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c-Fos-activated synthesis of nuclear phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] promotes global transcriptional changes

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c-Fos is a well-recognized member of the AP-1 (activator protein-1) family of transcription factors. In addition to this canonical activity, we previously showed that cytoplasmic c-Fos activates phospholipid synthesis through a mechanism independent of its genomic AP-1 activity. c-Fos associates with particular enzymes of the lipid synthesis pathway at the endoplasmic reticulum and increases the V_{max} of the reactions without modifying the K_m values. This lipid synthesis activation is associated with events of differentiation and proliferation that require high rates of membrane biogenesis. Since lipid synthesis also occurs in the nucleus, and different phospholipids have been assigned transcription regulatory functions, in the present study we examine if c-Fos also acts as a regulator of phospholipid synthesis in the nucleus. Furthermore, we examine if c-Fos modulates transcription through its phospholipid synthesis activator capacity. We show that nuclear-localized c-Fos associates with and activates PI4P5K (phosphatidylinositol-4-monophosphate 5-kinase), but not with PI4KIIIβ (type IIIβ phosphatidylinositol 4-kinase) thus promoting PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) formation, which, in turn, promotes transcriptional changes. We propose c-Fos as a key regulator of nuclear PtdIns(4,5)P_2 synthesis in response to growth signals that results in c-Fos-dependent transcriptional changes promoted by the newly synthesized lipids.

Key words: activator protein-1 (AP-1) transcription factor, c-Fos-dependent phospholipid synthesis, nuclear phospholipid synthesis, phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2], phosphatidylinositol 4-monophosphate 5-kinase (PI4P5K), transcription regulation.

INTRODUCTION

More than 30 years ago it was shown that the genome is packaged in a repetitive nuclear subunit structure of which the nucleosome is the fundamental unit [1,2]. The compacted chromatin structure is inherently repressive to processes such as RNA transcription, DNA replication and DNA repair, all of which require accessibility to the DNA strand. In addition to containing the genome, the nucleus has a high capacity for lipid metabolism: the presence of sphingolipids [3] and phospholipids [4–6] has been unequivocally demonstrated both in chromatin and in the NM (nuclear matrix). Many studies have established that in the nuclear compartment distinct lipid second messengers elicit reactions that regulate gene transcription as well as DNA synthesis and pre-mRNA splicing [7–11]. Although different phospholipids such as phosphatidylcholine, phosphatidylethanolamine and PtdSer (phosphatidylserine) have also been assigned nuclear functions, PtdInsPs (polyphosphoinositides) are by far the most extensively characterized nuclear lipids [12,13].

PtdInsPs can regulate protein–chromatin and protein–nucleic acid interactions [14]. Indeed, a proteomics study of PtdInsP-interacting nuclear proteins revealed the overrepresentation of functions related to RNA splicing, chromatin assembly and DNA topological changes [15]. Such is the case of the SWI/SNF-like chromatin remodelling complex BAF (Brg/Brm-associated factor) that, upon lymphocyte activation, is targeted to chromatin and to the NM by a PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate)-dependent mechanism [11]. PtdIns(4,5)P_2 also interacts with histone H1 and inhibits its ability to suppress basal transcription by RNA polymerase *in vitro*, a phenomenon abolished by histone H1 phosphorylation [5]. Recently, a mode of gene-specific transcriptional repression through nuclear PtdIns(4,5)P_2 was demonstrated. The transcriptional co-repressor BASP1 (brain-abundant membrane-attached signal protein 1) converts the transcriptional regulator WT1 from a transcriptional activator into a repressor. For this, BASP1 needs to be myristoylated and associated with PtdIns(4,5)P_2 to interact with HDAC1 (histone deacetylase 1) [16].

The concentration of lipids, and particularly of PtdInsPs, can be dynamically regulated in response to signalling cascades in the different subcellular compartments [17]. The first reports showing the presence of lipids in the nucleus together with the required enzymes and substrates for their synthesis appeared more than 20 years ago [13,18,19]. Biochemical and imaging studies later identified nuclear PtdIns(3)*P* (phosphatidylinositol 3-monophosphate), PtdIns(4)*P* (phosphatidylinositol 4-monophosphate), PtdIns(3,4)P_2 (phosphatidylinositol 3,4-bisphosphate), PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 (phosphatidylinositol 3,4,5-trisphosphate) and the specific nuclear pathways of inositol metabolism [11,20–23]. However, despite PtdIns(4,5)P_2 metabolism having been shown to play essential roles in several nuclear processes, the question of how nuclear PtdIns(4,5)P_2 metabolism

Abbreviations: Angptl4, angiopoietin-like 4; Anxa8, annexin A8; AP-1, activator protein-1; BAF, Brg/Brm-associated factor; BASP1, brain-abundant membrane-attached signal protein 1; CaXII, carbonic anhydrase 12; ER, endoplasmic reticulum; Fn1, fibronectin 1; IP, immunoprecipitation; Klf4, Kruppel-like factor 4; Mmp3, matrix metallopeptidase 3; NM, nuclear matrix; NP40, Nonidet P40; NSB, Nuclei Storage Buffer; Pdgfra, platelet-derived growth factor receptor α; PI4KIII, type III phosphatidylinositol 4-kinase; PI4P5K, phosphatidylinositol 4-monophosphate 5-kinase; Prkcc, protein kinase Cγ; PtdInsP, polyphosphoinositide; PtdIns(4)*P*, phosphatidylinositol 4-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(5)*P*, phosphatidylinositol 5-monophosphate; PtdSer, phosphatidylserine; Ptn, pleiotrophin; qPCR, quantitative real-time PCR; RT–PCR, reverse transcription–PCR.

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is regulated remains unanswered. We have shown previously that c-Fos, a well-known member of the AP-1 (activator protein-1) family of transcription factors, activates the extranuclear synthesis of phospholipids including PtdInsPs, in events related to cell growth and proliferation [24,25]. Interestingly, this latter activity of c-Fos is independent of its AP-1 transcription factor capacity [24,25], but requires the association of c-Fos with specific enzymes of phospholipid synthesis at the ER (endoplasmic reticulum) [26]. c-Fos/ER association/dissociation is highly regulated by the phosphorylation state of c-Fos tyrosine residues: tyrosine-phosphorylated c-Fos neither associates to the ER nor does it activate phospholipid synthesis [27]. c-Src is the kinase and TC45 the phosphatase that participate in this reversible phosphorylation/dephosphorylation cycle of c-Fos [28].

At least two concurrent and complex events occur in the nucleus: those leading to transcription and those to the synthesis of phospholipids. As c-Fos has been shown to participate in both of these events and nuclear phospholipid synthesis has been postulated to regulate transcription [10,29], the present study was directed towards determining if c-Fos regulates nuclear phospholipid synthesis and, if so, if this regulated phospholipid synthesis in its turn regulates gene expression. It was demonstrated that c-Fos is capable of activating the synthesis of PtdIns(4,5)P_2 within the nucleus, which, in its turn, promotes global transcriptional changes.

EXPERIMENTAL

Cell cultures, transfection and NM preparation

NIH 3T3 cells (A.T.C.C., Manassas, VA, U.S.A.) were grown under standard conditions in DMEM (Dulbecco's modified Eagle's medium; Life Technologies) plus 10 % FBS (Life Technologies). Upon desired confluence, growth was continued for 48 h without FBS to establish quiescence. For NM preparation, 20×10^6 quiescent cells were rinsed twice with ice-cold 10 mM PBS, incubated in 10 ml of NMPB [Nuclear Matrix Preparation Buffer; 20 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 20 mM NaCl, 100 mM sucrose and 0.25 % NP40 (Nonidet P40); unless otherwise specified, all reagents were acquired from Sigma–Aldrich], and left on ice for 10 min with hand-mixing several times. Preparations were spun at 216 ***g*** and the pellet resuspended in 100 μl of NSB [Nuclei Storage Buffer; 50 mM Tris/HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA and 45 % glycerol]. The purity control of the NM preparation is shown in Supplementary Figure S1 (http://www.biochemj.org/bj/461/bj4610521add.htm).

Transfections and stable cell lines were performed with Lipofectamine™ 2000 and the pGene/pSwitch Invitrogen system (Life Technologies) respectively, according to the manufacturer's protocol. The inducible Fos–YFP stable cell line was grown serum-deprived for 48 h to attain quiescence and then treated with 10 nM Mifepristone. Cells were washed twice in ice-cold 10 mM PBS and collected, at the times indicated, in TRIzol® reagent (Life Technologies) or RIPA buffer [20 mM Hepes (pH 7.4), 100 mM NaCl, 20 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT and 0.1 % NP40].

siRNA experiments

ON-TARGETplus PI4P5KIα (phosphatidylinositol 4-monophosphate 5-kinase iα)-specific and non-targeting siRNAs were obtained from Thermo Fisher. Upon reaching the desired confluence, NIH 3T3 cells were transfected using Lipofectamine™ 2000, at a final concentration of 25 nM siRNA, according to the manufacturer's protocol. After 24 h, growth was continued without serum for 48 h to establish quiescence and then cells were harvested and NMs prepared. After incubation with or without c-Fos (1 ng/μg of NM protein), RT–PCR (reverse transcription–PCR) and qPCR (quantitative real-time PCR) were performed as described below.

Preparation of recombinant Fos and Jun

His-tagged c-Fos or c-Jun were expressed and recovered from pDS56-HisFos- or pDS56-HisJun-transformed BL21 cells [30].

In vitro transcription

NMs isolated in NSB were incubated with 2× run-on reaction buffer {300 mM KCl, 10 mM $MgCl_2$, 2 mM cold (non-radioactive) rATP, rCTP, rGTP or rUTP (where r is recombinant) or 1 μC [^{32}P]UTP, specific activity 800 Ci/mmol; GE Healthcare) [31], ribonuclease inhibitor (100 units, Promega) with or without recombinant c-Fos (1 ng/μg of NM protein), and/or Wortmannin (1 μM) and/or PtdIns(4)*P* or PtdIns(4,5)P_2 (16.6 nM; Avanti Polar Lipids), as indicated. Samples were incubated at 37 °C for 60 min and reactions stopped with TRIzol® reagent.

In vitro phospholipid labelling

Phospholipid labelling was assessed by incubating 100 μg of NM protein for 15 min in a 100 μl final volume of a medium containing 20 mM Hepes buffer (pH 7.5), 10 mM $MgCl_2$ and 3 μCi of [γ^{32}P]ATP (PerkinElmer) [25] with or without recombinant Fos (1 ng/μg of NM protein) and lipid kinase activities measured [32] in a final volume of 200 μl containing 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM ATP, 10 μCi [γ^{32}P]ATP, 0.3 % Triton X-100 and 0.2 mg/ml, PtdIns/PtdSer or PtdIns(4)*P*/PtdSer (Avanti Polar Lipids) vesicles (2:1) and 30–50 mg of protein. Proteins were sonicated three times for 10 s on ice before use. Where indicated, Wortmannin (1 μM) was added using DMSO as a vehicle. Reactions were started by the addition of vesicles and carried out at 37 °C for 20 min. Under these conditions, lipid synthesis was linear with protein concentration and time. Reactions were stopped with chloroformmethanol (2:1) and lipids purified [33]. After partitioning with 0.2 vol. of H_2O, the organic phase was washed five times with chloroform/methanol/H_2O (3:48:47), dried and polyphosphoinositide lipids separated in a monodimensional two solvent system [34]. To detect kinase activity, chloroform/methanol/4.3 M NH_4OH (90:70:20) was used as the solvent mixture [35]. Labelled phosphoinositides were visualized by autoradiography and individual bands quantified using Scion Image Software.

Electrophoresis, Western blot and co-IP assays

For co-IP (immunoprecipitation) assays, 500 μg of total protein from cells treated as indicated was immunoprecipitated for 4 h at 4 °C with Protein G–Sepharose (GE Healthcare Biosciences) with the desired antibody, washed and immunodetection performed as described below. Cell lysates (10 μg) or IPs were fractionated through SDS-containing polyacrylamide gels (12 %) and electrotransferred at 300 mA for 1 h. Immunodetection was carried out at room temperature by blocking the membranes with 10 mM PBS containing 5 % (w/v) non-fat dried skimmed milk powder for 1 h, followed by incubation with an anti-Fos antibody (1:5000 dilution; Sigma–Aldrich), an anti-PI4P5K antibody (1:500 dilution; Santa Cruz Biotechnology), an anti-FLAG antibody (1:500 dilution: Sigma–Aldrich) or an anti-α-tubulin antibody (1:5000 dilution; Sigma–Aldrich) overnight at 4 °C in PBS/0.1 % Tween 20. Membranes were washed three

times (for 10 min) with PBS/0.1 % Tween 20 and incubated for 1 h at room temperature with an IRDye 800CW goat anti-mouse secondary antibody or an IRDye 800 goat anti-rabbit secondary antibody (1:25 000 dilution; LI-COR). Membranes were washed and immunodetection performed using an ODYSSEY Infrared Imaging System (LI-COR).

Immunofluorescence and microscopy

Cells grown on round acid-washed coverslips used to obtain NM preparations or as such were rinsed twice with ice-cold 10 mM PBS, fixed in 3 % (w/v) paraformaldehyde and 4 % (w/v) sucrose in PBS 10 mM at 37 °C for 10 min. Coverslips were blocked with blocking buffer [1 % (w/v) BSA in 10 mM PBS] for 2 h at room temperature in a humid chamber. Samples were incubated overnight at 4 °C in blocking buffer containing an anti-PtdInsPs antibody (1:50 dilution; catalogue number ab11039; Abcam), an anti-(histone H3 trimethylated at Lys[4]) antibody (1:500 dilution; Abcam), an anti-(histone H3 trimethylated at Lys[9]) antibody (1:500 dilution; Abcam), an anti-Fos antibody (1:500 dilution; Sigma–Aldrich) or an anti-PI4P5K antibody (1:100 dilution; catalogue number sc-11783; Santa Cruz Biotechnology), washed twice in 10 mM PBS/0.1 % Tween 20, incubated with Alexa Fluor® 546 and Alexa Fluor® 488 antibodies (1:1000 dilution; Life Technologies) for 2 h at room temperature, washed and mounted in FluorSave (Millipore). DAPI (Life Technologies) was used to visualize the nuclear structures. Imaging was performed on an Olympus FV300 confocal microscope. Scion Image Software was used to assess nucleus area.

RT–PCR and qPCR analysis

RNA (1 μg) purified using TRIzol® reagent by standard techniques was retrotranscribed with random primers and MMLV (Moloney murine leukaemia virus; Promega). qPCR experiments were performed using specific cDNA primers (final concentration 500 μM) from the Harvard Primer Database plus 2× Master Mix according to the manufacturer's protocol (Life Technologies). qPCR amplifications were carried out in triplicate on an 7500 A Applied Biosystems termocycler, using SyberGreen PCR Mastermix (Life Technologies). Relative gene expressions are calculated using the $\Delta\Delta C_T$ method. The normalized ΔC_T value of each sample was calculated using myoglobin as a housekeeping gene. Results are the average fold change (average $\Delta\Delta C_T$) for genes relative to the control samples. Selected genes, GenBank® accession numbers, descriptions and activation/repression states are listed in Supplementary Table S1 (http://www.biochemj.org/bj/461/bj4610521add.htm).

Microarray analysis using Affymetrix GeneChip

NIH 3T3 cells were incubated as indicated and RNA isolated using TRIzol® reagent and an RNeasy Kit (Qiagen). RNA was quantified using a NanoDrop ND-1000 spectrophotometer and tested for quality using an Agilent Bioanalyzer 2100 before use. cDNA was prepared using 50 ng of RNA and hybridized to an Affymetrix GeneChip Mouse Genome 2.0 array. Staining and post-hybridization washes were completed according to the manufacturer's recommendations (Affymetrix). Gene arrays were processed with a GeneChip Fluidics Station 450 and double staining captured using a Gene Array Scanner 3000.

Data analysis and bioinformatics

Hybridization intensities were quantified from the data image files using Gene Chip Operating Software algorithms (GCOS1.2; Affymetrix) with global scaling. Data analysis was performed using a Data Mining Tool (DMT 3.1; Affymetrix) and a GeneSpring 7.2 (Silicon Genetics). Cell intensity files were processed into expression values for all the 39 000 probe sets (transcripts) on each array following the respective normalization step. Differentially expressed genes were selected if they passed Welch's t test and parametric test and showed at least 2-fold changes between the control and treated sets. Heatmap images were generated using Nexus Expression Array tools.

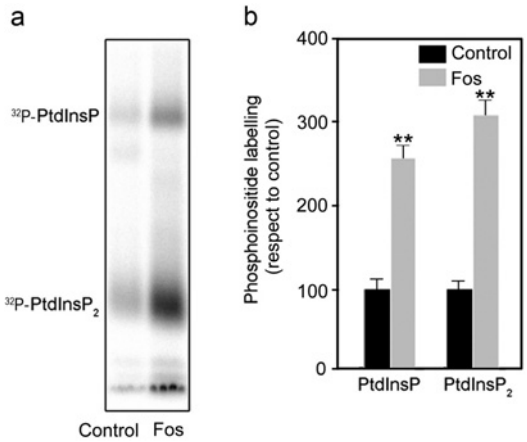

Figure 1 c-Fos activates PtdInsP and PtdInsP_2 synthesis in NMs

(**a**) Equal samples of isolated NMs (100 μg) were incubated with [γ^{32}P]ATP in the presence or the absence of recombinant c-Fos (1 ng/μg of protein), and [^{32}P]lipids separated by HP-TLC. Standards run in parallel denote the presence of labelled PtdInsP and PtdInsP_2, both of which showed a c-Fos-dependent increase in labelling. (**b**) Quantification of [^{32}P]lipids shown in (**a**). Results are expressed as the percentage incorporation with respect to the control which was taken as 100 % and are means ± S.D. for three independent determinations. $^{**}P < 0.001$ as determined by one-way ANOVA with Tukey's multiple comparison test.

RESULTS

c-Fos activates endogenous PtdInsP labelling and significantly increases NM size by a mechanism dependent on PtdInsP formation

As c-Fos activates the pathway of synthesis of PtdInsPs at the ER [24,26], we examined the capacity of c-Fos to activate the synthesis of the nuclear inositol family of phospholipids in NM preparations isolated from quiescent NIH 3T3 fibroblasts [31]. Initially, we confirmed that our NMs synthesize PtdInsPs from endogenous substrates by incubating these preparations in the presence of [γ^{32}P]ATP. As shown in Figure 1(a), NMs synthesize [^{32}P]PtdInsP and [^{32}P]PtdInsP$_2$, confirming the presence of both the required enzymes and the substrates of this pathway in these preparations [36–38]. Furthermore, addition of c-Fos to the assays (1 ng of c-Fos/μg of NM protein) significantly increases the labelling of these phospholipids. Quantification of these differences (~2-fold) is shown in Figure 1(b).

Next we examined the consequence of incubating NMs with c-Fos with respect to nuclear size, to the content of PtdInsPs and to the distribution of heterochromatin and euchromatin. Clearly, a significant increase in the mean size of NMs (2-fold) was promoted by the addition of c-Fos to the assays as evidenced in the DAPI-stained NMs of Figure 2(a) (compare panel a with panel g) and its quantification in Figure 2(b). Furthermore, c-Fos promotes an increase in the content of PtdInsPs in these NMs, as evidenced by immunolabelling of PtdInsPs (Figure 2a, h and b). Figure 2(a) and the quantification in Figure 2(b) show

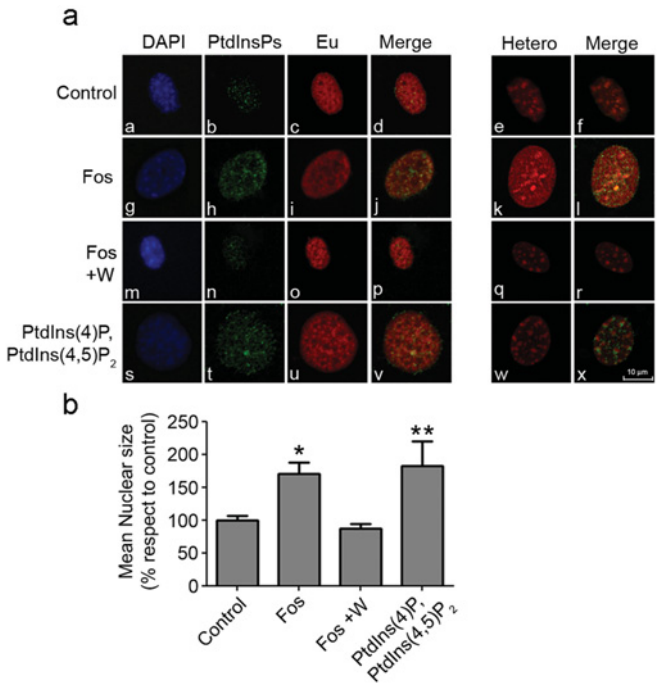

Figure 2 c-Fos-promoted increase of NM size is dependent on PtdInsP formation

(**a**) NMs were incubated in the absence (Control), the presence of c-Fos (1 μg/mg of NM protein) (Fos), the presence of c-Fos and Wortmannin (Fos + W) or of an equimolar mixture (16.6 nM each lipid) of PtdIns(4)*P* plus PtdIns(4,5)P_2 [PtdIns(4)P_1 PtdIns(4,5)P_2]. The NMs treated with c-Fos show an increase in the PtdInsP content and in NM size, similar to PtdInsPs incubation. No effect was observed when the NMs were incubated with Wortmannin or in the absence of c-Fos. NMs were stained with DAPI, for PtdInsPs, for euchromatin (Eu) or for heterochromatin (Hetero). The Merge column of the left-hand panel is the combination of the PtdInsPs and Eu columns, whereas the Merge column of the right-hand panel is the combination of the Hetero and PtdInsPs columns (this is not shown because PtdInsP immunolabelling showed a similar pattern of distribution, indicating no changes with respect to their localization with either euchromatin or heterochromatin). Scale bar, 10 μm. (**b**) Morphometric quantification of the NM area was performed using ImageJ software. Results are the means $\pm$ S.D. ($n = 50$ for the + c-Fos samples and $n = 65$ for the NMs incubated with the equimolar mixture of lipids) with respect to controls quantified from three different preparations in each case. $^*P < 0.01$ and $^{**}P < 0.001$ as determined by one-way ANOVA with Tukey's multiple comparison test.

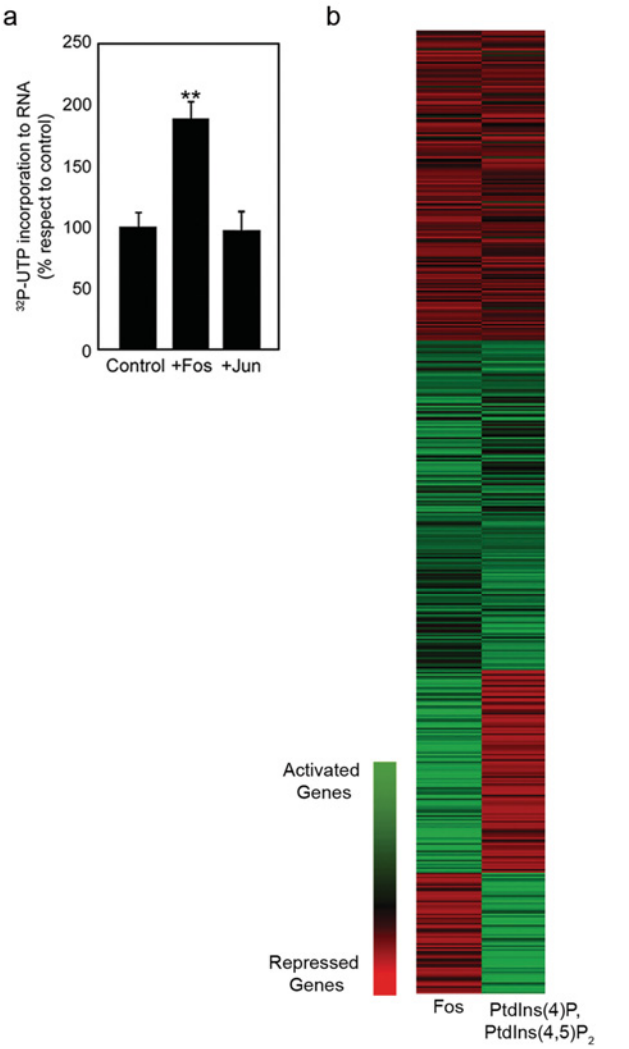

Figure 3 c-Fos increases [^{32}P]RNA labelling in NMs and promotes transcriptional changes in more than 1000 genes

(**a**) NMs were incubated with [α^{32}P]UTP in the absence (Control) or presence (1 ng/μg of NM protein) of c-Fos (+Fos) or c-Jun (+Jun) for 1 h at 37 °C. Total RNA was purified and [^{32}P]RNA quantified. Note the significant increase in [^{32}P]RNA labelling in c-Fos-treated NMs as compared with the non-treated or the c-Jun-treated NMs. $^{**}P < 0.001$ ($n = 3$) as determined by one-way ANOVA with Tukey's multiple comparison test. (**b**) RNA (50 ng) obtained from NMs incubated with or without c-Fos or with PtdInsP and PtdInsP_2 was retrotranscribed to cDNA, hybridized against an Affymetrix GeneChip Mouse Genome 2.0 array and processed with a GeneChip fluidics station 450. Double staining was captured using a gene array scanner 3000. Activated genes are pseudo-coloured green and repressed genes red, as indicated.

that no changes are observed in NM size between NMs incubated without c-Fos and those incubated with c-Fos plus Wortmannin, an inhibitor of PI4KIII (type III phosphatidylinositol 4 kinase) [39] that converts PtdIns into PtdIns(4)*P* [40]. To further confirm that the effect of c-Fos was mediated by the increased amount of the PtdInsPs, an equimolar mixture of PtdIns(4)*P* and PtdIns(4,5)P_2 (16.6 nM of each lipid) was exogenously added to the NM preparations. It is clear that the addition of these lipids to NMs promoted similar changes in the NM size as those observed after their incubation with c-Fos. The possible contribution of PI3Ks (phosphatidylinositol 3-kinases) was disregarded because these enzymes are strongly inhibited by the non-ionic detergents used in the NM preparation buffer [41]. Co-localization between both euchromatin and heterochromatin with PtdInsPs was observed without apparent changes in their distribution or interaction (Figure 2a, Merge). Taken together, these results indicate that the increase observed in the size of NMs is dependent on the c-Fos-promoted activation of PtdInsP synthesis. Changes in nucleus size have been previously linked to wide global rearrangements in genomic structure [42].

c-Fos induces AP-1 independent transcriptional changes in NM genes *in vitro*

To examine if c-Fos also promotes transcriptional changes in these preparations, nuclear run-on experiments were carried out. NMs from quiescent fibroblasts were incubated with [α^{32}P]UTP in the presence or the absence of added c-Fos. As the labelled nucleotide is incorporated into nascent RNA, the amount of [^{32}P]RNA obtained is a direct indication of transcriptional activity. Figure 3(a) shows a significant increase in [^{32}P]RNA labelling in c-Fos-treated NMs (+Fos) compared with the non-treated NMs (control). By contrast, the addition of c-Jun, another well-known member of the AP-1 family of transcription factors [29] that lacks the capacity to activate lipid synthesis [25], tested at the same

concentration as c-Fos (1 ng/μg of NM protein), had no effect on [^{32}P]RNA labelling (Figure 3a).

To further examine the changes in gene expression promoted by c-Fos, NMs prepared from quiescent fibroblasts were incubated with or without c-Fos (1 ng/μg of NM protein), RNA was extracted, retrotranscribed to cDNA and hybridized against a cDNA microarray. More than 1000 genes (listed in Supplementary Table S1) showed expression changes in Fos-treated compared with untreated arrays (Figure 3b). By contrast, incubation of NMs with c-Fos plus Wortmannin showed substantially no transcriptional changes compared with c-Fos NMs (results not shown), whereas incubation of NMs with an equimolar mixture of PtdIns(4)*P* and PtdIns(4,5)P_2 (16.6 nM of each lipid) promoted similar changes in >1000 genes, resembling those observed after incubating NMs with c-Fos (listed in Supplementary Table S2 at http://www.biochemj.org/bj/461/bj4610521add.htm). Approximately 25 % of the genes showed opposite effects upon addition of c-Fos compared with the addition of PtdIns(4)*P* and PtdIns(4,5)P_2 (Figure 3b, see genes shown in the bottom of the array): these genes showed no activation/repression upon addition of Wortmannin to the NMs and no further studies involving these genes were performed. Of the genes that showed similar changes upon c-Fos or PtdIns(4)*P* and PtdIns(4,5)P_2 addition, we identified some 200 genes that showed changes $\geqslant$5-fold in both treatments. Of these, we selected three representative genes from the group showing increased expression [*Angptl4* (angiopoietin-like 4), *Pdgfra* (platelet-derived growth factor receptor α) and *Ptn* (pleiotrophin)] and three of those that decreased in expression [*Anxa8* (annexin A8), *Klf4* (Kruppel-like factor 4) and *Prkcc* (protein kinase Cγ)], but taking into consideration the additional selection criteria that they did not contain an AP-1 consensus sequence. A third group of three genes that did not show transcriptional changes [*Fn1* (fibronectin 1), *Mmp3* (matrix metallopeptidase 3) and *CaXII* (carbonic anhydrase 12)] was also selected on the basis that they contained at least one AP-1 consensus sequence and are known to respond to AP-1 transcription factors [43].

qPCR amplifications of cDNA from NMs incubated with and without c-Fos were performed using specific primers for the nine genes selected. The results in Figure 4 confirm that treatment of NMs with c-Fos promotes both gene activation and repression of the previously selected genes with no changes observed in the non-changing third group. However, if c-Fos treatment was performed in the presence of Wortmannin, no activation or repression was observed in either of the first two groups indicating once again that PtdInsPs are mediating these transcriptional changes. To further examine this possibility, NMs were incubated in the presence of PtdIns(4)*P* and PtdIns(4,5)P_2 and qPCR performed. Similar changes in gene expression to those observed when incubating NMs with c-Fos were found in the first two groups with no significant changes in the third non-responsive group (Figure 4). These results are in line with those obtained with the microarray studies (Figure 3b) and indicate that the changes observed are independent of the well-known AP-1 transcription factor activity of c-Fos because in no case were changes observed in the genes containing an AP-1 consensus sequence.

PtdIns(4,5)P_2 formation is activated by c-Fos in NMs and promotes transcriptional changes

Next we examined if one or both lipids, PtdIns(4)*P* and PtdIns(4,5)P_2, promoted the observed transcriptional changes by incubating NMs separately with PtdIns(4)*P* or with PtdIns(4,5)P_2 and determining transcriptional levels by qPCR amplification.

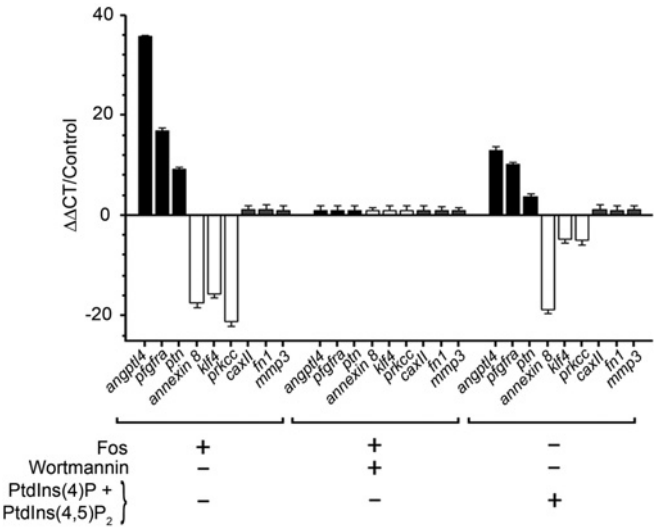

Figure 4 c-Fos and PtdInsPs promote similar transcription changes in particular genes

The previous activation/repression events observed in the array were confirmed by qPCR using the selected genes to determine the transcriptional changes. Note the absence of changes in the AP-1-responsive genes. NMs were incubated with or without c-Fos and/or Wortmannin or an equimolar mixture of PtdIns(4)*P* and PtdIns(4,5)P_2 as indicated. Total RNA was purified, retrotranscribed to cDNA and subjected to qPCR amplification with specific cDNA primers for the selected genes. Relative gene expression was calculated using the $\Delta\Delta C_T$ method. To calculate the normalized $\Delta\Delta C_T$ value of each sample, myoglobin was used as the housekeeping gene. Results are expressed as average fold change ($\Delta\Delta C_T$) for genes in treated relative to control samples and are the mean $\pm$ S.D. of the relative gene expression values from three independent experiments performed in triplicate.

Figure 5(a) shows that both PtdIns(4)*P* and PtdIns(4,5)P_2 promote transcriptional changes of the selected genes, although changes promoted by PtdIns(4,5)P_2 were in all cases greater than those promoted by PtdIns(4)*P*. Incubation of NMs with PtdIns(4,5)P_2 plus Wortmannin had no effect on transcriptional changes (Figure 5b). The simplest interpretation of these results is that PtdIns(4,5)P_2 is in fact the lipid promoting the described transcriptional changes. The changes observed upon PtdIns(4)*P* addition are probably due to its conversion into PtdIns(4,5)P_2 during incubation, and that the Wortmannin inhibition on transcriptional activity observed in Figure 4 could be due to the upstream pathway inhibition at the PI4KIII level.

The formation of PtdIns(4,5)P_2 by PI4P5K is activated by c-Fos

It has been shown previously that, at the ER, c-Fos physically interacts with the enzymes of the PtdInsPs synthesis that it activates [26]. PtdIns(4,5)P_2 can be synthesized by two different pathways, that is, through the formation of PtdIns(4)*P* or PtdIns(5)*P* (phosphatidylinositol 5-monophosphate). However, the nuclear mass level of PtdIns(4)*P* is at least 20-fold higher than that of PtdIns(5)*P* [39,40,44] and most nuclear PtdIns(4,5)P_2 is primarily synthesized through the PI4P5K pathway [45]. In NIH 3T3 cells, the conversion of PtdIns into PtdIns(4)*P* is catalysed mainly by PI4KIIIβ [32] and, as mentioned before, is inhibited by Wortmannin [39]. As shown in Figure 1, the labelling of both PtdInsP and PtdInsP$_2$ are increased upon incubation of NMs with c-Fos and, therefore, both enzymes could have been activated. However, as PtdIns(4)*P* is the substrate of the enzyme PI4P5K for PtdIns(4,5)P_2 synthesis, if only PI4P5K is activated by c-Fos, more PtdIns(4)*P* will be consumed for PtdIns(4,5)P_2 synthesis and, in consequence, more PtdIns(4)*P* must be generated by the

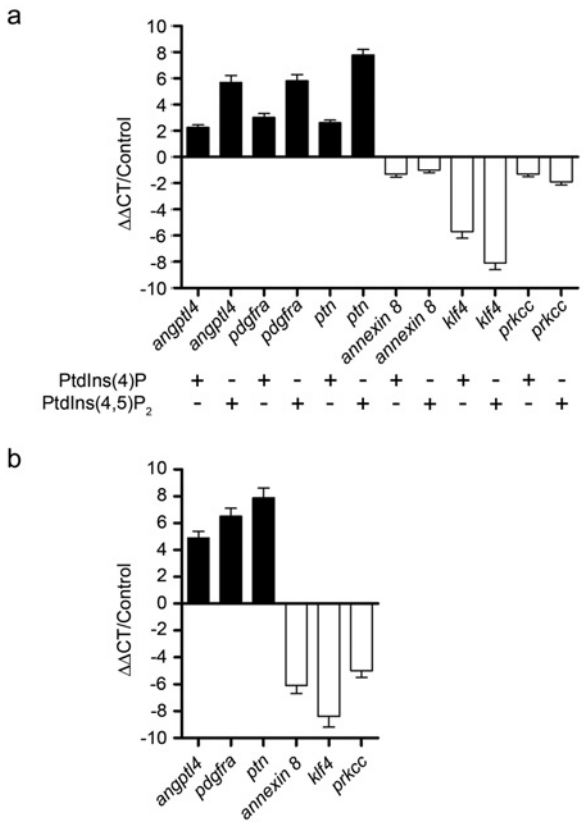

Figure 5 Addition of PtdIns(4,5)P_2 to NMs promotes similar transcriptional changes as c-Fos

(**a**) NMs were incubated in the presence of PtdIns(4)*P* or of PtdIns(4,5)P_2 as indicated and transcriptional levels determined as described in the legend of Figure 4. Results are the mean ± S.D. of the relative gene expression values from three independent experiments performed in triplicate. PtdIns(4,5)P_2 promotes changes of a greater magnitude than PtdIns(4)*P*. (**b**) NMs were incubated in the presence of PtdIns(4,5)P_2 plus Wortmannin and transcriptional levels determined as in (**a**). Note that the transcriptional changes are promoted by PtdIns(4,5)P_2 irrespective of the presence of Wortmannin in the incubations. Results are the mean ± S.D. of the relative gene expression values from three independent experiments performed in triplicate.

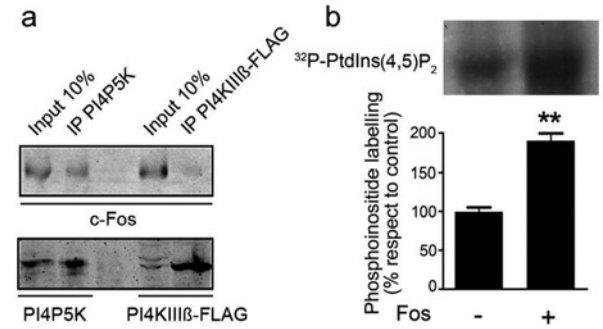

Figure 6 c-Fos interacts with the enzyme PI4P5K and increases PtdIns(4,5)P_2 formation

Quiescent cells were transfected with FLAG-tagged PI4KIIIβ and stimulated to re-enter growth by feeding with FBS for 1 h. (**a**) Lysates from transfected cells were immunoprecipitated using a mouse anti-PI4P5K antibody or anti-FLAG antibodies, as indicated. As can be observed, c-Fos co-immunoprecipitates with PI4P5K, but not with PI4KIIIβ. Western blotting with the anti-c-Fos antibody is shown in the top panel. In the bottom, the immunoprecipitates obtained for each enzyme were examined using anti-FLAG or anti-PI4P5K antibodies. Western blotting of enzyme inputs (10%) are indicated. (**b**) NMs were incubated with PtdIns(4)*P*/PtdSer vesicles in the presence of c-Fos (1 ng/μg of NM protein) as indicated the Experimental section. The rate of ^{32}P labelling of PtdIns(4,5)P_2 from [γ^{32}P]ATP increases with respect to c-Fos absence, as determined by autoradiography (upper panel) and quantified as the percentage with respect to NMs incubated in the absence of c-Fos (lower panel). Results are means ± S.D. for three independent experiments. $^{**}P < 0.001$ as determined by Students two-tailed *t* test.

phosphorylation of PtdIns to replenish the pool of PtdIns(4)*P*. As [^{32}P]ATP is the donor nucleotide, more labelled PtdIns(4)*P* would be found in the NMs despite PI4K not being activated. Consequently, we examined if a physical interaction between either of the enzymes PI4KIIIβ or PI4P5K with c-Fos could be shown using co-IP assays. For this, quiescent cells were transfected with FLAG-tagged PI4KIIIβ and stimulated to re-enter growth with FBS for 1h. Then NMs were prepared and co-IP experiments carried out using anti-PI4P5K or anti-FLAG antibodies to reveal PI4P5K and FLAG-tagged PI4KIIIβ respectively. Figure 6(a) shows that c-Fos co-immunoprecipitates with PI4P5K, but not with FLAG-tagged PI4KIIIβ.

To confirm that [^{32}P]PtdIns(4,5)P_2 formation is indeed activated by treatment of NMs with c-Fos, they were incubated with [^{32}P]ATP using PtdIns(4)*P* as the substrate, with or without c-Fos addition. The results of the autoradiogram shown in Figure 6(b) indicate that c-Fos promotes a significant increase in [^{32}P]PtdIns(4,5)P_2 formation compared with the amount labelled in the absence of c-Fos. This result further supports that PtdIns(4,5)P_2 synthesized in the nucleus following c-Fos activation promotes the observed transcriptional changes. It is also in agreement with the notion that c-Fos, irrespective of its subcellular localization, promotes lipid synthesis activation by interacting with the enzymes it regulates.

The most abundant form of PI4P5K in the nucleus is PI4P5KIα [11]. To further demonstrate that the activation of PI4P5KIα that forms PtdIns(4,5)P_2 promotes the transcriptional changes observed upon the addition of c-Fos, PI4P5KIα was specifically blocked in NIH 3T3 cells with a siRNA (Figure 7a). Then, NMs were obtained, incubated with or without c-Fos and qPCR amplification of cDNA was performed using specific primers for the six genes previously selected. As shown in Figure 7(b), treatment of cells with PI4P5KIα siRNA blocks the transcriptional changes observed upon incubation of NMs with c-Fos as compared with cells treated with non-targeting siRNA.

c-Fos co-localizes with PI4P5K in cultured cells and promotes AP-1-independent transcriptional changes

Monomeric c-Fos is highly mobile and can shuttle between the nucleus and the cytoplasm [46,47]. To confirm that c-Fos reaches a nuclear localization and interacts with PI4P5K, NIH 3T3 cells were stimulated to re-enter growth by the addition of FBS to the culture medium. Then they were stained with DAPI and immunolabelled for c-Fos and PI4P5K. As shown in Figure 7(c), FBS induces c-Fos expression (compare panel b with f) and c-Fos co-localizes with PI4P5K in the nucleus (see panels d and h).

To examine if the observed transcriptional changes promoted by c-Fos in isolated NMs also occur *in vivo*, we prepared a cell line that expresses YFP–c-Fos upon induction with Mifepristone (Supplementary Figure S2 at http://www.biochemj.org/bj/461/bj4610521add.htm). Quiescent cells that contain undetectable levels of AP-1 transcription factors, including c-Fos [24,48], were treated with Mifepristone for 3 h and compared with the non-treated cells or with cells treated with Mifepristone plus Wortmannin. Cells were harvested, RNA isolated and qPCR amplification of cDNA was performed for the genes previously examined (and shown in Figures 4a and 5). Figure 8 shows that the three genes from the group that previously

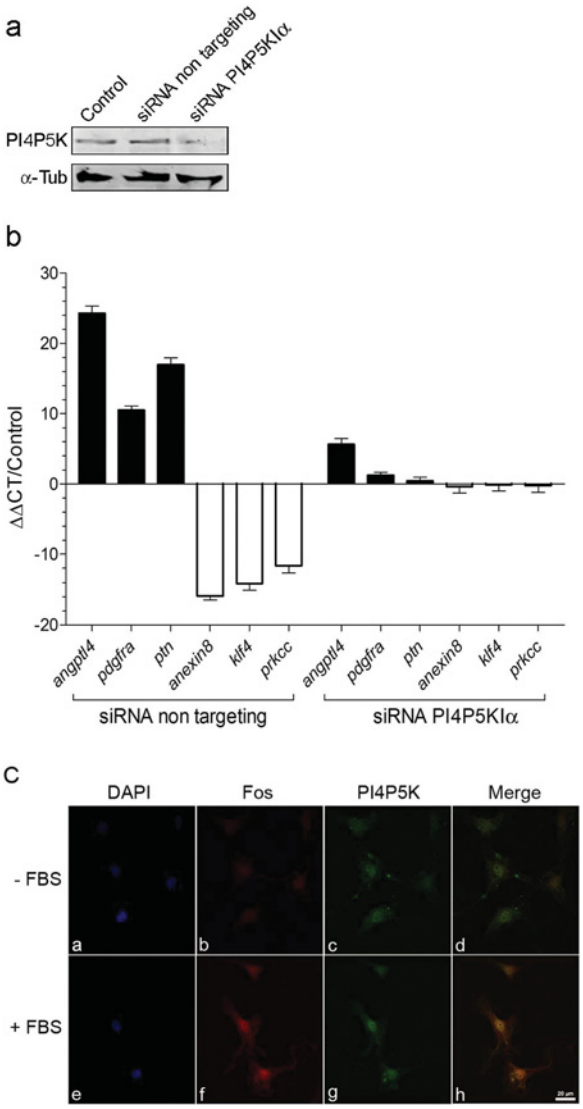

Figure 7 Transcriptional effects of blocking PI4P5KIα in NMs and subcellular localization in cultured cells

NIH 3T3 cells treated with PI4P5KIα siRNAs, non-targeting siRNAs or no siRNA (controls), were grown to establish quiescence, and then cells were harvested and NMs prepared. After incubation in the absence (controls) or the presence of c-Fos (1 ng/μg of NM protein) (siRNAs), RT-PCR and qPCR were performed as described in the legend of Figure 4. (**a**) Western blot of lysates obtained from non-transfected cells (control) or from cells transfected with non-targeting or with PI4P5KIα siRNAs, revealed with PI4P5KI antibody. Note the reduction in the expression of the enzyme after PI4P5KIα siRNA treatment. (**b**) Cells treated as in (**a**) were harvested, total RNA purified, retrotranscribed to cDNA and subjected to qPCR amplification with specific cDNA primers for the previously selected genes. Relative gene expression was calculated as described in the legend of Figure 4. Results are the mean ± S.D. of the relative gene expression values from three independent experiments performed in triplicate. (**c**) NIH 3T3 cells were stained for DAPI and immunostained using anti-c-Fos and anti-PI4P5K antibodies. Abundant c-Fos expression and c-Fos/PI4P5K co-localization was observed in the nucleus of NIH 3T3 cells cultured with FBS (lower row) as compared with quiescent (-FBS) cells (upper row). The merged images of c-Fos and PI4P5K labelling are shown in the fourth column. Scale bar, 20 μm.

showed increased expression (*Angptl4*, *Pdgfra* and *Ptn*) also increased after 3 h of Mifepristone treatment as compared with the control cells not treated with Mifepristone. In agreement, the three genes that previously showed decreased expression (*Anxa8*, *Klf4* and *Prkcc*) also decreased after 3 h of Mifepristone treatment, whereas the three AP-1-dependent genes examined (*Fn1*, *Mmp3* and *CaXII*) did not change following Mifepristone

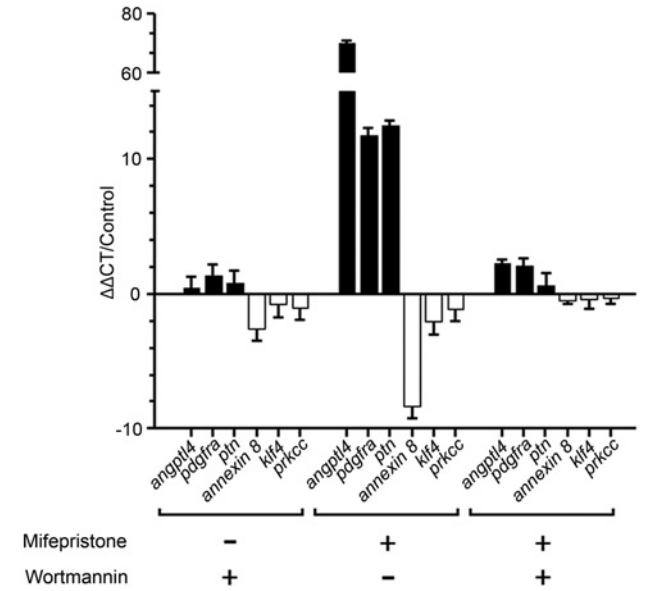

Figure 8 Wortmannin impairs transcriptional changes in AP-1-independent genes

Cells from a stable cell line that expresses c-Fos upon induction with Mifepristone were cultured in the presence or the absence of Mifepristone and in the presence or the absence of Wortmannin, as indicated, for 3 h. Total RNA was purified, retrotranscribed to cDNA and subjected to qPCR amplification with specific cDNA primers for the AP-1-independent genes previously examined in Figure 4. Note that Wortmannin completely impairs the Mifepristone-promoted changes. Results are expressed as average fold change ($\Delta\Delta C_T$) for genes in treated relative to control samples and are the mean ± S.D. of the relative gene expression values from three independent experiments performed in triplicate.

treatment. Induction with Mifepristone, but in the presence of Wortmannin, results in the abolishment of the observed changes (Figure 8). It should be noted that a 1.5 h treatment of cells with Mifepristone promoted similar, although smaller, changes than those observed after 3 h of treatment (Supplementary Figure S3 at http://www.biochemj.org/bj/461/bj4610521add.htm). These results further support the need for c-Fos-activated PtdIns(4,5)P_2 formation to promote the described transcriptional changes in cells in culture independently of its AP-1 activity.

Both AP-1-independent and -dependent transcriptional changes are observed in cultured cells induced to re-enter growth

It has been previously shown that in quiescent cells induced to re-enter growth by feeding with FBS, the expression of various members of the AP-1 family of transcription factors (i.e. c-Fos, c-Jun etc.) is induced [29,49]. Consequently, it was investigated whether the transcription of both the AP-1-independent and -dependent genes under examination were modified by the addition of FBS to the cell culture medium. Figure 9 shows the expected transcriptional changes promoted by FBS in the AP-1-dependent genes. In addition, the AP-1-independent genes that had increased (*Angptl4*, *Pdgfra* and *Ptn*) or decreased (*Anxa8*, *Klf4* and *Prkcc*) expression showed similar changes in cells treated with FBS. Our interpretation of these results is that, in cultured cells, heterodimeric and monomeric c-Fos co-exist so both mechanisms, c-Fos–AP-1-dependent and -independent regulation of gene expression occur when cells are induced to re-enter growth.

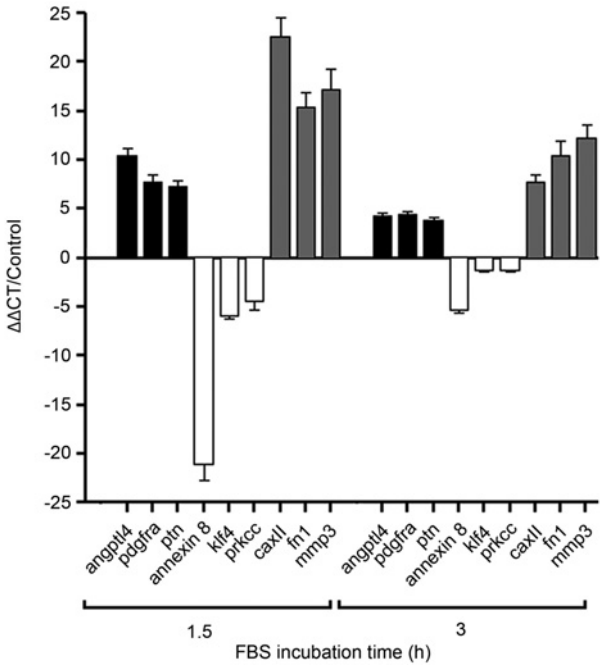

Figure 9 Induction of fibroblasts to re-enter growth promotes both AP-1-independent and -dependent transcriptional changes

Total RNA was purified from quiescent NIH 3T3 cells (-FBS) or from cells induced to grow by addition to the culture medium of 20 % FBS (+ FBS), retrotranscribed to cDNA and subjected to qPCR amplification with specific cDNA primers for the selected genes. In addition to the AP-1-independent genes, the levels of the genes responding to an AP-1 mechanism also increased. Relative gene expression was calculated using the $\Delta\Delta C_T$ method as described in the legend of Figure 4. Results are expressed as the average fold change ($\Delta\Delta C_T$) for genes in + FBS relative to -FBS samples and are the mean ± S.D. of the relative gene expression values from three independent experiments performed in triplicate.

DISCUSSION

Although the presence in the nucleus of phospholipids and particularly those of the PtdInsPs family has been extensively documented [40,50], their precise functions and the regulatory mechanisms that determine their concentration still need to be clarified. Emerging data indicate that nuclear PtdIns(4,5)P_2 regulates diverse processes including the stress response, cell-cycle control and mitosis, transcription, mRNA processing and export, DNA repair, chromatin remodelling and gene expression [9,51–55]. It is also becoming clear that specific nuclear PIPKs produce localized PtdIns(4,5)P_2 in response to different external and internal signals. It has been hypothesized that these nuclear PIPKs can be associated with organized nuclear scaffolds that contain other proteins, including effector proteins or even phospholipids. PtdIns(4,5)P_2 locally produced by PIPKs or delivered by phosphoinositide-carrier proteins could lead to conformational/stoichiometric changes in effector proteins that modulate nuclear biological processes [52]. In spite of the importance of the nuclear concentration of these lipids that, as shown in present and other studies [51], directly influence transcriptional responses, little is known about the mechanisms that regulate their nuclear production and content. Phosphoinositide metabolism uses many of the same enzymes and substrates in the nucleus as those found in the cytoplasm and plasma membrane [45]. Considering our previous observations that c-Fos activates polyphosphoinositide synthesis at the ER by associating with particular enzymes involved in their synthesis [26,56,57], we examined if the same mechanism operates in NMs. This is in fact the situation: c-Fos associates with and activates particular enzymes of the pathway of synthesis of a given lipid both at the ER and in the nucleus. In the light of these results, the notion that nuclear and extranuclear phosphoinositide metabolisms share enzymes and substrates should now also be extended to the mechanisms involved in regulating their production.

It has been well established that c-Fos forms hetero- but not homo-dimers that reach a nuclear localization as AP-1 transcription factors and activate/repress target genes that contain AP-1 consensus sites [48]. However, this canonical AP-1 activity of c-Fos fails to explain the activation/repression of many genes including some examined in the present study. In addition to the *in vitro* assays with NMs of Figures 3 and 4(a), examples of AP-1-independent c-Fos-dependent gene regulation are shown in Figure 8. In the cell line that expresses YFP–c-Fos upon Mifepristone induction (Figure 8), quiescent cells induced to express c-Fos, but not a c-Fos–AP-1 heterodimer partner, show similar changes in gene expression to those observed when inducing cells to re-enter growth by feeding cells with FBS. Furthermore, these genes do not contain an AP-1 consensus sequence. The simplest interpretation of these results is that c-Fos itself is sufficient to promote the regulation of the transcription of specific genes. However, to pinpoint this gene transcription regulation to c-Fos, c-Fos has to reach a nuclear localization and, if it is expected to fulfil a regulatory role, its concentration in the nucleus must be precisely regulated. This in fact seems to be the case: the cellular content of c-Fos is controlled at the level of transcription, RNA turnover and protein stability [29]; monomeric c-Fos has also been shown to be highly mobile, shuttling between the nucleus and the cytoplasm [46,47,58,59]. In addition to the control exerted on the cellular content of c-Fos, its entry to the nucleus is also controlled by at least two nuclear localization signals: its conventional basic nuclear localization signal and an unconventional nuclear localization signal located in the N-terminal moiety of the protein [46,47,58]. Heterodimerization of c-Fos with c-Jun results in a dramatic reduction in c-Fos intranuclear mobility, although by a mechanism not primarily mediated by binding to AP-1/TRE (tetradecanoylphorbal-13-acetate-responsive element) or CRE (cAMP-response element) target sequences, strengthening the idea of an AP-1-independent mechanism responsible for this reduction in c-Fos mobility [58].

The BAF complex mentioned above is dependent on PtdIns(4,5)P_2. This chromatin remodelling complex concomitantly induces decondensation of chromatin and an increase in the size of the nucleus, events that can be induced by the sole addition of PtdIns(4,5)P_2 to resting T-lymphocyte nuclei [8]. Several reports have shown that T-lymphocyte activation leads to an increase in nuclear c-Fos content [60]. Similarly, we showed than induction of quiescent NIH 3T3 fibroblasts to re-enter growth increases both nuclear size and PtdIns(4,5)P_2 content, the latter in a Fos-dependent way. Future studies are required to establish how ubiquitous this phenomenon is in different cell types as has been shown to occur, i.e. with the regulation of lipid synthesis by c-Fos, a mechanism that is shared by diverse cell types [57].

In conclusion, we propose c-Fos as a key regulator of two related phenomenon. One is the regulation of nuclear PtdInsP synthesis in response to growth signals that results in c-Fos-dependent transcriptional changes promoted by the newly synthesized lipids. The other is its canonical AP-1 transcription factor activity. It can be envisaged that both c-Fos–AP-1-independent and -dependent mechanisms will work co-ordinately when a cell re-enters the cell cycle.

AUTHOR CONTRIBUTION

Gabriel Ferrero planned and performed experiments, processed data, discussed the results and participated in the preparation of the paper. Marianne Renner planned and performed experiments, discussed the results and participated in the preparation of the paper. Germán Gil processed data, discussed the results and participated in the preparation of the paper. Lucia Rodriguez-Berdini planned and performed experiments, discussed the results and participated in the preparation of the paper. Beatriz Caputto planned experiments, discussed the results and participated in the preparation of the paper.

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online data

SUPPLEMENTARY ONLINE DATA

c-Fos-activated synthesis of nuclear phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] promotes global transcriptional changes

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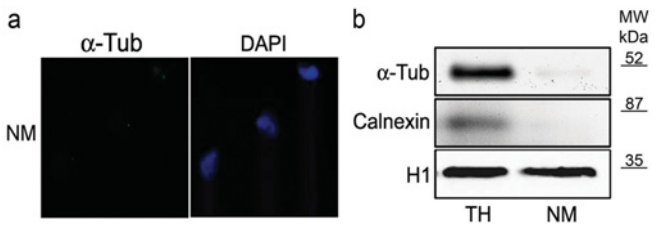

Figure S1 NM preparation and sample purification controls

(**a**) NMs obtained from NIH 3T3 cells were stained with DAPI and with an antibody against α-tubulin (α-Tub) and immunostained with Alexa Fluor® 488 to reveal the possible presence of α-tubulin. Note the absence of immunolabelling that is indicative of low or no contamination with cytosolic proteins. (**b**) Western blot of total homogenate of NIH 3T3 cells (TH) and of NMs obtained from the same cells (NM). Mebranes were revealed with antibodies against calnexin (ER marker) and α-tubulin. The absence of both of these markers indicates the high purity of the NM preparations. Histone 1 (H1) was used as a loading control.

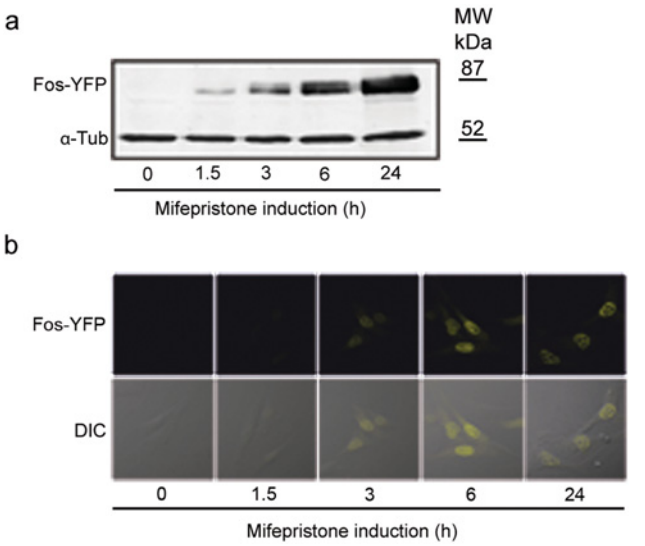

Figure S2 Inducible c-Fos–YFP expression and subcellular localization

A stable cell line was obtained that expresses c-Fos–YFP upon the addition of Mifepristone to the culture medium. (**a**) c-Fos expression upon Mifepristone induction at different times as indicated, shown by Western blotting. Total cell homogenates were immunostained for c-Fos and α-tubulin (α-Tub) as indicated in the Experimental section of the main text. (**b**) c-Fos–YFP expression upon Mifepristone induction in the stable cell line at different times in culture as indicated, shown by fluorescence microscopy. Differential interference contrast microscopy (DIC) shows the nuclear localization of c-Fos–YFP. Scale bar, 20 μm

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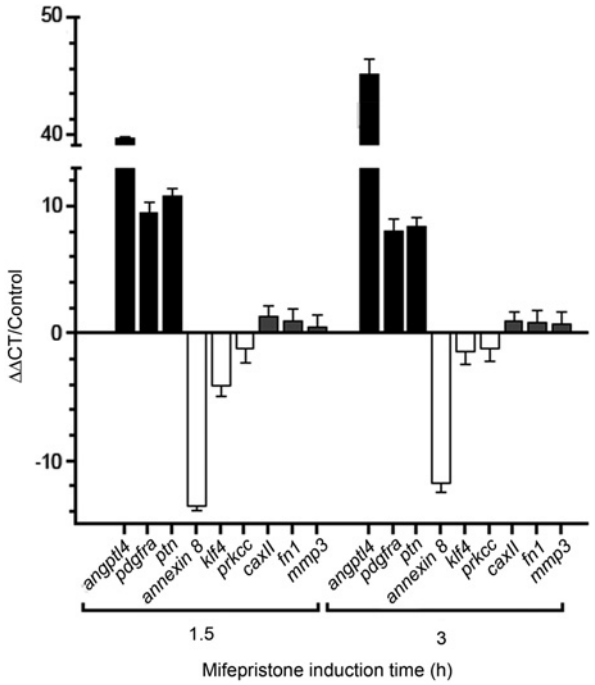

Figure S3 Transcriptional changes of the selected genes in the c-Fos–YFP stable cell line upon Mifepristone induction

Total RNA purified from the c-Fos–YFP stable cell line induced with Mifepristone for 1.5 or 3 h and from non-induced cells was retrotranscribed to cDNA and subjected to qPCR amplification with specific cDNA primers for the selected genes. Relative gene expression was calculated using the $\Delta\Delta C_T$ method. To calculate the normalized $\Delta\Delta C_T$ value of each sample, myoglobin was used as the housekeeping gene. Results are expressed as the means±S.D. fold change ($\Delta\Delta C_T$) for the genes in the treated relative to the control samples from three independent experiments performed in triplicate.

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