

# Binding of Elongation Factor eEF1A2 to Phosphatidylinositol 4-Kinase $\beta$ Stimulates Lipid Kinase Activity and Phosphatidylinositol 4-Phosphate Generation\*

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Eukaryotic protein translation elongation factor 1  $\alpha$  2 (eEF1A2) is an oncogene that transforms mammalian cell lines and increases their tumorigenicity in nude mice. Increased expression of eEF1A2 occurs during the development of breast, ovarian, and lung cancer. Here, we report that eEF1A2 directly binds to and activates phosphatidylinositol 4-kinase III  $\beta$  (PI4KIII $\beta$ ), an enzyme that converts phosphatidylinositol to phosphatidylinositol 4-phosphate. Purified recombinant eEF1A2 increases PI4KIII $\beta$  lipid kinase activity *in vitro*, and expression of eEF1A2 in rat and human cells is sufficient to increase overall cellular phosphatidylinositol 4-kinase activity and intracellular phosphatidylinositol 4-phosphate abundance. siRNA-mediated reduction in eEF1A2 expression concomitantly reduces phosphatidylinositol 4-kinase activity. This identifies a physical and functional relationship between eEF1A2 and PI4KIII $\beta$ .

eEF1A2<sup>2</sup> is one of two members of the eEF1A family of proteins (eEF1A1 and eEF1A2). During protein translation elongation, eEF1A proteins bind amino-acylated tRNA and facilitate their recruitment to the ribosome (1). Aside from their canonical role in protein translation, eEF1A proteins have other functions, including binding actin and inducing rearrangements of the actin and tubulin cytoskeleton (2, 3). The inactivation of the mouse eEF1A2 homolog, *Eef1a2*, leads to immunodeficiency and death by 30 days of age (4, 5).

Mammalian eEF1A2 mRNA can be detected only in normal mammalian heart, brain, and skeletal muscle tissues (6–8). However, high levels of eEF1A2 protein and mRNA are observed in a 30–60% fraction of ovarian, breast, and lung tumors (9–12). We have previously reported that eEF1A2 has

transforming properties; ectopic expression of wild type human eEF1A2 in mammalian cells enables anchorage-independent growth and enhances tumorigenicity in nude mice (9). Thus, eEF1A2 has an important role in promoting tumor development. However, the mechanism by which eEF1A2 promotes oncogenicity remains unclear.

It has previously been reported that an eEF1A-like protein purified from carrots, PIK-A49, binds and activates carrot phosphatidylinositol 4-kinase (PI4K) (13, 14). This suggests an important relationship between translation elongation and phosphatidylinositol generation. Phosphatidylinositols are negatively charged, membrane-bound phospholipids that serve as regulators of multiple signaling pathways (15–18). Phosphatidylinositols are composed of an inositol ring covalently bound to a lipid phosphatidic acid backbone by a phosphodiester bond at the inositol D1 carbon. Inositol phosphorylation occurs at the D3, D4, or D5 carbons. Specific kinase families are responsible for phosphorylation at each of these sites. Phosphatidylinositol 3-kinases, PI4K, and phosphatidylinositol 5-kinases phosphorylate the D3, D4, and D5 inositol carbons, respectively (15–17).

PIK-A49 showed *in vitro* translation elongation factor activity and an ability to activate *in vitro* PI4K lipid kinase activity (13, 14). However, PIK-A49 does not have complete amino acid sequence identity with wild-type carrot eEF1A and has yet to be cloned as a full-length cDNA. It is therefore unclear whether PIK-A49 is a *bona fide* eEF1A protein; nor is it known whether wild-type carrot eEF1A or eEF1A proteins from nonplant species participate in PI4K activation. In addition, there are three identified subfamilies of PI4K proteins, phosphatidylinositol 4-kinase III  $\alpha$ , phosphatidylinositol 4-kinase III  $\beta$  (PI4KIII $\beta$ ), and PI4KII (19, 20), and it is unclear which PI4K isoform(s) is activated by PIK-A49 or other eEF1A proteins. Moreover, *in vitro* PI4K activation by PIK-A49/eEF1A has unknown physiological significance.

Here we report that human eEF1A2 can directly bind and activate PI4KIII $\beta$ . Ectopic expression of eEF1A2 in rodent and human cells increases overall PI4K activity and cellular phosphatidylinositol 4-phosphate (PI4P) generation. Furthermore, eEF1A2 ablation reduces endogenous PI4K activity. This suggests that eEF1A2 is a physiological regulator of PI4KIII $\beta$ .

## EXPERIMENTAL PROCEDURES

**Cell Lines**—MCF7, BT549, and Rat2 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown according to their instructions.

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<sup>2</sup> The abbreviations used are: eEF1A2, eukaryotic elongation factor 1  $\alpha$  2; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; siRNA, short interfering RNA; GFP, green fluorescent protein; C/EBP $\beta$ , CAAT/enhancer-binding protein  $\beta$ ; GST, glutathione S-transferase; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PI(3,4,5)P<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate.

**Adenoviral Vectors**—eEF1A2 was subcloned into the pShuttle-IRES plasmid (EcoRV/XhoI) with a FLAG epitope tag. eEF1A2 and GFP virus were manufactured by the Adenoviral Core Facility of the University of Ottawa. For viral transduction, BT549 and Rat2 cells were infected with Ad-eEF1A2 or Ad-GFP at a multiplicity of infection (MOI) of 200 (BT549) or 500 (Rat2) in complete media. Cells were incubated with virus for a minimum of 24 h.

**Antibodies**—Antibodies used for experiments were as follows: human PI4KIII $\beta$  (Upstate Cell Signaling Solutions, Charlottesville, VA),  $\beta$ -actin (Sigma), horseradish peroxidase-conjugated goat anti-mouse IgG (Upstate Cell Signaling Solutions), and horseradish peroxidase-conjugated anti-rabbit IgG, (Cell Signaling Technology, Danvers, MA). The generation of the rabbit polyclonal eEF1A2 antibody and its validation in Western blotting, immunoprecipitation, and immunohistochemistry is described elsewhere (10).

**GST Fusion Proteins**—eEF1A2 cDNA was cloned into the EcoRI/NotI site of pGEX-4T2 (Amersham Biosciences). GST-eEF1A2 was transformed into *Escherichia coli* BL21DE3 and grown in Luria-Bertani media with 100  $\mu$ g/ml ampicillin to  $A_{600} \sim 0.7$ . 0.5 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside was added for 2 h at 25  $^{\circ}$ C. Bacteria were lysed in 25 mM HEPES, pH 7.9, 100 mM KCl, 2 mM EDTA, 20% glycerol, 2 mM dithiothreitol, and 1 $\times$  protease inhibitor mixture (Roche Applied Science). Glutathione-Sepharose 4B beads (Amersham Biosciences) were equilibrated in lysis buffer and mixed with sonicated suspensions. PI4KIII $\beta$  in pGEX-6P-3 was a gift of T. Balla (21, 22). The GST-PI4KIII $\beta$  fusion protein was purified as described (22). GST-C/EBP $\beta$  was a kind gift from N. Wipbergeron (University of Ottawa). To remove the GST moiety, 100  $\mu$ g of GST-eEF1A2 was incubated overnight with 1 unit of thrombin (Amersham Biosciences) in 1 $\times$  PBS at room temperature. PI4KIII $\beta$  was generated by cleaving 100  $\mu$ g of GST-PI4KIII $\beta$  with 1 unit of PreScission Protease (Amersham Biosciences) in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, at 4  $^{\circ}$ C.

**Lipid Kinase Assay**—10  $\mu$ l of recombinant protein in PBS, at concentrations indicated in the figure legends, was added to 35  $\mu$ l of kinase buffer (1 mM EDTA, 30 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, and 0.2% Triton X-100), 3 mM PI (or as indicated in the figure legend), and 5  $\mu$ l of 10 mM ATP containing 10  $\mu$ Ci of [<sup>32</sup>P]ATP, and incubated for 60 min. The reaction was stopped by the addition of 60  $\mu$ l of 1 N HCl. Phospholipids were extracted by adding 160  $\mu$ l of CHCl<sub>3</sub>/MeOH (1:1, v/v). After a brief vortex, samples were centrifuged for 10 min at 10,000  $\times$  g. Aliquots of the organic phase (10–20  $\mu$ l) were spotted onto TLC plates (Sigma) and placed in a pre-equilibrated tank containing CHCl<sub>3</sub>/acetone/MeOH/HOAc/water (46:17:15:14:8, v/v/v/v/v). Prior to use, TLC plates were precoated with 1% potassium oxalate, 3 mM EDTA in methanol/water (2:3, v/v) for 1 h and allowed to air dry overnight. Plates were activated by baking for 1 h at 110  $^{\circ}$ C. Phosphatidylinositol standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). PI4P spots were scraped and dissolved in 1–2 ml of water. Aliquots were then diluted in BetaMax scintillation fluid (ICN Biomedicals, Irvine, CA), and scintillation counts were taken using the Wallac 1414 liquid scintillation counter (Fisher). Values of  $K_m$ ,  $V_{max}$ , and  $K_{cat}$

were determined using the GraphPad Prism software (San Diego, CA). For assays involving cell lysates, recombinant eEF1A2 and the cell lysate were added in a total volume of 10  $\mu$ l.

**Cross-linking Studies**—Purified eEF1A2 and/or PI4KIII $\beta$  (without GST) were incubated in PBS and cross-linked with 30 mM dimethyl pimelimidate (Pierce) in 0.2 M ethanolamine, pH 8.0, at room temperature. Reactions were stopped by the addition of glacial acetic acid at a 1:4 (v/v) ratio to the sample. Proteins were concentrated to a final volume of 50  $\mu$ l with Microcon centrifugal filter devices (Millipore, Billerica, MA), solubilized using 5% (w/v) sucrose in water, and electrophoresed in a continuous, nondenaturing 5% (29:1 acrylamide/bisacrylamide) polyacrylamide gel using 100 mM sodium phosphate (pH 7.0) running buffer as previously described (23).

**Cell Lysis and Co-immunoprecipitation**—For co-immunoprecipitation, cells were grown to 80–95% confluence in 100-mm cell culture plates. Cells were lysed by sonication on ice in detergent-free buffer (137 mM NaCl, 8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.5), 2.7 mM KCl, 2.5 mM EDTA, 1% aprotinin, 1 mg/ml leupeptin, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 10,000  $\times$  g for 20 min to remove membranes. Supernatants were collected, and protein levels were quantified using a Bradford assay (Bio-Rad) according to the manufacturer's instructions. 100  $\mu$ g of total protein was precleared with protein G-Sepharose (Amersham Biosciences) for 1 h at 4  $^{\circ}$ C. Following this, 2–4  $\mu$ g of PI4KIII $\beta$ , FLAG, or eEF1A2 antibody coupled to beads were added and incubated overnight at 4  $^{\circ}$ C. Beads were washed three times in PBS, centrifuged, and boiled for 5 min in sample buffer, and the supernatant was subjected to SDS-PAGE. The covalent coupling of the eEF1A2 antibody to protein A-agarose beads was performed using a previously described protocol (24). Western blot detection was according to the manufacturer's instructions or as described for the eEF1A2 antibody (10).

For Western blotting, cells were lysed in radioimmune precipitation buffer (50 mM Tris-Cl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, pH 7.0, 150 mM NaCl, 1% aprotinin, 1 mg/ml leupeptin, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml pepstatin in ethanol, and 1 mM phenylmethylsulfonyl fluoride in Me<sub>2</sub>SO).

**siRNA Transfections**—Sequences of the eEF1A2 siRNAs are 5'-UGGUCCUUUGUCAAUACCTc-3' (siRNA 1) and 5'-UCGAACUUCUCAUUGGUCctt-3' (siRNA 2). The negative control siRNA was purchased from Ambion (catalog number 4611; Austin, TX). siRNA transfections were performed using siPORT Lipid (Ambion) according to the manufacturer's instructions.

**Immunofluorescence**—To detect PI4P levels, Rat2 and BT549 cells were transfected with either eEF1A2-pcDNA3.1 (9) or GFP-pcDNA3.1 using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. 5 h post-transfection, transfection medium was removed, and complete medium was added. The next day, cells were fixed and permeabilized as above. Cells were then blocked with 10% goat serum, PBS for 30 min at 37  $^{\circ}$ C, and anti-PI4P IgM antibody was added (1:100 in PBS overnight; Echelon Biosciences Inc., Salt Lake City, UT). Goat anti-mouse IgM, (R)-phycoerythrin (Caltag Laboratories, Carlsbad, CA) was then added at 1:100 in PBS for 30 min.

## eEF1A2 Activates PI4KIII $\beta$

eEF1A2 was detected with a monoclonal anti-V5 antibody (1:500 in PBS, 1 h; Sigma) followed by an AlexaFluor 488 goat anti-mouse IgG (1:450 in PBS, 1 h; Invitrogen). Cell nuclei were stained with Hoescht 33258 (20  $\mu\text{g}/\text{ml}$ ; Sigma) for 10 min at room temperature. Slides were viewed with either a Leica DM-1L fluorescence microscope and deconvolved using Improvision 3.1 software (Richmond Hill, Canada) or an Olympus FluoView FV1000 laser-scanning confocal microscope. Fluorescence was quantified with the confocal microscope using Olympus software (FV1000, version 01.04a, Center Valley, PA).

### RESULTS

**eEF1A2 Increases PI4KIII $\beta$  Lipid Kinase Activity**—To determine whether eEF1A2 could activate PI4KIII $\beta$ , we purified recombinant GST-eEF1A2 and GST-PI4KIII $\beta$ . The proteins were isolated both with and without their GST moiety. A Coomassie-stained gel of the purified proteins is shown in Fig. 1A. The predicted molecular mass of full-length, wild-type eEF1A2 is  $\sim 54$  kDa, and that of PI4KIII $\beta$  is  $\sim 100$  kDa.

We first determined whether eEF1A2 could increase PI4K activity in cell lysates. As shown in Fig. 1B, the addition of GST-eEF1A2 to a cell lysate of BT549 breast carcinoma cells reproducibly doubled *in vitro* PI4P generation compared with the addition of GST alone or GST coupled to the C/EBP $\beta$  transcription factor. A representative TLC plate is also shown in the right panel of Fig. 1B.

Bacterially expressed PI4KIII $\beta$  has previously been reported to be active in *in vitro* lipid kinase assays (21, 22). As shown in Fig. 1C, our GST-PI4KIII $\beta$  was active *in vitro*, and its kinase activity was inhibitable by 0.2  $\mu\text{M}$  wortmannin. Wortmannin inhibition indicates that we are measuring the lipid kinase activity of a type III PI4K, the family of PI4Ks to which PI4KIII $\beta$  belongs. To investigate whether purified eEF1A2 could directly activate PI4KIII $\beta$ , we next added recombinant eEF1A2 (without GST) to purified PI4KIII $\beta$  (without GST). As shown in Fig. 1D, eEF1A2 increased PI4KIII $\beta$  lipid kinase activity in a dose-dependent manner. PI4KIII $\beta$  activity was increased  $\sim 2$ -fold. 100–200 nM eEF1A2 is required to maximally activate a 100 nM solution of PI4KIII $\beta$ . No enhancement of PI4KIII $\beta$  activity was observed in the presence of bovine serum albumin.

In order to further determine how eEF1A2 affected PI4KIII $\beta$  activity, we experimentally determined the PI4KIII $\beta$   $K_m$ ,  $V_{\text{max}}$ , and  $K_{\text{cat}}$  for phosphatidylinositol with and without eEF1A2. Bovine albumin served as the control. As shown in Fig. 1E, eEF1A2 (without GST) reproducibly increased the  $V_{\text{max}}$  of PI4KIII $\beta$  (without GST) from 0.62 to 1.11  $\mu\text{mol}/\text{min}/\text{mg}$ . This doubling of  $V_{\text{max}}$  was also mirrored in the increase of  $K_{\text{cat}}$  from 0.82 to 1.48  $\text{s}^{-1}$ . The PI4KIII $\beta$   $K_m$  was unchanged.

**eEF1A2 Binds PI4KIII $\beta$** —We next tested whether eEF1A2 could directly interact with PI4KIII $\beta$  in a cell-free system. As shown in Fig. 2A, purified PI4KIII $\beta$  (without GST) was immunoprecipitated by GST-eEF1A2, and eEF1A2 (without GST) was immunoprecipitated by GST-PI4KIII $\beta$ . Neither PI4KIII $\beta$  nor eEF1A2 was immunoprecipitated by GST alone. We next investigated the stoichiometry of eEF1A2/PI4KIII $\beta$  interaction. We incubated recombinant eEF1A2 and PI4KIII $\beta$  (both without GST) and chemically cross-linked the resulting complex.

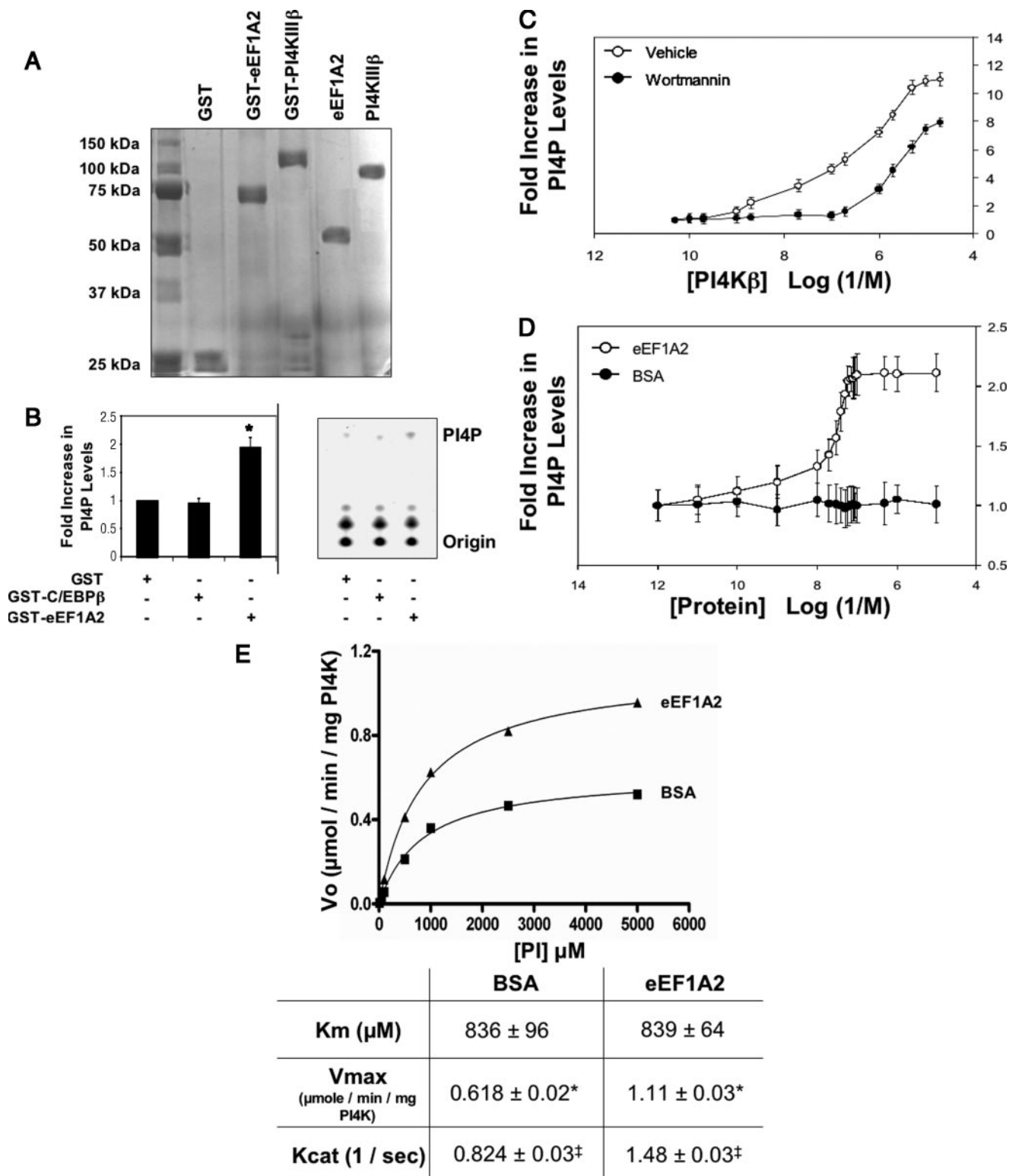
As seen in Fig. 2B, after 1 h of incubation, most of the added PI4KIII $\beta$  and eEF1A2 were found in a complex with an apparent molecular mass of 165 kDa. The formation of the complex was time-dependent, and a greater amount of complex was detected at 60 min of incubation relative to the 30 min time point. eEF1A2 has an apparent molecular mass of  $\sim 60$  kDa, and that of PI4KIII $\beta$  is  $\sim 100$  kDa. The  $\sim 165$ -kDa molecular mass of the complex indicates that eEF1A2 and PI4KIII $\beta$  associate with a 1:1 stoichiometry. Neither eEF1A2 nor PI4KIII $\beta$  formed detectable multimers on their own. We were unable to detect protein complexes with a molecular mass greater than 165 kDa, indicating that multimers containing more than one molecule each of eEF1A2 or PI4KIII $\beta$  do not form.

**eEF1A2 Interacts with PI4KIII $\beta$  in Mammalian Cell Lines**—To determine whether eEF1A2 and PI4KIII $\beta$  associate in cells, we first identified mammalian cells that express eEF1A2. In rodents and humans, eEF1A2 shows detectable mRNA and protein expression only in the tissues of the heart, brain, and skeletal muscle (6–8). We have also observed eEF1A2 expression in many breast cancer cell lines (10). As shown in Fig. 3A, we detected eEF1A2 protein expression in the MCF7 human breast carcinoma cell line but neither in BT549 breast carcinoma cells nor in the nontransformed Rat2 rat fibroblast cell line.

We then used co-immunoprecipitation to determine whether wild-type eEF1A2 and PI4KIII $\beta$  associate in MCF7 cells. We used detergent-free cell lysis for these experiments. As shown in Fig. 3B, eEF1A2 detectably immunoprecipitated with PI4KIII $\beta$ , and PI4KIII $\beta$  reciprocally immunoprecipitated with eEF1A2. Neither protein bound to the FLAG antibody control. Although immunoprecipitation is only semiquantitative, we estimate that  $\sim 10\%$  of endogenous PI4KIII $\beta$  associated with eEF1A2 under detergent-free lysis conditions. A similar ratio of eEF1A2 was associated with PI4KIII $\beta$ .

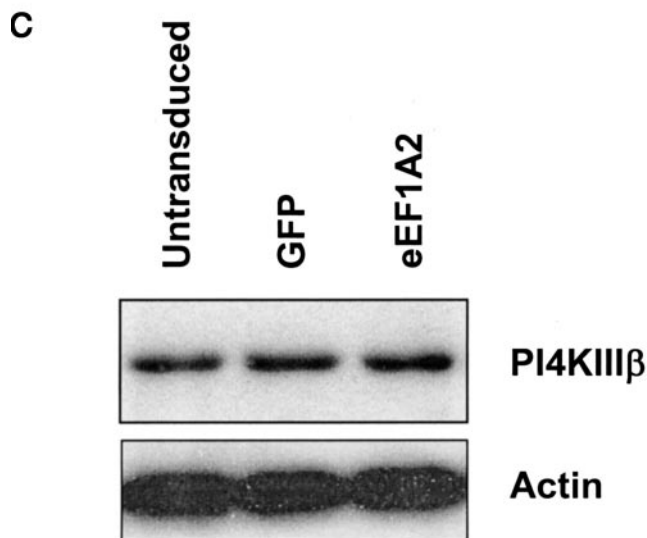
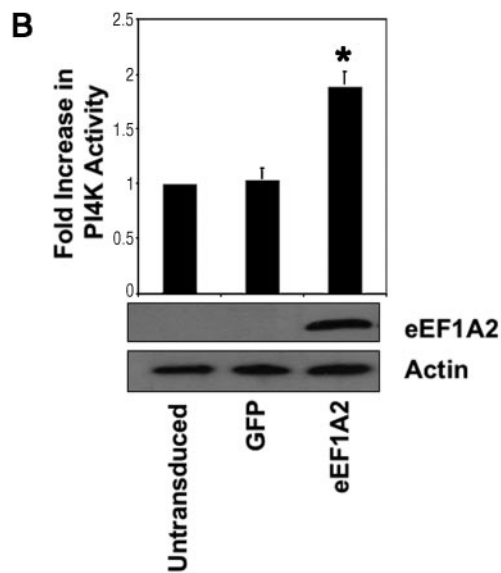
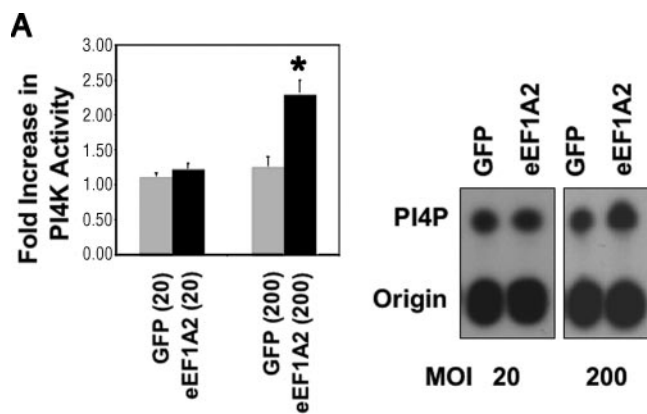
Both PI4KIII $\beta$  and eEF1A2 have been previously reported to be diffusively cytoplasmic, although PI4KIII $\beta$  has some concentration in the Golgi apparatus (9, 19, 20). We attempted to visualize eEF1A2 and PI4KIII $\beta$  co-localization via immunofluorescence confocal microscopy but were unable to detect significant co-localization between PI4KIII $\beta$  and eEF1A2 (not shown). Co-localization detection is complicated by the diffuse cytoplasmic staining pattern of the two proteins and the limited extent to which the two proteins co-precipitate. Nonetheless, the detectable amount of eEF1A2 that co-precipitates with *in vivo* PI4KIII $\beta$  indicates that PI4KIII $\beta$ -eEF1A2 complexes do exist in MCF7 cells.

**eEF1A2 Is Sufficient to Increase Cellular PI4K Activity**—To determine whether eEF1A2 expression was sufficient to increase PI4K activity in mammalian cells, we used an adenovirus to express eEF1A2 in BT549 and Rat2 cells. Neither cell line detectably expresses eEF1A2 without transduction (Fig. 3A). At a low MOI of 20 virus particles/cell, infection had little effect on PI4K activity; lipid phosphorylation was similar between eEF1A2-infected cells and those infected with GFP (Fig. 4A). However, increasing the MOI to 200 reproducibly doubled cellular PI4K activity in eEF1A2-infected cells relative to those infected with GFP (Fig. 4A). Similarly, infection of Rat2 cells with the eEF1A2 adenovirus doubled PI4K activity relative to

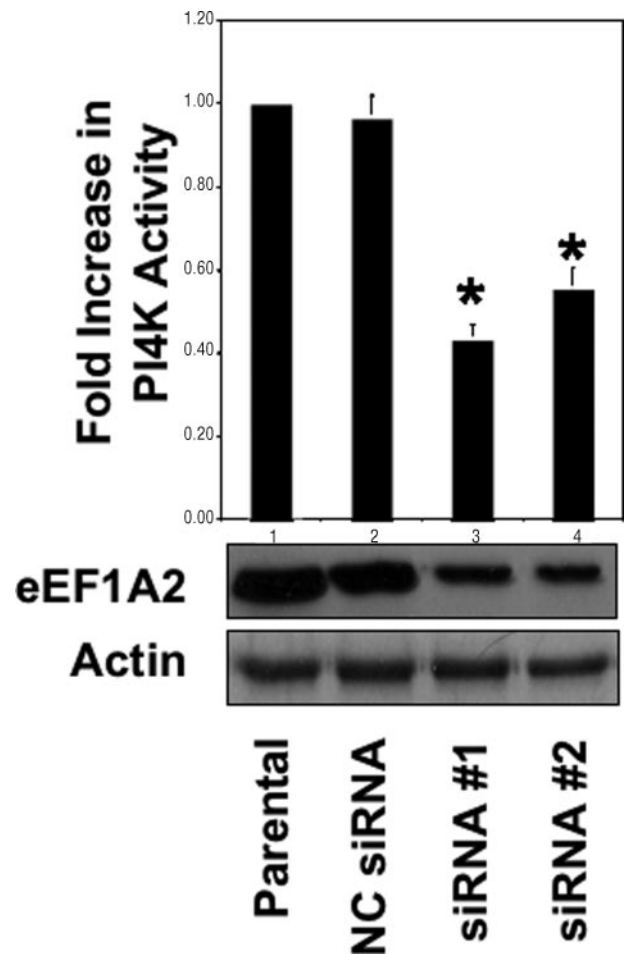


**FIGURE 1. eEF1A2 activates PI4KIII $\beta$  lipid kinase activity.** *A*, Coomassie stain of purified recombinant eEF1A2 and PI4KIII $\beta$  proteins with and without their GST moiety. 30  $\mu$ g of each sample were loaded on a 10% SDS-polyacrylamide gel. *B*, *left*, the addition of 10  $\mu$ M GST-eEF1A2 to a protein lysate from BT549 cells increases total PI4K lipid kinase activity relative to the addition of GST alone or GST-C/EBP $\beta$ . Activation is calculated relative to the GST alone control and is the mean and S.D. of three independent experiments with triplicate scintillation counts. Significant activation ( $p < 0.05$ , Student's *t* test) is indicated by an asterisk. *Right*, a representative TLC plate from one of the kinase assays. *C*, purified recombinant GST-PI4KIII $\beta$  protein has lipid kinase activity (open circles) that is inhibited by 0.2  $\mu$ M wortmannin (closed circles). -Fold increase is calculated relative to no protein control. The reaction contained 20 nM phosphatidylinositol. *D*, PI4KIII $\beta$  (GST-free) activity is increased by eEF1A2 (GST-free) addition (open circles) but not by bovine albumin (closed circles). Lipid kinase assays are the mean and S.D. of triplicate experiments with 3 mM phosphatidylinositol and 100 nM PI4KIII $\beta$  (GST-free). *E*, eEF1A2 increases the  $V_{max}$  of the reaction but not the  $K_m$ . *Top*, a representative kinase assay using 100 nM PI4KIII $\beta$  (GST-free) and either 2.5  $\mu$ M eEF1A2 (GST-free) or 2.5  $\mu$ M bovine albumin. *Bottom*, summary of PI4KIII $\beta$  kinetic parameters. Values are the means and S.D. from three independent experiments. eEF1A2 significantly increases the  $V_{max}$  and  $K_{cat}$  values (\* and ‡, respectively;  $p < 0.0005$ , unpaired *t* test with Welch correction) but not the  $K_m$ . BSA, bovine serum albumin.





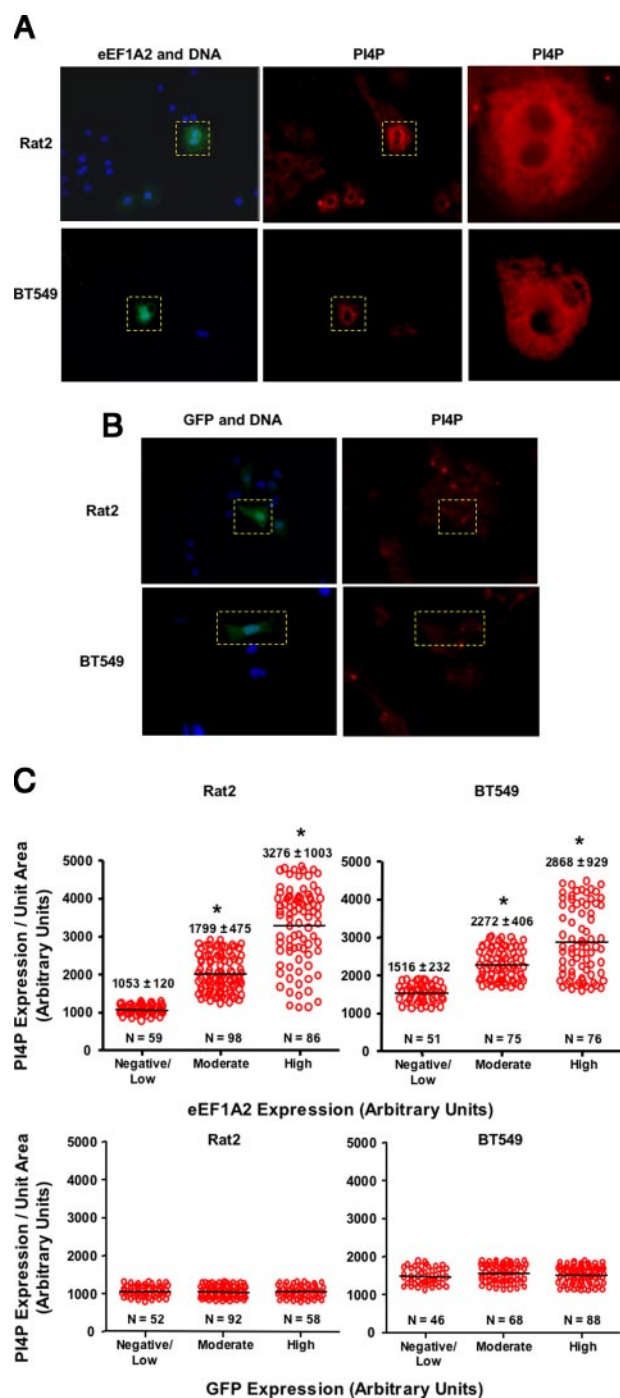
**FIGURE 4. eEF1A2 expression increases overall cellular PI4K activity.** *A, left*, BT549 cells were transduced with Ad-eEF1A2 or Ad-GFP at an MOI of either 20 or 200 plaque-forming units/cell. At an MOI of 20, there is no significant change in PI4P generation. At an MOI of 200, there is a significant increase in PI4K activity in eEF1A2-transduced cells relative to GFP-transduced cells ( $p < 0.05$ ; Student's *t* test). -Fold activation is calculated relative to untransduced cells and is the mean and S.D. of three independent experiments. *Right*, a representative TLC plate. *B*, transduction of Rat2 cells with Ad-eEF1A2 at an MOI of 500 similarly increases PI4K activity ( $p < 0.05$ ; Student's *t* test). Protein levels of eEF1A2 and actin are shown. Control cells are



**FIGURE 5. siRNA-mediated eEF1A2 down-regulation decreases overall cellular PI4K activity in MCF7 cells.** Two siRNAs directed against eEF1A2 decrease its steady state protein level in MCF7 cells (*bottom panel*; Western blot) relative to untreated cells or those treated with a negative control (NC) siRNA. Down-regulation of eEF1A2 significantly decreased overall PI4K activity ( $p < 0.05$ ; Student's *t* test). -Fold activation is calculated relative to untreated cells and is the mean and S.D. of three independent experiments.

mammalian PI4KIII $\beta$ , *PIK1*, has been studied in great detail (33–38). *Pik1p*, the protein encoded by *PIK1*, is essential for normal secretion, Golgi and vacuole membrane dynamics, and endocytosis (33–38). *Pik1p* localizes to the nucleus and the *trans*-Golgi (34, 38). *Pik1<sup>ts</sup>* cells exhibit a defect in secretion of Golgi-modified secretory pathway cargoes (36). Mammalian PI4KIII $\beta$  is concentrated in the Golgi apparatus (39, 40), although detectable protein is found in the non-Golgi cytoplasm. However, we have not seen appreciable eEF1A2 protein in the Golgi, and we have observed no consistent Golgi defects in eEF1A2-expressing cells (data not shown). This suggests that the capacity of eEF1A2 to increase PI4P formation is regulating some non-Golgi aspect of cell physiology. Active PI4K is observed in the non-Golgi cytosol (41), and we find that eEF1A2 expression increases PI4P generation throughout the cell.

untransduced. *C*, BT549 cells transduced with Ad-eEF1A2 do not show an increase in steady-state PI4KIII $\beta$  protein levels as seen by Western blotting. Actin was used as a loading control, and cells were transduced as in Fig. 5A at an MOI of 200.



**FIGURE 6. Ectopic expression of eEF1A2 increases cellular PI4P abundance.** *A*, Rat2 and BT549 cells were transiently transfected with an eEF1A2 expression plasmid and stained for DNA (blue), eEF1A2 (green), and PI4P (red). A higher magnification view of an eEF1A2-expressing cell (yellow box) is shown in the far right panels. *B*, Rat2 and BT549 cells were transiently transfected with a GFP expression plasmid and stained for DNA (blue) and PI4P (red). A GFP-expressing cell is marked by a yellow box. *C*, quantification of eEF1A2-mediated increase in cellular PI4P levels. *Top*, PI4P levels in eEF1A2-transfected Rat2 and BT549 cells expressing no/low, moderate, or high levels of eEF1A2. Cells expressing moderate or high levels of eEF1A2 each show significantly higher PI4P staining (both  $p < 0.0001$ , Mann-Whitney test, asterisk) than those expressing no/low eEF1A2. *Bottom*, quantification of PI4P staining in GFP-transfected Rat2 and BT549 cells expressing no/low, moderate, and high levels of GFP. eEF1A2 staining was categorized as negative/low when the eEF1A2 fluorescence/unit area ratio was  $< 1400$ , as moderate when this ratio was between 1400 and 3500 for Rat2 or between 1400 and 2500 for BT549, and as high when the ratio exceeded 3500 or 2500 for Rat2 and BT549, respectively.

The molecular mechanism by which eEF1A2 activates PI4KIII $\beta$  is currently unknown. eEF1A proteins bind and hydrolyze GTP and bind tRNA. They have not been shown to have phosphotransferase activity or other capacity to covalently modify proteins. Thus, it is unlikely that eEF1A2 affects PI4KIII $\beta$  activity by post-translationally modifying it. Because eEF1A2 expression does not increase steady-state PI4KIII $\beta$  protein levels (Fig. 4C), it is unlikely that eEF1A2 expression increases cellular PI4P generation by increasing the amount of PI4KIII $\beta$  message being translated. We propose that direct interaction between eEF1A2 and PI4KIII $\beta$  leads to a conformational change in PI4KIII $\beta$  that increases its catalytic activity. This hypothesis is consistent with our observations that eEF1A2 increases the overall PI4KIII $\beta$  catalytic rate ( $K_{cat}$ ) and makes PI4KIII $\beta$  a more efficient kinase ( $V_{max}/K_m$ ). Although the possibility of this conformational change remains an open one, we do not believe that eEF1A2 is a general phosphatidylinositol kinase activator. We have been unable to detect physical interaction between eEF1A2 and phosphatidylinositol 3-kinases, nor have we observed activation of this kinase by eEF1A2 (data not shown).

Our *in vitro* results suggest that eEF1A2 and PI4KIII $\beta$  bind in a 1:1 stoichiometry, and the majority of recombinant eEF1A2 and PI4KIII $\beta$  are competent to physically interact with each other. However, up to a 2-fold molar excess of recombinant eEF1A2 is required to maximally increase *in vitro* PI4KIII $\beta$  activity. Thus, it is possible that some post-translational modification of eEF1A2 may enhance its ability to activate PI4KIII $\beta$ . Yang and Boss (14) showed that dephosphorylation of PIK-A49 prevented it from activating PI4KIII $\beta$  and that activation could be restored by *in vitro* phosphorylation of PIK-A49 by a calcium-dependent protein kinase. The possibility of post-translational modification of eEF1A2 enhancing its ability to enhance PI4KIII $\beta$  activity is intriguing. However, no post-translational modifications of eEF1A2 have yet been identified, and our results indicate that post-translational modification of eEF1A2 is not obligatory for PI4KIII $\beta$  activation. Post-translational modification could also regulate PI4KIII $\beta$ /eEF1A2 interaction. Because only a small fraction ( $< 10\%$ ) of total eEF1A2 is in a complex with PI4KIII $\beta$  in MCF7 cells (and *vice versa*), post-translational modification of eEF1A2 could enhance cellular complex formation and therefore PI4P generation.

eEF1A2 is highly expressed in a 30–60% fraction of breast, ovary, and lung tumors (9–12). We have previously reported that eEF1A2 has transforming properties (9) and that its inactivation in mice increases lymphoid apoptosis (42). This identifies eEF1A2 as an important human oncogene. We hypothesize that eEF1A2 activates oncogenesis through PI4K activation and PI4P generation. Our observation that eEF1A2 expression is sufficient to increase both PI4K activity and PI4P levels indicates that high eEF1A2 levels in tumors probably leads to an overall increase in cellular PI4P production. We propose that increased production of PI4P could increase the abundance of PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>. Both of these phospholipids are second messengers that regulate actin cytoskeletal organization, intracellular vesicular trafficking, and proliferation (15, 20, 43–47). Of particular importance to oncogenesis would be PI(3,4,5)P<sub>3</sub>, the abundance of which controls cell growth, apo-

ptosis, and cell invasiveness. Although phosphatidylinositol 3-kinase activity has long been thought to be the rate-limiting step in the production of PI(3,4,5)P<sub>3</sub>, this may not be the case in tumors that have high phosphatidylinositol 3-kinase activity through oncogenic mutation of the kinase or have inactivating mutations in the phosphatase and tensin homolog (PTEN) lipid phosphatase (48). In those tumors with constitutively active phosphatidylinositol 3-kinase or inactive PTEN, increased PI4K activity would be predicted to increase overall PI(3,4,5)P<sub>3</sub> abundance and activate PI(3,4,5)P<sub>3</sub>-dependent signaling. This idea is consistent with a recent report by Pendairens *et al.* (49), who show that the phosphatidylinositol 5-phosphate produced by the *Shigella* pathogen is sufficient to activate Akt, a serine threonine kinase whose activity is dependent on PI(3,4,5)P<sub>3</sub> abundance (50). No PI4K has been reported to activate PI(3,4,5)P<sub>3</sub> production; nor have PI4K genes been reported to be transforming or to be highly expressed during oncogenesis. However, this idea has not been fully explored.

eEF1A proteins have a well characterized ability to bind and bundle F-actin (2, 3). It has been proposed that eEF1A2 couples the protein translation machinery to actin cytoskeleton assembly. Because we find that eEF1A2 directly binds PI4KIII $\beta$ , it is probable that eEF1A2 links protein translation to phosphatidylinositol generation and the actin cytoskeleton.

In summary, we have identified eEF1A2 as a direct activator of PI4KIII $\beta$ . Ectopic expression of eEF1A2 increases cellular PI4K activity and increases cellular PI4P abundance. Furthermore, siRNA-mediated eEF1A2 inactivation decreases PI4K activity. This is consistent with the idea that eEF1A2 functionally and physiologically regulates PI4K function. We propose that PI4KIII $\beta$  activation has an important role in eEF1A2-mediated tumorigenesis and transformation.

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