The Translation Factor eIF3a Regulates the Extracellular Signal regulated Kinase (ERK) Pathway

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ABSTRACT

The extracellular signal regulated kinase (ERK) pathway participates in the control of numerous cellular processes including cell proliferation. As its activation kinetics are critical for to its biological effects, they are tightly regulated. We report that the protein translation factor, eukaryotic translation initiation factor 3, subunit A (eIF3a) binds to SHC and Raf-1, two components of the ERK pathway. The interaction of eIF3a with Raf-1 is increased by β–arrestin2 expression and transiently decreased by EGF stimulation in a concentration dependent manner. The epidermal growth factor (EGF) induced decrease in Raf-1 – eIF3a association kinetically correlates with the timecourse of ERK activation. eIF3a interferes with Raf-1 activation and eIF3a downregulation by siRNA enhances EGF induced ERK activation, early gene expression and DNA synthesis. Thus, eIF3a serves to negatively modulate the activation of the ERK pathway by EGF.

INTRODUCTION

The ERK pathway is involved in many fundamental cellular processes, including cell proliferation and transformation (3, 7). The activation sequence of the core components of the pathway is well characterised (24, 31). The pathway is typically activated by receptor tyrosine kinases, such as the EGF receptor (EGFR), which auto-phosphorylate at their intracellular kinase domains upon ligand binding. These phospho-tyrosines serve as docking sites for adaptor proteins and signal transducers which activate the downstream pathways that mediate the biological effects of the ligands. The ERK pathway is initiated by the translocation of the guanine nucleotide exchange factor (GEF) SOS from the cytosol to the plasma membrane via the adaptor proteins SHC and Grb2 binding to specific phospho-tyrosines at the EGFR. SOS

activates Ras, which thereby bringing SOS in the vicinity of Ras proteins at the plasma membrane. SOS activates Ras, which binds to Raf kinases recruiting them to the membrane for activation. Raf activation is a complex process that is still not fully elucidated, and slightly different between the three Raf isoforms A-Raf, B–Raf and Raf-1. A critical step in Raf-1 activation is the Ras induced dephosphorylation of the inhibitory phospho-S259, which is required for the phosphorylation of the key activating site S338 (9). Active Raf-1 phosphorylates MEK, which in turn phosphorylates ERK. ERK has >150 substrates in the cytosol and nucleus (37). This large number of substrates enables the pathway to carry out its highly pleiotropic functions, although it is still rather enigmatic as to how specificity in signalling and biological responses is produced. Nevertheless, it is thought that the activation kinetics, spatial organisation, crosstalk and binding to scaffold proteins contribute to the generation of signaling specificity (3, 31). Thus, although the core pathway is well mapped, identification and analysis of the proteins that modulate these parameters is required in order to understand the functional diversity of the pathway.

We report here the identification of eIF3a as a protein that modulates the activation kinetics of the ERK pathway. eIF3a (also called eIF3θ, p150, p170) is a component of eIF3, a multi-subunit factor involved in mRNA translation (4, 13, 18). eIF3 participates in forming the pre-initiation complex and preventing the premature binding of the 40S to the 60S ribosomal subunits (13, 18). eIF3a can regulate cell cycle progression and proliferation, presumably by controlling the translation of mRNAs encoding the cell cycle inhibitor $p27^{kip1}$ and the M2 subunit of ribonucleotide reductase, which is a rate limiting enzyme in DNA synthesis (11, 12). Similarly, overexpression of certain eIF3 subunits, including eIF3a, can transform NIH 3T3 cells by enhancing global protein synthesis and, in particular, the synthesis of proteins that stimulate

proliferation, such as cyclinD1, c-Myc, fibroblast growth factor-2 and ornithine decarboxylase (38). Intriguingly, however, in mammalian cells eIF3a is not deemed essential for the function of eIF3, and not all of eIF3a is associated with ribosomes indicating that it may have functions unrelated to protein translation (28). Indeed, eIF3a has been reported to bind to actin (26), cytokeratin 7 (21), and microtubules (16, 29) and the TrkA receptor (23), although the functional consequences of these interactions remain to be ascertained.

We have found that eIF3a can regulate the ERK pathway by binding to SHC and Raf-1. Critically, the interaction with Raf-1 is enhanced by the signalling scaffold protein β–arrestin2 and, in doing so, it serves to interferes with Raf-1 activation. Consistent with these observations, the downregulation of eIF3a results in prolonged ERK activation, induction of the nuclear ERK target c-Fos and enhanced proliferation.

MATERIALS AND METHODS

Materials. Doxycycline, monoclonal mouse anti-VSV-G antibody and anti-VSV-G agarose beads were from Sigma-Aldrich (Gillingham, UK), EGF was from Promega (Southampton, UK). Lipofectamine 2000 transfection reagent was from Invitrogen (Paisley, UK). Protease inhibitor cocktail tablets were from Roche Diagnostics (West Sussex, UK). Polyclonal goat eIF3a antibody, polyclonal rabbit EGFR antibody, monoclonal mouse SOS1 antibody, polyclonal rabbit c-Fos antibody and monoclonal mouse β-actin antibody were from Santa Cruz (Heidelberg, Germany). Polyclonal rabbit SHC antibody and monoclonal mouse Ras antibody were from Upstate (Watford, UK). Monoclonal mouse Raf-1 antibody and monoclonal mouse Grb2 antibody were from BD (Oxford, UK). Monoclonal, rabbit β-arrestin2 antibody, monoclonal mouse B–Raf antibody, polyclonal rabbit eIF3a antibody, polyclonal rabbit MEK1/2

antibody, Monoclonal rabbit p44/42 MAPK (ERK1/2) and polyclonal rabbit phospho-p44/42 MAPK (phospho-ERK1/2) antibodies were from Cell Signalling Technology (Hitchin, Hertfordshire, UK). Polyclonal rabbit anti-VSV-G antibody was generated in house.

Generation of stable Flp-In T-REx HEK293 cells inducibly expressing VSV-β-arrestin2. VSV-β-arrestin-2 was generated by PCR and inserted into the KpnI-ApaI sites of pcDNA5/FRT/TO. The primers were CGAT GGT ACC GCC ACC ATG TAC ACC GAT ATA GAG ATG AAC CGC CTT GGA AAG GGG GAG AAA CCC GGG ACC AGG GT (forward with VSV-G sequence and KpnI site, underlined) and TGAT GGG CCC TCA ACA GAA CTG GTC GTC ATA GTC CTC GT (reverse with ApaI site, underlined). To generate stable Flp-In T-REx HEK293 cells inducibly expressing VSV-β-arrestin2, cells were transfected with a mixture containing VSV-β-arrestin2 cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using Lipofectamine 2000 transfection reagent from Invitrogen, according to the manufacturer's instructions. Resistant clones were selected by replacing zeocin with 200 μg/ml hygromycin B and the expression of VSV-β-arrestin2 was examined by Western blotting using anti-VSV antibody. Clones were pooled to avoid clonal variation and maintained in Dulbecco's modified Eagle's medium without sodium pyruvate, 4500 mg/liter glucose, and L-glutamine supplemented with 10% (v/v) FBS, 1% antibiotic mixture, 100 μg/ml zeocin and 10 μg/ml blasticidin. To induce the expression of VSV-β-arrestin2, cells were treated with 1 μg/ml doxycycline for 24.

Identification of β-arrestin2 binding proteins by mass spectrometry. VSV-β-arrestin2 was induced by 1 μg/ml doxycycline in Flp-in T-REx HEK293 cells. 24 hours after induction cells were harvested and resuspended in immunoprecipitation buffer (150 mM NaCl, 0.01 mM $NaPO_4$, 2 mM EDTA, 0.5% Triton X-100, and 5% glycerol plus protease inhibitor cocktail tablets). Non-induced Flp-In T-REx 293 cells were used as negative control. The cell pellets were

lysed, centrifuged for 15 minutes at 20,000*g* at 4°C, and the supernatant transferred to fresh a tube with Protein G/A beads (Sigma) to preclear the samples. After 1 hour at 4°C, the samples were re-centrifuged at 20,000*g* for 1 minute. Equal amounts of protein were incubated with anti-VSV-G agarose beads (Sigma) at 4°C for 2 hours on a rotating wheel. Samples were subsequently washed four times with immunoprecipitation buffer. Proteins were eluted from beads by 0.2 mg/ml VSV peptide (Sigma) three times in immunoprecipitation buffer. The combined eluates were precipitated with 30% TCA at 4°C overnight. Precipitates were washed three times with acetone and air dried. Then the precipitates were resuspended in 6M urea plus 0.1 % N-octyl glucoside, diluted with 10 volumes of 25mM ammonium bicarbonate and digested with 20μg/ml sequencing grade trypsin (Promega) in 25mM ammonium bicarbonate with 10mM DTT overnight. The digestion was stopped by adding formic acid to 1%. Tryptic peptides were separated by a two-dimensional liquid chromatography (LC) system using stepwise elution from a cation exchange column and sequential fractionation of eluates on a Pepmap C18 reversed phase column (LC Packings, Bath, UK) by a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min. The flow rate was maintained at 0.2 μl / min. The fractions were analysed by online electrospray ionisation (ESI) mass spectrometry (MS) on a Q-STAR® Pulsar-i MS/MS system. MS analysis was performed using a 3 second survey scan followed by up to four MS/MS analyses of the most abundant peptides (3 seconds per peak) in Information Dependent Acquisition (IDA) mode, choosing 2+ to 4+ ions above threshold of 30 counts, with dynamic exclusion for 180s. Data generated from the Q-STAR® Pulsar-i hybrid were analysed using Applied Biosystems Analyst QS (v1.1) software and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein identifications were assigned using the Mascot search engine. In all cases variable methionine oxidation was allowed in searches. An MS tolerance of 1.2 Da

for MS and 0.4 Da for MS/MS analysis was used. The MS plus MS/MS data were searched against the NCBInr public database. A match was considered significant if the MOWSE score was equal to or greater than 75. The proteins identified in both non-induced and induced samples were considered as no specific binding.

siRNA mediated gene silencing. siRNA duplex oligonucleotides were purchased from Ambion (Warrington, UK). The siRNA sequences targeting human eIF3a were: CGAACCAAUUAUGUUGAAA-dTdT (sense), UUUCAACAUAAUUGGUUCG-dTdG (Antisense). The siRNA sequences targeting human β-arrestin-2 were: the mixture of AAGUCUCUGUGAGACAGUA-dTDT (sence), UACUGUCUCACAGAGACUU-dTdG; and CGAACAAGAUGACCAGGUA-dTdT (sence), UACCUGGUCAUCUUGUUCG-dAdG (antisence). The Silencer control siRNA1 from Ambion was used as negative control. siRNAs were transfected using Lipofectamine 2000 reagent according to the supplier's protocol.

Co-immunoprecipitation studies - Cells were harvested in co-immunoprecipitation buffer (150mM NaCl, 0.01mM $NaPO_4$, 2mM EDTA, 1mM N-ethylmaleimide, 0.5% Triton X-100, and 5% glycerol plus protease inhibitor cocktail tablets) and processed as described above. Instead of elution with VSV peptide samples were resolved by SDS-PAGE and subsequently immunoblotted to detect proteins of interest.

Bromodeoxyuridine (BrdU) incorporation. Cells seeded on poly-lysine coated coverslips were transfected with human eIF3a siRNA or control siRNA. 24 hours after transfection the cells were serum starved for 48 hours and stimulated with 20ng/ml EGF plus 20μM BrdU for another 6 hours. Then, cells were washed with PBS and fixed (90% ethanol, 5% acetic acid, 5% H_2O) for 20 minutes. DNA was denatured with 2M HCl at 37^0C for 20 minutes and neutralized in 0.1M $Na_2B_4O_7$, pH 8.5 for 2 minutes. After three washes with PBS, cells were blocked and

permeabilized in 3% non-fat dry milk plus 0.15 % Triton X-100 for 30 minutes, and incubated with 1:500 diluted mouse anti-BrdU antibody (Sigma) in blocking buffer for 1h. After another three PBS washes cells were incubated with 1:500 anti-mouse Alexa-594 (Molecular Probes) plus 10μg/ml Hoechst stain for 1 hour and subsequently washed with PBS for three times. Coverslips were mounted with Immu-mount medium (Thermo). Images were collected with an epifluorescent microscope, and BrdU incorporation was quantified. BrdU positive nuclei per total number of nuclei were counted from at least 9 random microscope fields in each experiment. The statistical data come from three independent experiments.

Statistical analysis was done using Student's t-test.

RESULTS

β–arrestin2 interacts with eIF3a – We identified eIF3a as a β–arrestin2 associated protein in a proteomic screen, where we used human Flp-In T-REx HEK293 cells engineered to express a doxycycline inducible VSV-tagged β–arrestin2 in order to identify binding partners of β–arrestin2. Immunoprecipitation of VSV-β–arrestin2 co-precipitated eIF3a (Fig. 1A), and *vice versa* immunoprecipitation of eIF3a co-precipitated VSV-β–arrestin2 (Fig. 1B). The co-precipitations were strictly dependent on the induction of VSV-β–arrestin2 expression supporting the specificity of the interaction.

Knockdown of β–arrestin2 or eIF3a prolongs EGF induced ERK activation – We had previously reported that β–arrestin2 mutants that are impaired to dimerize also show a diminished ability to bind to ERK and mediate ERK activation (36). Therefore, we tested the effects of β–arrestin2 downregulation on ERK activation (Fig. 2). We stimulated HEK293 cells

with EGF and monitored ERK activation by blotting with phospho-specific antibodies detecting the activating phosphorylation sites (Fig. 2A). The downregulation of β–arrestin2 expression slightly augmented and significantly extended ERK activation by EGF (Fig. 2B).

In order to assess whether eIF3was involved in this effect we downregulated eIF3a and examined ERK activation (Fig. 3A). Similar to β–arrestin2, eIF3a knockdown caused a slight increase in the peak of ERK activity and a significant extension of the duration of ERK activation (Fig. 3B). These results suggested that eIF3a may be involved in mediating the suppressive effects of β–arrestin2 on the EGF stimulated ERK pathway.

β–arrestin2 modulates binding of eIF3a to selected components of the ERK pathway – As eIF3a modulated the activation of ERK in response to EGF, we tested whether eIF3a could interact with components of the ERK pathway. For this purpose we immunoprecipitated endogenous eIF3a and assessed co-precipitation of the EGFR, SHC, Grb2, SOS, Ras, Raf-1, B–Raf, MEK and ERK (Fig. 4A). An unrelated, species-matched antibody was used as control. Specific interactions were observed between eIF3a and SHC, and eIF3a and Raf-1. Interestingly, B–Raf did not interact with eIF3a suggesting that the interaction is Raf isoform specific. We then examined the influence of β–arrestin2 on the interaction of eIF3a with SHC and Raf-1 (Fig. 4B). While induction of β–arrestin2 expression had no influence on the interaction between eIF3a and SHC, it enhanced the interaction between eIF3a and Raf-1, suggesting that eIF3a may sequester Raf-1 from EGF activation.

eIF3a binding to Raf-1 is regulated by EGF – The hypothesis that eIF3a may sequester Raf-1 from EGF mediated activation predicts that EGF stimulation should diminish this interaction. This was indeed the case (Fig. 5A). Importantly, EGF caused an early dissociation of Raf-1 from eIF3a and a re-association at later time points, which closely corresponded to the timecourse of ERK activation. In addition, the dissociation of Raf-1 from eIF3a was dependent on the EGF dose (Fig. 5B). Again, the progressive disruption of the eIF3a - Raf-1 complex with increasing EGF doses was mirrored by an increase in ERK activation (Fig. 5B). Interestingly, the fraction of Raf-1 that co-immunoprecipitated with eIF3a was phosphorylated on S259 and stayed phosphorylated on this site after EGF treatment (Fig. 5C). S259 phosphorylation is inhibitory for Raf-1 kinase activity and needs to be dephosphorylated for Raf-1 activation to take place (8). The dephosphorylation of S259 precedes the phosphorylation of S338 (9), a phosphorylation event which is essential for Raf-1 kinase activation (10). Raf-1 associated with eIF3a did not become phosphorylated on S338, while S338 phosphorylation was readily observable in lysates in response to EGF treatment (Fig. 5C). These data further corroborate the hypothesis that eIF3a binding impedes the activation of Raf-1 and the downstream ERK pathway.

eIF3a regulates nuclear and biological effects of ERK signalling – In response to stimulation of angiotensin II type 1a receptors β–arrestins have been shown to function as scaffolds that selectively promote the activation of the ERK pathway in the cytosol, but not in the nucleus (34). Therefore, we investigated whether eIF3a has a similarly compartmentalized effect on ERK signalling. We first assayed the effect of eIF3a on the induction of c-Fos, an early gene that is a classic transcriptional target of ERK signalling (19). Down-regulation of eIF3a increased the

basal levels c-Fos protein and enhanced the induction of c-Fos protein expression by EGF (Fig. 6A) indicating that eIF3a down-regulation also promotes ERK nuclear signalling.

In order to test whether the enhanced ERK signalling enabled by eIF3a down-regulation also has a biological effect, we examined DNA synthesis. While basal DNA synthesis was unchanged, eIF3a down-regulation caused a small, but significant increase in EGF stimulated DNA synthesis. These data confirm that eIF3a has a suppressive effect on signalling through the ERK pathway and the biological outcomes thereof.

DISCUSSION

Our results suggest that β–arrestin2 can functionally interfere with the activation of the ERK pathway by EGF through enhancing the binding of eIF3a to Raf-1. These data add two new aspects to the regulation of Raf and ERK signalling, namely in revealing novel regulatory roles for β–arrestin2 and eIF3a.

β–arrestins1,2 were originally discovered as proteins that terminate GPCR signalling , but have turned out to be multifunctional proteins that play critical roles in coordinating GPCR and EGFR dependent signalling pathways (2, 5, 6, 27). The relationship mainly has been explored from the role β–arrestins play in crossregulating EGFR signalling after stimulation of a GPCR. The results suggest a complicated interplay, where β–arrestins1/2 can have different and antagonistic roles in the regulation of the ERK pathway (1, 14, 15). Our results suggest that β–arrestin2 can impede ERK pathway activation by EGF, while down-regulation of β–arrestin2 enhances ERK activation. This regulation pertains to the duration of ERK signalling rather than to peak activation, and while statistically significant it is quite subtle. These properties may explain why it has not been observed in previous studies.

Differences in spatial and temporal components of ERK activation can lead to profoundly distinct functional effects (20, 25, 30). Our results also suggest that eIF3a can exert specific regulatory effects on the kinetics of ERK activation through its interaction with β–arrestin2. Specifically, this is mediated by β–arrestin2 binding to eIF3a, which serves to enhances the binding of eIF3a to Raf-1. As β–arrestin2 can act as scaffold that facilitates the assembly of the Raf-1 – MEK – ERK kinase signalling cascade (22), and eIF3a also can bind Raf-1, then the enhancing effects of β–arrestin2 on the eIF3a – Raf-1 interaction may simply be due to the formation of a higher order complex where binding affinities are cooperatively stabilised. However, while β–arrestin2 promotes ERK activation (22), eIF3a interferes with it. Thus, the interactions between Raf-1, β–arrestin2, and eIF3a may occur in different protein complexes, different subcellular compartments or on different timeframes. Distinguishing between these possibilities is technically extremely challenging and will require future evaluation as and when technologies develop. Nevertheless, our data provide key mechanistic insight in showing that the mechanism of ERK pathway inhibition by eIF3a is the interference with Raf-1 activation, as reflected by a lack of dephosphorylation of the inhibitory residue S259 and failure to phosphorylate S338 in Raf-1. These changes in phosphorylation are Ras dependent and take place at the membrane (8-10), suggesting that eIF3a interference operates at the membrane and not at the endosome level, where β–arrestin2 is thought to scaffold the Raf-1 – MEK – ERK pathway (22). Interestingly, eIF3a localisation has been described to include the plasma membrane (26), which is clearly compatible with a mechanism of interference with Raf-1 activation at the plasma membrane.

In addition to its role in protein translation eIF3a has been suggested to have other functions, especially in cancer, although the findings are contradictory as to whether eIF3a promotes or

counteracts cellular transformation (28). eIF3a is certainly overexpressed in cancers of breast, lung, cervix, oesophagus, stomach and colon (17, 28, 32). In keeping with a cancer promoting role, overexpression of several eIF3 subunits, including eIF3a, was shown to transform NIH 3T3 fibroblasts (38). However, further studies in cervical, oesophageal and gastric cancer demonstrated that eIF3a overexpression was associated with a better prognosis (28) suggesting that the role of eIF3a in malignancy is more differentiated. Intriguingly, the processes eIF3a is thought to be involved in, such as regulation of the cell cycle and cell differentiation (28), overlap with main functions of the ERK pathway. Thus, eIF3a may play a role in regulating the ERK pathway in both physiological and pathophysiological conditions. This hypothesis is supported by our observation that the downregulation of eIF3a expression stimulates the expression of nuclear ERK target genes, such as *c-fos*, and DNA synthesis. Thus, eIF3a can regulate proliferation induced by EGF via the ERK pathway. As overexpression of members of the EGF receptor family and its ligands is frequently observed in cancer (33, 35), eIF3expression may modulate the activity of the EGF stimulated ERK pathway in cancers.

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REFERENCES

1. **Ahn, S., H. Wei, T. R. Garrison, and R. J. Lefkowitz.** 2004. Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. J Biol Chem **279:**7807-11.

2. **Baillie, G. S., and M. D. Houslay.** 2005. Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes. Curr Opin Cell Biol **17:**129-34.

3. **Calvo, F., L. Agudo-Ibanez, and P. Crespo.** 2010. The Ras-ERK pathway: understanding site-specific signaling provides hope of new anti-tumor therapies. Bioessays **32:**412-21.

4. **Damoc, E., C. S. Fraser, M. Zhou, H. Videler, G. L. Mayeur, J. W. Hershey, J. A. Doudna, C. V. Robinson, and J. A. Leary.** 2007. Structural characterization of the human eukaryotic initiation factor 3 protein complex by mass spectrometry. Mol.Cell Proteomics. **.**

5. **Defea, K.** 2008. Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. Br J Pharmacol **153 Suppl 1:**S298-309.

6. **DeWire, S. M., S. Ahn, R. J. Lefkowitz, and S. K. Shenoy.** 2007. Beta-arrestins and cell signaling. Annu Rev Physiol **69:**483-510.

7. **Dhillon, A. S., S. Hagan, O. Rath, and W. Kolch.** 2007. MAP kinase signalling pathways in cancer. Oncogene **26:**3279-90.

8. **Dhillon, A. S., S. Meikle, Z. Yazici, M. Eulitz, and W. Kolch.** 2002. Regulation of Raf-1 activation and signalling by dephosphorylation. EMBO J **21:**64-71.

9. **Dhillon, A. S., A. von Kriegsheim, J. Grindlay, and W. Kolch.** 2007. Phosphatase and feedback regulation of Raf-1 signaling. Cell Cycle **6:**3-7.

10. **Diaz, B., D. Barnard, A. Filson, S. MacDonald, A. King, and M. Marshall.** 1997. Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol Cell Biol **17:**4509-16.

11. **Dong, Z., L. H. Liu, B. Han, R. Pincheira, and J. T. Zhang.** 2004. Role of eIF3 p170 in controlling synthesis of ribonucleotide reductase M2 and cell growth. Oncogene **23:**3790-801.

12. **Dong, Z., and J. T. Zhang.** 2003. EIF3 p170, a mediator of mimosine effect on protein synthesis and cell cycle progression. Mol Biol Cell **14:**3942-51.

13. **Dong, Z., and J. T. Zhang.** 2006. Initiation factor eIF3 and regulation of mRNA translation, cell growth, and cancer. Crit Rev.Oncol.Hematol. **59:**169-180.

14. **Gesty-Palmer, D., M. Chen, E. Reiter, S. Ahn, C. D. Nelson, S. Wang, A. E. Eckhardt, C. L. Cowan, R. F. Spurney, L. M. Luttrell, and R. J. Lefkowitz.** 2006. Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. J Biol Chem **281:**10856-64.

15. **Gesty-Palmer, D., H. El Shewy, T. A. Kohout, and L. M. Luttrell.** 2005. beta-Arrestin 2 expression determines the transcriptional response to lysophosphatidic acid stimulation in murine embryo fibroblasts. J Biol Chem **280:**32157-67.

16. **Hasek, J., P. Kovarik, L. Valasek, K. Malinska, J. Schneider, S. D. Kohlwein, and H. Ruis.** 2000. Rpg1p, the subunit of the Saccharomyces cerevisiae eIF3 core complex, is a microtubule-interacting protein. Cell Motil Cytoskeleton **45:**235-46.

17. **Haybaeck, J., T. O'Connor, R. Spilka, G. Spizzo, C. Ensinger, G. Mikuz, T. Brunhuber, A. Vogetseder, I. Theurl, W. Salvenmoser, H. Draxl, R. Banziger, F. Bachmann, G. Schafer, M. Burger, and P. Obrist.** 2010. Overexpression of p150, a part of the large subunit of the eukaryotic translation initiation factor 3, in colon cancer. Anticancer Res **30:**1047-55.

18. **Hinnebusch, A. G.** 2006. eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem.Sci. **31:**553-562.

19. **Karin, M.** 1996. The regulation of AP-1 activity by mitogen-activated protein kinases. Philos Trans R Soc Lond B Biol Sci **351:**127-34.

20. **Kholodenko, B. N., J. F. Hancock, and W. Kolch.** 2010. Signalling ballet in space and time. Nat Rev Mol Cell Biol **11:**414-26.

21. **Lin, L., T. Holbro, G. Alonso, D. Gerosa, and M. M. Burger.** 2001. Molecular interaction between human tumor marker protein p150, the largest subunit of eIF3, and intermediate filament protein K7. J Cell Biochem. **80:**483-490.

22. **Luttrell, L. M., F. L. Roudabush, E. W. Choy, W. E. Miller, M. E. Field, K. L. Pierce, and R. J. Lefkowitz.** 2001. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. Proc Natl Acad Sci U S A **98:**2449-54.

23. **MacDonald, J. I., J. M. Verdi, and S. O. Meakin.** 1999. Activity-dependent interaction of the intracellular domain of rat trkA with intermediate filament proteins, the beta-6 proteasomal subunit, Ras-GRF1, and the p162 subunit of eIF3. J Mol Neurosci **13:**141-58.

24. **McKay, M. M., and D. K. Morrison.** 2007. Integrating signals from RTKs to ERK/MAPK. Oncogene **26:**3113-21.

25. **Murphy, L. O., and J. Blenis.** 2006. MAPK signal specificity: the right place at the right time. Trends Biochem Sci **31:**268-75.

26. **Pincheira, R., Q. Chen, Z. Huang, and J. T. Zhang.** 2001. Two subcellular localizations of eIF3 p170 and its interaction with membrane-bound microfilaments: implications for alternative functions of p170. Eur.J Cell Biol. **80:**410-418.

27. **Rajagopal, S., K. Rajagopal, and R. J. Lefkowitz.** 2010. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov **9:**373-86.

28. **Saletta, F., Y. S. Rahmanto, and D. R. Richardson.** 2010. The translational regulator eIF3a: the tricky eIF3 subunit! Biochim Biophys Acta **1806:**275-86.

29. **Severin, F. F., N. A. Shanina, A. Shevchenko, O. B. Solovyanova, V. V. Koretsky, and E. S. Nadezhdina.** 1997. A major 170 kDa protein associated with bovine adrenal medulla microtubules: a member of the centrosomin family? FEBS Lett **420:**125-8.

30. **Shankaran, H., and H. S. Wiley.** 2010. Oscillatory dynamics of the extracellular signal-regulated kinase pathway. Curr Opin Genet Dev **20:**650-5.

31. **Shaul, Y. D., and R. Seger.** 2007. The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim Biophys Acta **1773:**1213-26.

32. **Silvera, D., S. C. Formenti, and R. J. Schneider.** 2010. Translational control in cancer. Nat Rev Cancer **10:**254-66.

33. **Takeuchi, K., and F. Ito.** 2010. EGF receptor in relation to tumor development: molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors. FEBS J **277:**316-26.

34. **Tohgo, A., K. L. Pierce, E. W. Choy, R. J. Lefkowitz, and L. M. Luttrell.** 2002. beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. J Biol Chem **277:**9429-36.

35. **Wilson, K. J., J. L. Gilmore, J. Foley, M. A. Lemmon, and D. J. Riese, 2nd.** 2009. Functional selectivity of EGF family peptide growth factors: implications for cancer. Pharmacol Ther **122:**1-8.

36. **Xu, T. R., G. S. Baillie, N. Bhari, T. M. Houslay, A. M. Pitt, D. R. Adams, W. Kolch, M. D. Houslay, and G. Milligan.** 2008. Mutations of beta-arrestin 2 that limit self-association also interfere with interactions with the beta2-adrenoceptor and the ERK1/2 MAPKs: implications for beta2-adrenoceptor signalling via the ERK1/2 MAPKs. Biochem J **413:**51-60.

37. **Yoon, S., and R. Seger.** 2006. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors **24:**21-44.

38. **Zhang, L., X. Pan, and J. W. Hershey.** 2007. Individual overexpression of five subunits of human translation initiation factor eIF3 promotes malignant transformation of immortal fibroblast cells. J Biol.Chem. **282:**5790-5800.

FIGURE LEGENDS

Figure 1. Identification of eIF3a as a β–arrestin2 binding protein. *A*, Expression of VSV-tagged β–arrestin2 in Flp-In T-REx VSV-β–arrestin2 cells was induced by 1μg/ml doxycycline for 24hours. VSV-β–arrestin2 was immunoprecipitated with mouse anti-VSV-G antibody covalently bound to agarose beads (Sigma), and blotted for associated eIF3a and immunoprecipitated VSV-β–arrestin2 as indicated. *B*, aliquots of the lysates were immunoprecipitated with a goat anti eIF3a antibody, and blotted for associated VSV-β–arrestin2 and immunoprecipitated eIF3a.

Figure 2. Knockdown of β–arrestin-2 causes sustained ERK activity in response to EGF. *A*, β–arrestin-2 was knocked down in HEK293 cells with siRNA. The β–arrestin-2 knockdown efficiency was monitored by Western blotting using β–actin as loading control. Cells were treated with EGF for the indicated time points, and ERK activation was examined by blotting with a phospho-specific antibody. *B*, the ratio of phospho-ERK to total ERK (pERK/ERK) was quantified by densitometric evaluation of Western blots. Data represent the means +/- S.E.M. of six independent experiments, *$p<0.05$, **$p < 0.01$.

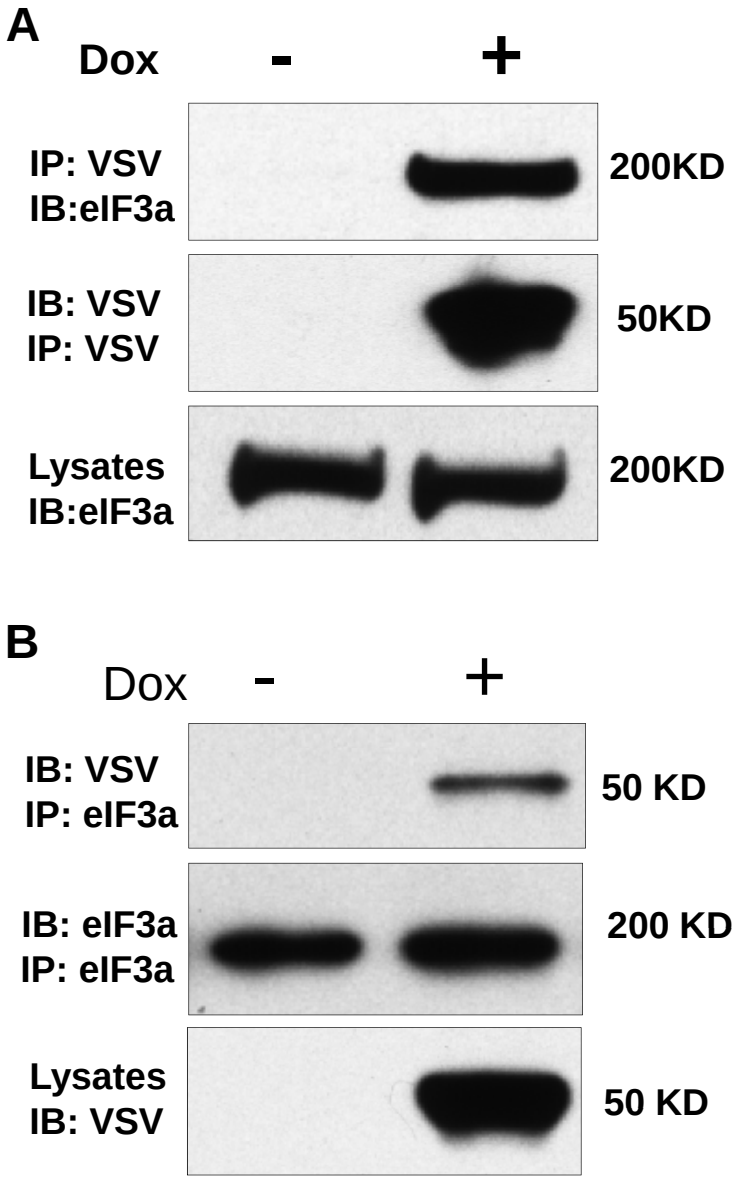
Figure 3. Knockdown of eIF3a causes sustained ERK activity in response to EGF. *A*, eIF3a was knocked down in HEK293 cells with siRNA, and knockdown efficiency was monitored by Western blotting using β–actin as loading control. Cells were treated with EGF for the indicated time points, and ERK activation was examined by blotting with a phospho-specific antibody. *B*, the ratio of phospho-ERK to total ERK (pERK/ERK) was quantified by densitometric evaluation of Western blots. Data represent the means +/- S.E.M. of six independent experiments, **p < 0.01.

Figure 4. Binding of eIF3a to protein components of the EGFR-ERK pathway. *A*, endogenous eIF3a was immunoprecipitated from HEK293 cells with a goat anti-eIF3 antibody and examined for associated proteins using the indicated antibodies. Goat IgG (Sigma) was used as control. The proteins examined and their positions in the pathway are schematically shown on the left with the proteins co-precipitating with eIF3a indicated in red. The bands labelled by asterisks in the Grb2 blot are IgG light chains detected by the secondary antibody. *B*, β–arrestin2 increases eIF3a binding to Raf-1 but not to SHC**.** Flp-In T-REx VSV-β–arrestin2 cells were induced with 1μg/ml doxycycline (Dox) or vehicle control for 24hours. Induction of VSV-β–arrestin2 was monitored by Western blotting with anti-VSV antibody. eIF3a immunoprecipitates were examined for co-precipitating SHC (left panel) and Raf-1 (right panel). A densitometric quantitation (arbitrary units) of Raf-1 binding to eIF3a is shown below, representing the means +/- S.E.M. from three independent experiments, *p < 0.05.

Figure 5. EGF regulation of eIF3a - Raf-1 association correlates with ERK pathway activation. *A*, HEK293 cells were serum starved overnight and treated with 20ng/ml EGF for the times indicated. Endogenous eIF3a was immunoprecipitated and examined for associated Raf-1 by Western blotting. The blots shown are representative of three independent experiments. *B*, serum starved HEK293 cells were stimulated with increasing doses of EGF. The eIF3a – Raf-1 association and ERK activation was assessed by Western blotting. Data are representative of three independent experiments. *C*, eIF3a was immunoprecipitated from EGF stimulated HEK293 cells. Raf-1 bound to eIF3a was examined for phosphorylation of the inhibitory S259 and activating S338 sites.

Figure 6. Knockdown of eIF3a enhances EGF induced c-Fos expression and DNA synthesis. *A*, HEK293 cells were transfected with siRNA against eIF3a, serum starved and treated with 20ng/ml EGF for the indicated time points. Induction of c-Fos protein and levels of eIF3a were assessed by Western blotting. β–actin served as loading control. Data are representative of three independent experiments. B, the effects of eIF3a knockdown on DNA synthesis in serum starved and EGF treated HEK293 cells was measured by BrdU incorporation. The experiment was performed three times, and over 400 cells were counted in each case. Data are means +/- S.E.M., *$p < 0.05$. eIF3a knockdown efficiency was monitored by Western blotting.

Fig.1.


A
Dox
-
+
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200KD
IB: VSV
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50KD
Lysates
IB:eIF3a
200KD
B
Dox
-
+
IB: VSV
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50 KD
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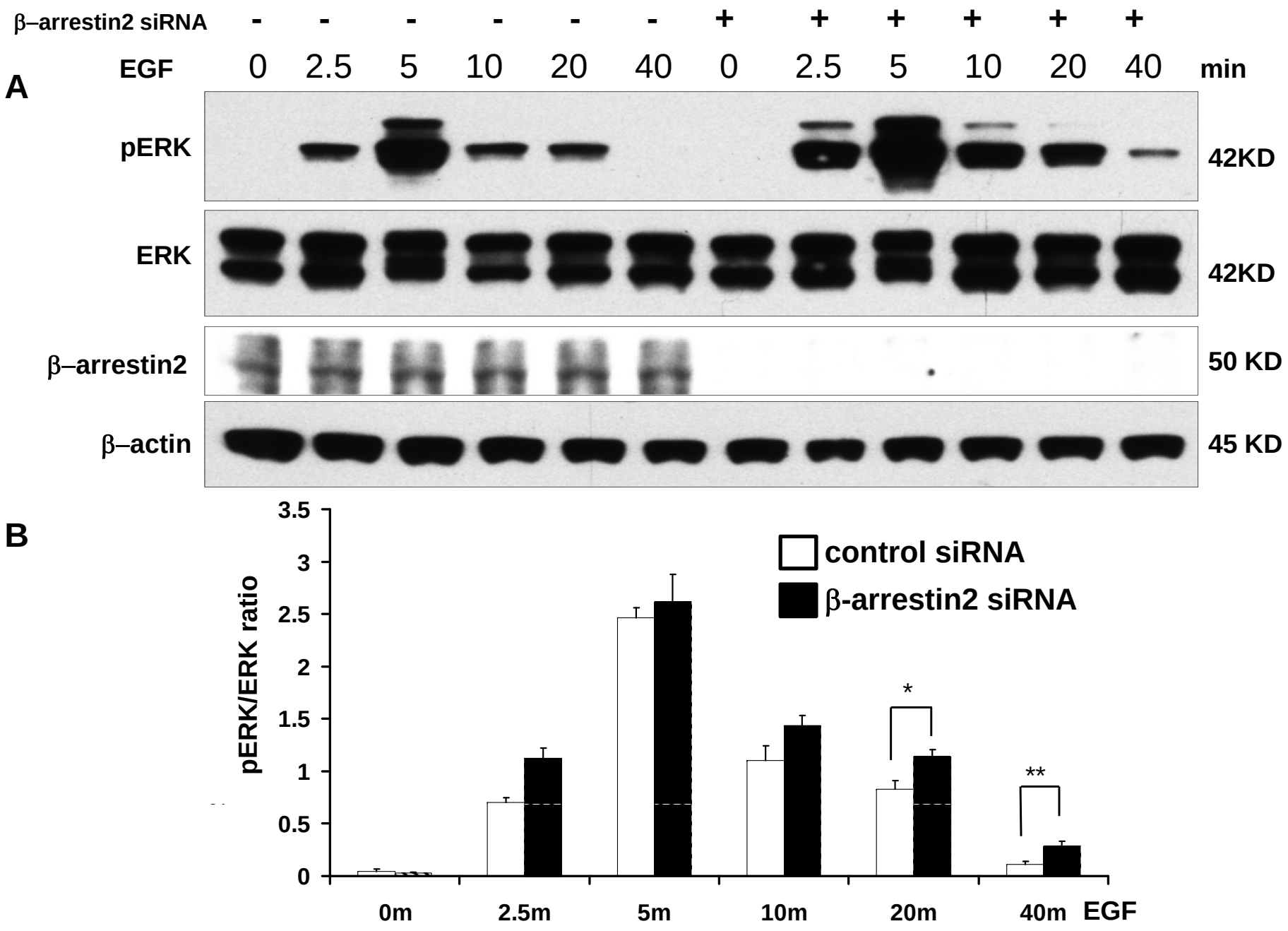
IP: eIF3a
200 KD
Lysates
IB: VSV
50 KD

Fig. 2.


β–arrestin2 siRNA - - - - - - + + + + + +
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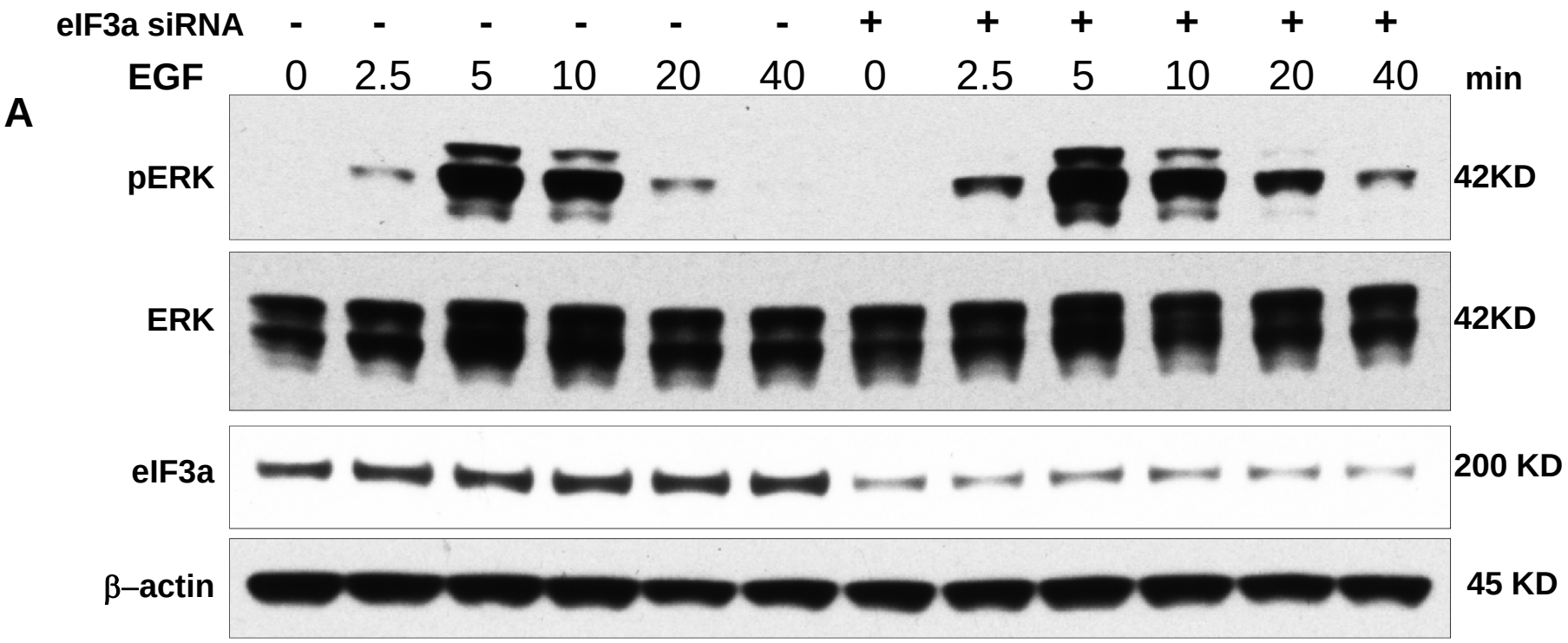
pERK
42KD
ERK
42KD
β–arrestin2
50 KD
β–actin
45 KD
B
pERK/ERK ratio
3.5
3
2.5
2
1.5
1
0.5
0
control siRNA
β-arrestin2 siRNA
*
**
0m
2.5m
5m
10m
20m
40m
EGF

Fig. 3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eIF3a siRNA - - - - - - + + + + + +
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pERK
42KD
ERK
42KD
eIF3a
200 KD
β–actin
45 KD

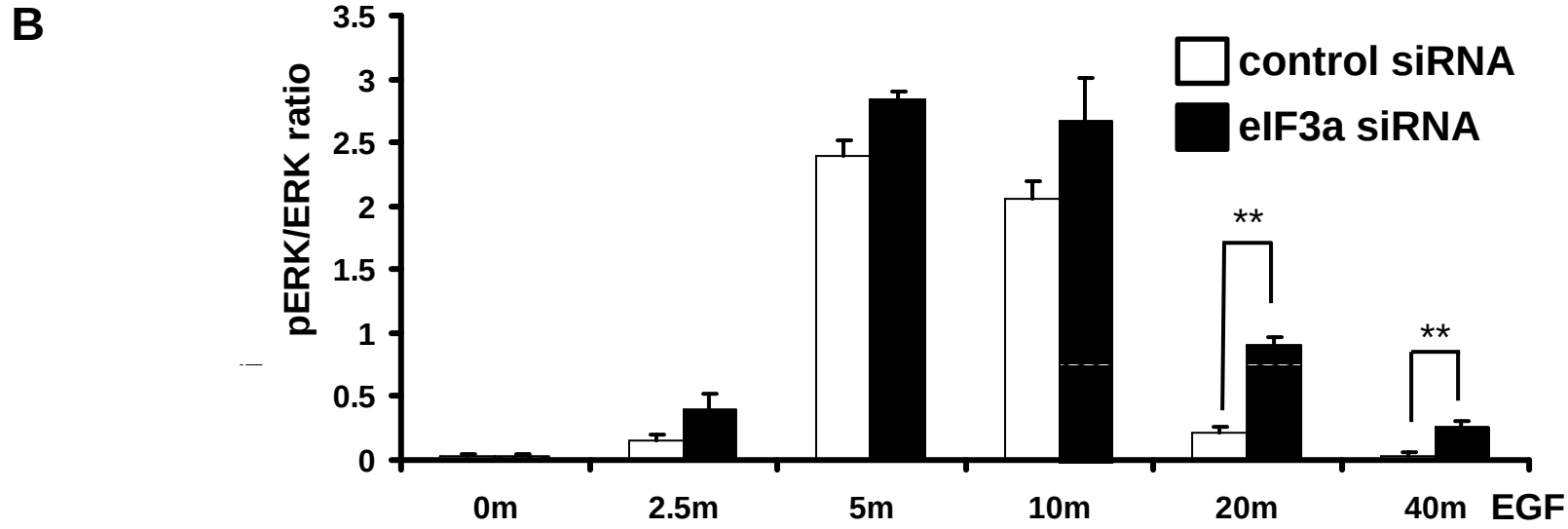
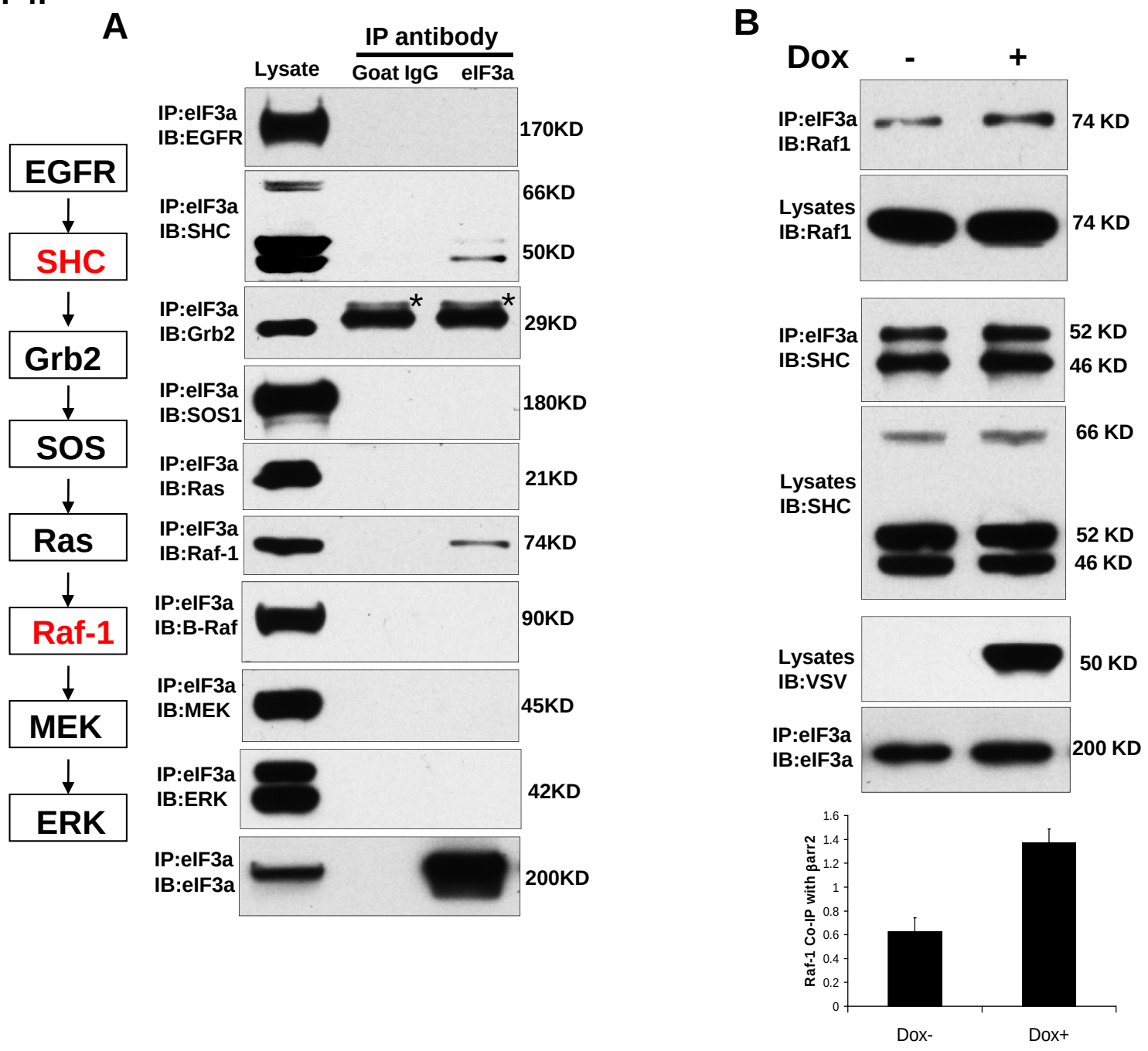

B
pERK/ERK ratio
3.5
3
2.5
2
1.5
1
0.5
0
control siRNA
eIF3a siRNA
**
**
0m
2.5m
5m
10m
20m
40m
EGF

Fig. 4.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IP antibody
Lysate
Goat IgG
eIF3a
EGFR
SHC
Grb2
SOS
Ras
Raf-1
MEK
ERK
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170KD
IP:eIF3a IB:SHC
66KD
50KD
IP:eIF3a IB:Grb2
29KD
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180KD
IP:eIF3a IB:Ras
21KD
IP:eIF3a IB:Raf-1
74KD
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90KD
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45KD
IP:eIF3a IB:ERK
42KD
IP:eIF3a IB:eIF3a
200KD
B
Dox
-
+
IP:eIF3a IB:Raf1
74 KD
Lysates IB:Raf1
74 KD
IP:eIF3a IB:SHC
52 KD
46 KD
Lysates IB:SHC
66 KD
52 KD
46 KD
Lysates IB:VSV
50 KD
IP:eIF3a IB:eIF3a
200 KD
Raf-1 Co-IP with βarr2
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Dox-
Dox+

Fig. 5.

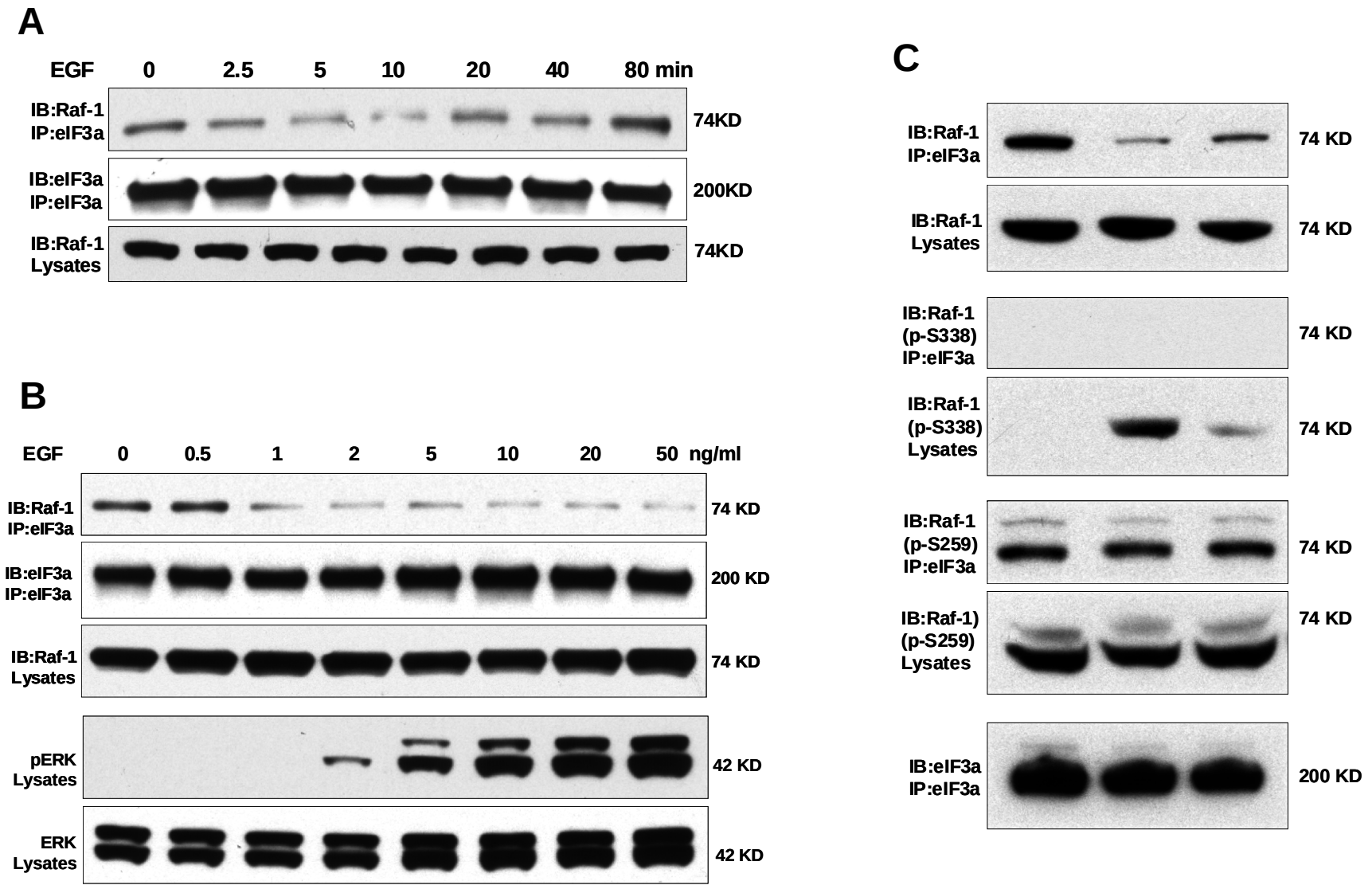
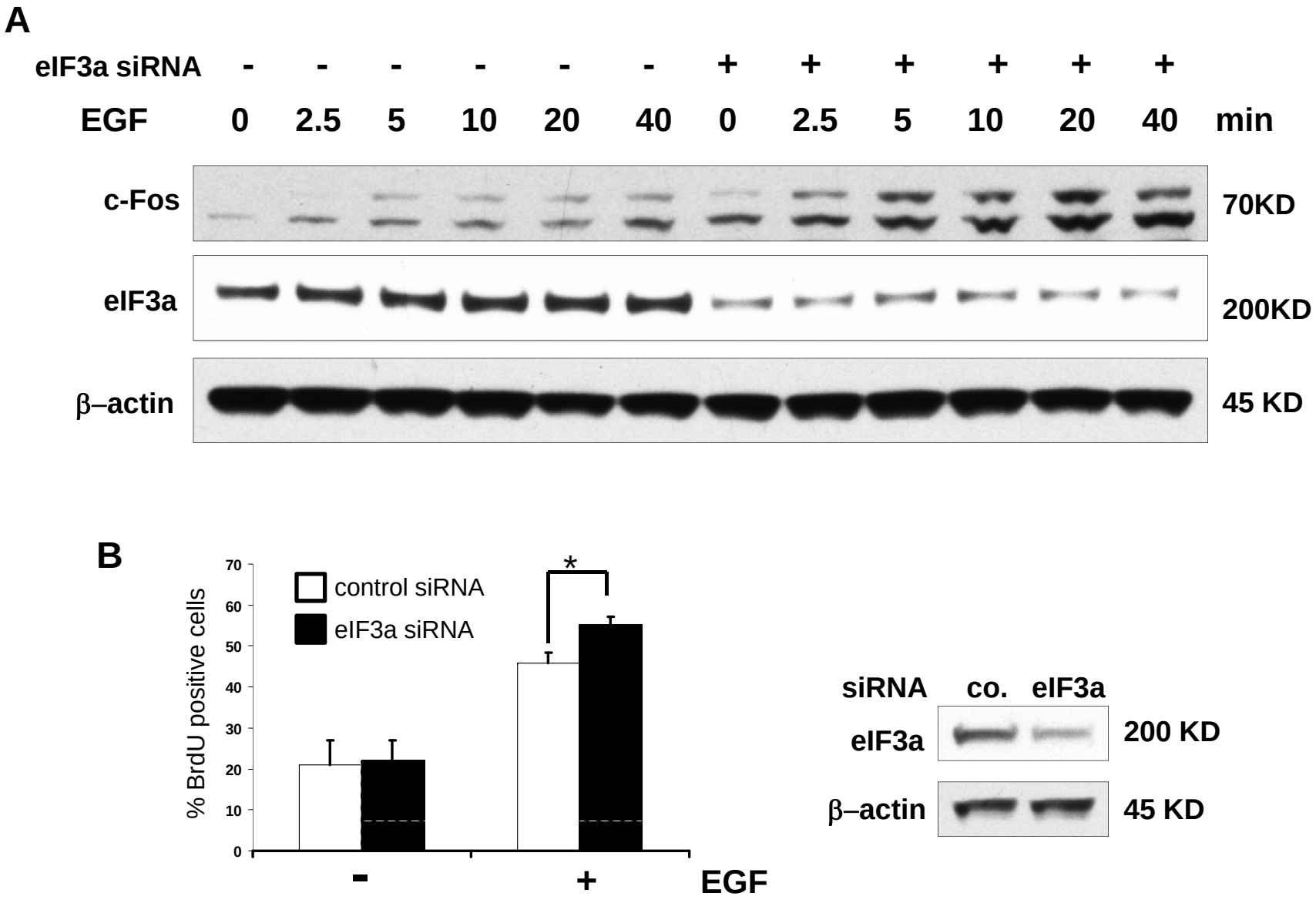

A
EGF 0 2.5 5 10 20 40 80 min
IB:Raf-1
IP:eIF3a
74KD
IB:eIF3a
IP:eIF3a
200KD
IB:Raf-1
Lysates
74KD
B
EGF 0 0.5 1 2 5 10 20 50 ng/ml
IB:Raf-1
IP:eIF3a
74 KD
IB:eIF3a
IP:eIF3a
200 KD
IB:Raf-1
Lysates
74 KD
pERK
Lysates
42 KD
ERK
Lysates
42 KD
C
IB:Raf-1
IP:eIF3a
74 KD
IB:Raf-1
Lysates
74 KD
IB:Raf-1
(p-S338)
IP:eIF3a
74 KD
IB:Raf-1
(p-S338)
Lysates
74 KD
IB:Raf-1
(p-S259)
IP:eIF3a
74 KD
IB:Raf-1)
(p-S259)
Lysates
74 KD
IB:eIF3a
IP:eIF3a
200 KD

Fig. 6.


A
eIF3a siRNA - - - - - - + + + + + +
EGF 0 2.5 5 10 20 40 0 2.5 5 10 20 40 min
c-Fos
70KD
eIF3a
200KD
β–actin
45 KD
B
% BrdU positive cells
control siRNA
eIF3a siRNA
*
EGF
siRNA co. eIF3a
eIF3a
200 KD
β–actin
45 KD