian cells is tissue- and development-specific for reasons that are not well understood. From our results above, we postulate that the selective expression of *EEF1A2* in normal skeletal muscle, cardiomyocytes and neurons is largely unrelated to protein synthesis and is a direct consequence of the key roles played by this protein in specific cellular contexts, primarily close to membranes and membrane contact sites (MCS).^[80] We propose that the genetically programmed developmental switch from *EEF1A1* to *EEF1A2* expression in several types of excitable and secretory cells is related to the fact that they possess distinct MCS and intracellular Ca^{2+} regulation mechanisms that set them apart from cells in other tissues. Cells that have intrinsic electrical excitability utilize changes in membrane potential to communicate with neighboring cells and

initiate signaling cascades. Excitable cells like neurons and myocytes have evolved highly specialized subcellular architectures, such as SR/ER-PM MCS, to translate electrical signals into cellular events. These MCS are positioned by specific membrane-membrane tethering proteins and contain a manifold of other macromolecules that organize information transfer across the junctional space.^[81] In fact, molecular details of MCS were first obtained from striated muscle cells, where they were shown to play essential roles in the regulation of excitation-contraction coupling and in the control of lipid-dependent ion channels.^[80] The high degree of interconnection between the mitochondrial network and both PM and SR invaginations in these cells argues for specific roles of eEF1A2 in membrane trafficking and protein recruitment that are surely worth further studies.

In line with this reasoning, the specific localization and functions of eEF1A2 in normal acini and lumps of invasive cancer cells can be related to the existence of this protein as membrane-anchored dimers and tetramers, a possibility that is supported by the present results and numerous, albeit scattered, experimental findings. Importantly, the close relationship between membrane-bound eEF1A2 and PI4KB in normal and cancer cells^[12], together with the involvement of eEF1A2 in promoting Cdc42-dependent filopodia and lamellipodia formation,^[11] strongly support a unique role of this protein in the GC because PI4P is a key lipid for the identities of the Golgi compartment and the TGN, in addition to being a precursor of (i) membrane-forming and protein-recruiting PIP_2 –and therefore the second messenger IP_3 –, and (ii) the Akt/mTOR signaling pathway activator PIP_3 .^[7] In this respect, it is important to note that the presence of PIP_2 at neuronal synapses is known to be essential for coupling exo- and endocytosis, two distinct processes that place different demands on the biochemical machinery involved in either fusing a small, highly curved synaptic vesicle with the flat PM or reforming a vesicle, respectively.^[82]

“Non-canonical” roles for eEF1A1 and eEF1A2 should come as no surprise because the comparatively less evolved prokaryotic EF1A (aka EF-Tu) has long been known to be able to execute diverse functions, including some on the extracellular surface of both prokaryotic and eukaryotic cells, where it interacts with membrane receptors and the extracellular matrix of plant and animal cells.^[18] Furthermore, the strict conservation of the PTM-free interfacial residues in eEF1A1 and eEF1A2 (SI Figure S1) attests to the feasibility of homo- and heterodimer formation, both in the cytoplasm and at the PM.^[83] Consequently, these dimers add an additional layer of complexity in the molecular behavior of these two “sister proteins” as they could perform distinct functions and be differentially regulated in separate subcellular locations depending on particular subsets of PTMs, as suggested for the monomers themselves.^[84]

Deciphering how different kinds of PTMs modulate the conformation and spatial arrangement of the three structural domains of eEF1A1/2 is crucial to understand the numerous functions of these two moonlighting proteins –simply “eEF1A” in many bibliographic sources– and the network of molecular interactions they are involved in. The positioning of domains II and III in relation to domain I clearly emerges as a crucial balancing factor to differentiate between two of the major independent functions of eEF1A, namely, translation elongation and cytoskeletal organi-

zation. Domain I in eEF1A2 is a good example of a folded domain characterized as an ideal effector, that is, a GTPase module that participates in a well-documented binary on/off switch. Arg240, in the SLiM connecting domain I to domain II, is involved in a strong ionic interaction with Asp110 in the GTP conformation^[41c] but its exposure to the solvent, as in the dimer presented and discussed here, would be critical for recognition and binding to the SH3 domains of PLC γ 1 and other adapters and effectors. In this respect, the recent delineation of Nck1- and Nck2-specific signaling networks in human embryonic kidney 293T cells^[85]—which are not expected to express *EEF1A2*—has revealed that the Nck2 network is enriched for proteins related to the actin cytoskeleton and eEF1A1 was identified as one of them. Moreover, the adapter protein intersectin-1 acts as a GEF for Cdc42, a PM-associated small GTPase involved in eEF1A2-mediated filopodia formation and maintenance.^[11]

Because eEF1A2 appears to sustain the progression/aggressiveness of several cancers, the simultaneous quantification of eEF1A1 and eEF1A2 proteins in cancer cells is receiving increasing attention as they are presently considered potential therapeutic targets and/or prognostic markers. Future investigations will need to consider that, given the large number of PTM sites in eEF1A1/2,^[84] the potential molecular diversity of these two nuanced proteins is likely to be not only huge but also spatiotemporally regulated.

Finally, eEF1A2 is considered to be the pharmacological target of plitidepsin (Aplidin®),^[86] an anticancer cyclic depsipeptide approved by Australia's Therapeutic Goods Administration in 2018 for the treatment of relapsed or refractory MM, in combination with dexamethasone,^[87] and currently undergoing clinical trials as a potential medicine for treating patients infected with SARS-CoV2^[88] in view of its extremely high antiviral potency *in vitro*. Plitidepsin binds tightly to eEF1A2 within a hydrophobic pocket located between domains I and III, thereby stabilizing the monomeric GTP conformation and hampering eEF1A2 dimerization.^[41c] In addition to activating the JNK1 pathway in cancer cells, plitidepsin exerts a profound inhibitory effect on autophagy,^[86] which hints at a possible interference with the biochemical machinery responsible for attaching the PE moiety to eEF1A1/2 and/or LC3.^[75]

Further structural studies directed at characterizing eEF1A1/2 complexes with other binding partners (and decoding the particular subset of PTMs that are likely to be involved) will be necessary to clarify, in molecular terms, the plethora of events in which these two elongation factors participate beyond their (more) “canonical” (or better studied and accepted) role during mRNA translation at the ribosome. From studies in yeast, it is clear that eEF1A1 methylation is dispensable for translation-related activities but necessary for engagement in crucial protein–protein interactions that are required for local actin translation, nucleation of actin polymerization, and β -actin mRNA binding and targeting. Organization of the actin cytoskeleton, in turn, is regulated by Rho GTPases, including Rac1, which has been shown to be targeted to the PM of MDA-MB-231 breast cancer cells upon treatment with plitidepsin in a cholesterol-dependent fashion.^[89]

We hope that the present results will foster further attempts to improve our understanding, in structural terms, of the intricate

eEF1A1/2 interactomes and open up fruitful new avenues of research.

Experimental Section

eEF1A2 purification from rabbit skeletal muscle

eEF1A2 protein was purified from rabbit skeletal muscle as previously described.^[24] Briefly, ca. 100 g was minced in dry ice and further homogenized in 180 mL of 30 mM potassium phosphate buffer pH 7.5 with 1 mM MgCl₂, 15% (v/v) glycerol, 6 mM 2-mercaptoethanol and a protease inhibitor cocktail (100 μ M *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 μ g/mL leupeptin, 2 μ g/mL pepstatin and 5 μ g/mL aprotinin) using a Dounce tissue grinder. Debris was removed by centrifugation at 3,000 x g for 20 min followed by a further centrifugation of the supernatant at 12,000 x g during 45 min. The resulting supernatant was filtered through four layers of sterile gauze and finally centrifuged at 100,000 x g for 60 min. The final supernatant (called “S-100”) was loaded onto a DEAE FF 10/10 column equilibrated in the same buffer as above at a 4 mL/min flow rate and the breakthrough was collected and loaded onto a SP HP 16/10 column equilibrated in the same buffer at 2.5 mL/min. After discarding the breakthrough (2 column volumes) the column was washed with 6 volumes of 10 mM KCl followed by 5 volumes of a 10–350 mM KCl gradient. Finally, the column was washed with 4 volumes of 1 M KCl, always using the same buffer described above. Fractions were collected and their eEF1A2 content was analysed by Western blotting. Fractions containing the protein were pooled, concentrated in a Vivaspin centrifugal concentrator (MWCO 10 kDa) and finally loaded onto a Superdex 200 16/600 gel filtration column equilibrated in the same buffer as above. The chromatography was run at a constant flow rate of 1 mL/min and fractions eluting at a position corresponding to the MW of eEF1A2 (ca. 50 kDa) were collected, checked by Western blotting and pooled.

eEF1A2 crystallization

Prior to crystallization, the protein was extensively dialyzed against 25 mM MES pH 5.6, 5 mM MgCl₂, 2 mM DTT, 5% glycerol, concentrated to around 7 mg/mL and finally supplemented with 100 μ M GDP. eEF1A2 concentration was determined spectrophotometrically using ϵ =280 45,380 M⁻¹ cm⁻¹ as deduced from its amino acid sequence (UniProtKB entry Q71V39). A crystallization grid was carried out across precipitant concentrations of 2–2.9 M ammonium sulphate and pH range of 5–7 at 0.1 M and 0.5 intervals, respectively. The buffer was varied to its buffer range accordingly. Positive results were obtained using the hanging-drop vapor diffusion method, mixing 1 μ L protein solution with 1 μ L reservoir solution (50 mM Na citrate pH 5/MES pH 5.6, 2.2–2.6 M ammonium sulphate, 10 mM Mg acetate) and equilibrating the drops against 150 μ L crystallization solution in the reservoir. The crystals obtained (Supplementary Figure S12) were mounted in Hampton 0.2 mm cryoloops and flash cooled in liquid nitrogen for data collection.

X-ray data collection and structural determination

Single wavelength (λ = 0.97926 Å) diffraction data sets were collected at 100 K using a Pilatus 6M detector on beamline BL-13 (XALOC) of the ALBA Synchrotron (Barcelona, Spain). Collected data sets were processed using iMOSFLM^[90] and AIMLESS^[91] for scaling and merging the data from the CCP4 program suite,^[92] reserving 5% of the diffraction data for cross-validation. A small anisotropy issue in the diffraction data led us to fix the maximum resolution used for integration at 2.7 Å so as to obtain similar statistics along the three crystallographic axes. The structure was determined by molecular replacement (MOLREP)^[93] using eEF1A2 (PDB

ID: 4C0S)^[24] as the search model. The geometries of N- and C-capped post-translationally modified residues were optimized using a 6-31G* basis set and density functional theory, as implemented in program Gaussian 09 (rev. D.01); water effects were taken into account by means of the IEF-SCRF continuum solvent method.^[94] The optimized coordinates were converted to restraints in Crystallographic Information File (CIF) format^[95] using eLBOW^[96] to be used thereafter in structure refinement against the electron density maps using COOT^[97] v. 0.8.2 and Phenix^[98] v. 1.18rc-3794. Crystallographic parameters, data collection and relevant statistics are listed in Supplementary Table S1.

Bioinformatic tools.

The crystallographic eEF1A2 dimer was analysed with the VoroMQA^[34a] and PROtein binDing enerGY prediction (PRODIGY)^[36] web servers for model quality assessment and dimerization energy evaluation, respectively. Dali^[60] was used to perform the structure-based protein superpositions and multiple sequence alignments. The Eukaryotic Linear Motif (ELM) resource^[58] and the LMDIPred server^[59] were utilised as exploratory tools for motif predictions. The molecular graphics program PyMOL (v. 1.8, Schrödinger LLC) was employed for structure visualization, molecular editing and figure preparation.

Computational chemistry and simulation methods

Computation of the protonation state of titratable groups at pH 5.6 were carried out using the H++ 3.0 Web server.^[29] The updated Random Coordinate Descent loop closure algorithm (RCD+)^[31] was employed to enrich the sampling distribution toward near-native conformations of the most recalcitrant loops prior to crystallographic refinement. The interpolation between dimeric GDP-bound and monomeric GTP-bound conformations of eEF1A2 to illustrate feasible transition pathways between them (Movie S1) was carried out by the iMODS^[49] web server using the coordinates of chain B from the present structure and those generated for the eEF1A2 sequence from the data deposited in PDB entry 1SYW for eEF1A1.^[41b] The AMBER force field^[99] was used for progressive energy refinement of crystallographic eEF1A2 dimers and tetramers in explicit solvent and counterions (Movies S2 and S3), as well as the GTP-bound monomeric conformation (Movie S4), followed by molecular dynamics simulations according to the same protocol previously reported for eEF1A1.^[41c] The latter was completely unrestrained but in the former two cases, the C α atoms of all α -helices and β -strands were restrained to their crystallographically determined positions using a harmonic potential of 1 kcal·mol⁻¹·Å⁻² whereas all loop atoms were free to move. Atom-centred RESP charges for nonstandard protein residues were obtained by means of the *sqm* program^[100] and the AM1-BCC model.^[101] Consistent covalent and non-bonded parameters, by analogy or through interpolation, from those already present for protein atoms in the ff14SB parameter set^[102] in AMBER^[103] (<http://ambermd.org/>). Per-residue solvent-corrected binding energies for intermonomer eEF1A2 association were estimated by means of the MM-ISMSA method.^[35] For the actin models, the cryo-EM structure of rabbit muscle F-actin (PDB ID: 3G37)^[104] and different structurally aligned ABP templates (PDB IDs: 2D1K, 2A3Z, 3SJH and 1YVN) were used.

Mass spectrometry data acquisition

An overnight in-gel digestion of eEF1A2 protein was carried out under reducing and alkylating conditions (10 mM dithiothreitol, 55 mM iodoacetamide) with 12.5 ng/μL of bovine trypsin (Roche, 20 mM ammonium bicarbonate pH 8.5, 37 °C). The resulting peptides were freeze-dried in SpeedVac and resuspended in buffer A (2% acetonitrile, 0.1% formic acid). The analysis was performed using an EASY-nLC 1000 System

coupled to the Q-Exactive HF mass spectrometer (1.8 Kv ion spray voltage and 250 °C transfer temperature) through the Nano-Easy spray source. Separation was achieved by means of an Acclaim PepMap 100 Trapping column (Thermo Scientific) and an NTCC analytical column (100 Å; 3 μm; 150x0.75 mm; Nikkoy Technos Co. Ltd.). A 5-35% gradient of buffer B (100% acetonitrile, 0.1% formic acid) in buffer A was performed over 80 min, followed by a 30-45% gradient over 10 min at a constant flow rate of 250 nL/min. Both full-MS data-dependent acquisition (300–2800 Da m/z mass range, 100 ms scan time and a normalized collision energy of 27%) and semitarget parallel reaction monitoring (isolation of target peptide with a 2 m/z window from the inclusion list according to different charged states of the PTMs) were employed in positive mode using Xcalibur 4.0 software. Injection times of 200 ms for MS/MS were used at a mass resolution of 45000 Da.

Mass spectrometry data analysis

The two acquired MS/MS datasets were analyzed with both Proteome Discoverer software v.2.2 (Thermo Scientific), with search engines MAS-COT 2.6 (MatrixScience, London, UK) and Sequest HT –which was used to identify the peptides against a home-made database with a FASTA sequence (Q71V39) and contaminant database of 247 sequences– and Peaks Studio v. 8.5 software (Bioinformatics solution Inc., trial version),^[105] to identify peptide/protein *de novo* sequencing and PTM analysis. Protein Discoverer searches were performed assuming trypsin digestion with up to 2 missed cleavage sites, a fragment ion mass tolerance of 0.02 Da and an ion precursor tolerance of 10 ppm. Carbamidomethylcysteine was specified as a fixed modification and acetylation (K), methylation (E, D), glycerylphosphoethanolamination (E), phosphorylation (S, T, Y), oxidation (M) and acetylation or methylation-loss-acetylation at the N-terminus were set as variable modifications. The ptmRS node was used to calculate the probability of each modification studied and the threshold for acceptance was set at 75%. The PTM identification acceptance criteria were a false discovery rate (FDR) <1% and at least one unique peptide was identified with high confidence (CI>95%, p<0.05). Only modified peptides with a site probability ≥75 % were reported.

The Peaks Studio database module performed the search against a database equivalent to that of Protein Discoverer and used the same fixed and variable modifications. The Decoy-Fusion approach was selected for FDR estimation and the tools Peaks-*de novo* and Peaks-PTM were used to extract peptide sequence from MS/MS spectra without any database and identified peptide sequences with unspecified modifications. The acceptance criteria for confidence peptides in database searches were (peptide-to-spectrum match)-FDR <0.5; peptide -10logP ≥23.7; protein -10logP ≥20 with at least one unique peptide. *De novo* sequences or tags were filtered by an average local confidence (ALC) score ≥80 and peptide modified by AScore ≥20, *i.e.* the probability of a specific modification occurrence at the reported position compared to other possible positions is equal to a p-value of 0.01.

Abbreviations

ABP, actin-binding proteins; ARTD, ADP-ribosyltransferase diphtheria toxin-like family; BAR, Bin/Amphiphysin/Rvs; eEF1A, elongation factor 1A; DMV, double-membrane vesicles; EPG, ethanolamine phosphoglycerol; ER, sarcoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, Golgi complex; GEF, guanine nucleotide exchange factor; GPI, glycosylphosphatidylinositol; JNK, c-Jun amino-terminal kinase; LC3, microtubule-associated protein light chain 3; MAPK, mitogen-activated protein kinase; MCS,

membrane contact sites; MEP, molecular electrostatic potential; MM, multiple myeloma; OSBP, oxysterol-binding protein; PE, phosphatidylethanolamine; PI4KB, phosphatidylinositol-4-kinase III β ; PLC γ 1, phospholipase C γ 1; PRM, Pro-rich motif; PTM, post-translational modification; RdRp, RNA-dependent RNA polymerase; rmsd, root-mean-squared deviation; RO, replication organelles; SH2, Src Homology 2; SH3, Src Homology 3; SLiMs, short linear motifs; SR, sarcoplasmic reticulum; TBSV, Tomato bushy stunt virus; TGN, trans-Golgi network; VRC, viral replication complexes; WASP, Wiskott-Aldrich syndrome protein; WH2, WASP-homology domain 2.

Accession Number: The atomic coordinates and structure factors for the eEF1A2:GDP complex have been deposited with the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) with entry code 6RA9.

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Entry for the Table of Contents**FULL PAPER**

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**Structural cues for
understanding
eEF1A2 moonlighting**

Text for Table of Contents

Beyond translation elongation: The X-ray crystal structure of the G protein eEF1A2 purified from rabbit skeletal muscle, together with additional bioinformatic and modeling work, help understand the roles of this ancient, multifaceted, and articulated protein at the crossroads of autophagy, oncogenesis, and viral replication.