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**INTEGRIN SIGNALLING REGULATES THE NUCLEAR
LOCALISATION AND FUNCTION OF THE LYSOPHOSPHATIDIC
ACID RECEPTOR-1 (LPA₁) IN MAMMALIAN CELLS**

**Catherine M. Waters¹, Bahman Saatian², Noreen. A Moughal¹, Yutong Zhao²,
Gabor Tigyi³, Viswanathan Natarajan², Susan Pyne¹ and Nigel J. Pyne¹**

**From ¹Department of Physiology and Pharmacology, Strathclyde Institute for
Biomedical Sciences, University of Strathclyde, 27 Taylor St, Glasgow, G4 0NR, UK.**

**²Department of Medicine, University of Chicago, Center for Integrative Science
Building, Room 408B, 929, E.57th Street, Chicago, IL 60637. ³Department of
Physiology, University of Tennessee Health Science Center Memphis, 894 Union
Avenue, Memphis, USA**

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Address correspondence to: Nigel Pyne, Department of Physiology and Pharmacology,
Strathclyde Institute for Biomedical Sciences, University of Strathclyde, 27 Taylor St,
Glasgow, G4 0NR, UK. Tel. 44-141-5482659; FAX 44-141-5522562; e-mail:
n.j.pyne@strath.ac.uk

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ABSTRACT--We show here that the lysophosphatidic acid receptor-1 (LPA₁) is constitutively localised in the nucleus of mammalian cells. LPA₁ also traffics from cell membranes to the nucleus in response to LPA. Several lines of evidence also suggest an important role for cell-matrix interaction in regulating the constitutive nuclear localisation of LPA₁. First, RGDS, which blocks cell matrix-induced integrin clustering and cytoskeletal rearrangement, reduced the number of cells containing LPA₁ in the nucleus. Second, a higher proportion of cells contained nuclear LPA₁ when adhesion on fibronectin coated glass was compared with adherence to polylysine coated glass. Third, pre-treatment of cells with the Rho kinase inhibitor (Y27632) or the MLCK inhibitor (ML9) reduced the number of cells containing nuclear LPA₁. The addition of LPA and/or Ki16425 (which binds to LPA₁) to isolated nuclei containing LPA₁ induced the phosphorylation of several proteins with molecular masses of 34, 32, 14 and 11kDa. These findings demonstrate that trafficking of LPA₁ to the nucleus is influenced by cell-matrix interactions and that nuclear LPA₁ may be involved in regulating intranuclear protein phosphorylation and signalling.

ABBREVIATIONS

CHO, Chinese hamster ovary cells; HBEC, human bronchial epithelial cells; p42/p44 MAPK, p42/p44 mitogen-activated protein kinase; MLCK, myosin light chain kinase; LPA, lysophosphatidic acid; LPA₁, lysophosphatidic acid receptor-1; PI3K, phosphoinositide 3-kinase; PTX, pertussis toxin;

INTRODUCTION

The bioactive lipid, lysophosphatidic acid (LPA) is produced by a lysophospholipase D (autotaxin) and can be dephosphorylated by lipid phosphate phosphatases [1, 2]. LPA binds to a family of G-protein coupled receptors. Three of these are members of the endothelial differentiation gene family (termed EDG receptors, and now renamed LPA₁₋₃), whereas GPR23 (LPA₄) is a member of the purinergic GPCR cluster [1, 3]. The LPA₁ gene is located on chromosome 9p31.3-32 and encodes a 364 amino acid protein [3, 4]. LPA₁ is coupled to effectors via the heterotrimeric G-protein G_i to inhibit adenylyl cyclase and to stimulate activation of the p42/p44 mitogen activated protein kinase (p42/p44MAPK) pathway linked to mitogenesis [1]. This receptor can also activate Rho-dependent signalling to modulate actin stress fibre formation [5]. LPA₁ is widely expressed in embryonic neural tissue, where it may be involved in regulating neurogenesis. Targeted deletion of LPA₁ caused neonatal lethality due to decreased suckling, possibly due to olfaction defects, and craniofacial abnormalities [6].

Gobeil et al. [7] have shown that LPA₁ is constitutively localized in the nucleus of porcine cerebral microvascular endothelial cells, LPA₁ stably transfected rat hepatoma HTC4 cells and rat liver cells. Nuclear LPA₁ may function in an intracrine manner to regulate inducible nitric oxide synthase and cyclo-oxygenase expression. These LPA-stimulated responses are inhibited by PLA₂ inhibitors, suggesting a requirement for arachidonic acid metabolites and/or LPA formed by the action of PLA₂ [7]. In addition, Gobeil et al. [7] have shown that LPA₁ co-localises with caveolin 1 in a nuclear cell free system [7]. We have also shown significant constitutive localisation of LPA₁ in the nucleus of PC12 cells [8] and have reported that LPA stimulates the translocation of LPA₁ to the nucleus of these cells [8].

In this paper, we have defined the mechanisms regulating the constitutive and LPA-induced nuclear localisation of the LPA₁ and investigated whether the receptor has an intranuclear signalling function.

MATERIALS AND METHODS

Materials--All biochemicals including LPA were from Sigma Chemical Co. (Dorset, U.K.). CHO and PC12 cell culture supplies were from Invitrogen (Paisley, U.K.). Anti-LPA₁ antibody was from Upstate Biotechnology (USA), Affiniti (UK), Merck Biosciences (UK). Anti-phospho serine/threonine and anti-phosphotyrosine antibodies were from Cell Signalling Technologies. Anti-phospho-p42/p44 MAPK (polyclonal), and anti-p42 MAPK antibodies were from New England Biolabs (UK). Ki16425 was a kind gift from Kirin Brewery (Japan).

Cell Culture--PC12 and CHO cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) foetal calf serum and 10% (v/v) horse serum. Unless otherwise stated, cells were placed in serum free DMEM for 24 hours before experimentation. Primary human bronchial epithelial cells (HBEC) were isolated following typical procedures, as previously described [9, 10]. Following overnight digestion of the tissues at 4°C in 0.1% protease Sigma Type XIV (Sigma, St. Louis, MO) in Ham's F-12 medium containing penicillin (100 U/ml, Gibco, Gaithersburg, MD), streptomycin (100 µg/ml, Gibco), amphotericin B (2.5 µg/ml, Gibco), and gentamicin (50 µg/ml, Gibco), the protease was neutralized by the addition of 10% foetal calf serum (Invitrogen) and the epithelial cells were freed from the tissue by agitation and isolated by centrifugation. The washed P₀ HBEC were then seeded on Vitrogen 100-coated (1:75 in sterile water, Cohesion, Palo Alto, CA) P-100 dishes in Bronchial Epithelium Growth Medium (BEGM), as described by Bernacki and colleagues [11]. Upon reaching confluence, HBE cells were transferred to glass coverslips and grown to ~60% confluence with BEGM.

Nuclear Fractionation--Serum deprived PC12 cell pellets were resuspended in buffer containing 10mM-Tris/HCl, pH 7.4, 10mM-NaCl and 3mM-MgCl₂, 100µg/ml soybean trypsin inhibitor and 1mM-PMSF and homogenised with a Dounce tissue grinder. The cell homogenate was then centrifuged at 880 g for 10 minutes at 4°C. The low speed nuclear pellet was re-suspended in buffer containing 10mM Tris/HCl, pH 7.4, 10mM-

NaCl and 3mM-MgCl₂, 100µg/ml soybean trypsin inhibitor, 1mM-PMSF and 0.1% (v/v) NP-40 for 5-10 minutes at 4°C. After this time the nuclei were sedimented at 880 g for 10 minutes at 4°C. The pellet was washed a further three times in 10mM Tris/HCl, pH 7.4, 10mM-NaCl and 3mM-MgCl₂, 100µg/ml soybean trypsin inhibitor and 1mM-PMSF before use. The low speed supernatant was centrifuged at 15000 g for 15 minutes at 4°C. The subsequent supernatant was finally centrifuged at 118000 g for 60 minutes at 4°C. The subsequent high speed pellet and supernatant were then taken for analysis.

HBEC were cultured on P-100 dishes and grown to ~90% confluence with BEGM medium. The PBS/phosphatase inhibitors, 1x hypotonic buffer and complete lysis buffer (10mM DTT, Lysis Buffer AM1, protease inhibitor cocktail) provided in Active Motif Nuclear Extract Kit (Carlsbad, USA) were prepared according to manufacturer's instruction. Cells were washed with PBS/phosphatase inhibitor and collected by gentle scraping with a cell lifter. The cell homogenate was centrifuged at 500 rpm for 5 min at 4°C and the supernatant discarded. The cell pellet was re-suspended in 1x hypotonic buffer, incubated on ice for 15 min and then detergent added. The suspension was vortexed for 10 seconds at the highest setting before centrifugation at 14000 g for 30 seconds at 4°C. The supernatant fraction (S) from the nuclear extract was transferred to pre-chilled tube and stored at -80°C for analysis. The nuclear pellet was re-suspended in complete lysis buffer, vortexed for 10 seconds and then incubated on ice for 30 min on a rocking platform set at 150 rpm. The nuclei were again vortexed for 30 seconds and finally centrifuged at 14000 g for 10 min at 4°C. The nuclear pellet (N) was transferred to new tube and stored at -80°C for analysis. Purity of the nuclear pellets was confirmed by detection of lamin B and the absence of lactate dehydrogenase.

Transfection--Smartpool human LPA₁ RNA duplexes were purchased from Dharmacon Research Inc. (Lafayette, CO). HBEC (P1) were cultured either in flasks or on 12 mm glass coverslips. At 60% confluence, transient transfection of LPA₁ siRNA was carried out using Transmessenger Transfection Reagent[®] (Qiagen, Chatsworth, CA). LPA₁ siRNA (200 nM) was condensed with Enhancer R and formulated with Transmessenger reagent, according to the manufacturer's instruction. The transfection complex was

diluted in 450 μ l of BEGM medium and added directly to the cells. After 3 hours of incubation at 37°C the transfection mixture was replaced with fresh DMEM. Cells were taken for preparation of nuclear pellets or fixed 72 hours after transfection for immunofluorescence staining.

Blotting--Immunoblotting was performed as described by us previously [8]. Immunoreactive proteins were visualised using enhanced chemiluminescence detection.

Immunofluorescence--Cells were grown on 12mm glass coverslips to either 50 or 100% confluency. Cells were fixed in 3.7% formaldehyde in PBS for 10 minutes then permeabilised in 0.1% Triton X100 in PBS for 1 minute. Non-specific binding was reduced by pre-incubating cells in blocking solution containing 5% FCS, 1% BSA in phosphate buffered saline for 1 hour. Cells were incubated in primary antibodies (1:100 dilution in blocking solution) for 1 hour at room temperature (or overnight at 4°C) and then incubated with the appropriate FITC or TRITC conjugated secondary antibodies (1:100) for 1 hour. Cells were mounted on glass slides using Vectashield mounting medium with DAPI and visualised using a Nikon E600 epi-fluorescence microscope.

Flow cytometry--HBEC (60 - 70% confluence) were challenged with 1 μ M of LPA for 30 min, and then suspended using Accutase (Innovative Cell Technologies, Inc). Suspended cells were washed by PBS and incubated with anti-LPA₁ N-terminal extracellular domain antibody (LifeSpan Inc, USA) in phosphate-buffered saline containing 1 % fetal calf serum and 0.04% azide at 4 °C for 30 min. Cells were washed and stained with allophycyanin-conjugated goat anti-rabbit Fab fragments (Molecular Probes, USA) at 4 °C for 30 min. Analysis was performed on a FACScan (Becton Dickinson, USA) with CellQuest software.

RESULTS

Nuclear Localisation of LPA₁--We have previously shown that our PC12 cells express endogenous LPA₁, but not LPA₂ [8] and have demonstrated that anti-sense-mediated down-regulation of the endogenous cell surface LPA₁ reduced LPA-stimulated activation

of p42/p44 MAPK [8]. Therefore, these cells express functionally active LPA₁. Immunofluorescent staining with antibody raised against the C-terminus of LPA₁ (Upstate Biotechnology, amino acids 328-344 of human LPA₁ CQRSENPTGPTTESSDRS) revealed that the receptor antigen is constitutively present in the nucleus of PC12 cells (Fig. 1a). The staining is nuclear diffuse throughout the nucleoplasm and excluded from nucleoli. This is the most common type of localisation of nuclear gene trap proteins [12]. Moreover, many transcription factors have a nuclear diffuse localisation, the largest class of which are the C2H2 zinc finger proteins which account for more than 12% of all nuclear diffuse gene-trap proteins [12]. These findings suggest that nuclear LPA₁ may be localised in zones of high transcriptional activity. Immunofluorescent staining of LPA₁ was not detected when cells were incubated with secondary antibody alone (Fig 1a). The constitutive localisation of LPA₁ in the nucleus of PC12 cells was confirmed by Western blotting low speed nuclear pellets with anti-LPA₁-328-344 antibody which detected a single major immunoreactive protein band corresponding to native LPA₁ (Fig. 1a). Nuclear LPA₁ migrated with an Mr=36-38kDa. The electrophoretic mobility of LPA₁ in PC12 cells was slightly faster than the predicted molecular mass of LPA₁ (42kDa), suggesting that LPA₁ in these cells might be subject to post-translational modification.

Stimulation of PC12 cells with LPA resulted in an increase in the amount of LPA₁ in the low speed nuclear pellet (Fig. 1b). A commensurate reduction in the amount of LPA₁ was detected in high-speed membrane pellet containing plasma membranes (Fig. 1b). These findings suggest that LPA₁ may traffic to the nucleus from cell membranes upon ligand activation.

Endogenous LPA₁ is also constitutively localised in the nucleus of HBEC detected by immunofluorescent staining of cells (Fig. 1c) and Western blotting analysis of hypotonic-treated nuclear pellets with anti-LPA₁ antibody (Fig. 1c). Identification of nuclear LPA₁ in HBEC was confirmed by infecting HBEC with LPA₁ siRNA, which substantially reduced the amount of LPA₁ in cell lysates and hypotonic-treated nuclear pellets (Fig. 1d). The reduction in nuclear LPA₁ was also evident by immunofluorescent analysis

using anti-LPA₁ antibody. siRNA LPA₁ does not completely abolish the expression of LPA₁, but there was a visible decrease in the intensity of anti-LPA₁ immunoreactivity in the nucleus of LPA₁ siRNA-treated HBEC compared with control cells (Fig. 1d).

To eliminate the possibility of epitope cross-reactivity, two additional anti-LPA₁ antibodies were used. These included another antibody raised against amino acids 328-344 of human LPA₁ CQRSENPTGPTESSDRS (Merck-Biosciences) and an antibody raised against amino acids 348-364 of LPA₁ corresponding to LNHTILAGVHSNDHSVV (Affinti Bioreagents). Immunofluorescent staining with these antibodies demonstrated the presence of LPA₁ in the nucleus of PC12 cells (Fig. 1e). Localisation of LPA₁ is again nuclear diffuse and excluded from nucleoli. Thus, antibodies which are raised against different C-terminal regions of LPA₁ produced similar results. We also detected nuclear localisation of LPA₁ by confocal analysis of cells immunostained with anti-LPA₁-348-364 antibody (Fig. 1f). Using both confocal and epifluorescent microscopy, we did not observe any immunostaining of the plasma membrane of PC12 cells or HBEC with anti-LPA₁ antibody. However, significant plasma membrane localisation of LPA₁ was detected when HBEC were treated with LPA or PC12 cells treated with NGF to induce cortical actin localisation and membrane ruffling (Fig. 1c). The increased LPA₁ immunoreactivity detected in the plasma membrane of HBEC was not due to increased LPA₁ number, determined by FACS analysis using N-terminal anti-LPA₁ antibody. Indeed, LPA induced a decrease in cell surface LPA₁ number (% LPA₁ positive cells; control, 42.4-54%; LPA (1 μ M, 30 min), 22.6-36.5%, n=2). These data therefore suggest that significant LPA- or NGF-induced changes in plasma membrane organisation may concentrate LPA₁, possibly in micro-domains, which may allow improved access of the LPA₁ antigen to antibody.

The Localisation of LPA₁ in the Nucleus Depends on Cell Density--Anti-LPA₁ immunoreactivity was not detectable in the nuclei from PC12 cells grown at high density, whereas it was present in the nuclei from cells grown at low density (Fig. 2a). An identical relationship between cell density and the nuclear localisation of endogenous LPA₁ was also observed in CHO cells (Fig. 2b) and rat cerebellar granule neurones (Fig.

2c). These data were confirmed by Western blot analysis with anti-LPA₁-348-364 antibody using low speed nuclear and high speed membrane pellets of PC12 cells. The amount of LPA₁ was decreased in the high speed membrane pellet isolated from cells grown at low density compared with high density (Fig. 2d). This was associated with an increase in the amount of LPA₁ in the low speed nuclear pellet from cells grown at low density compared with high density (Fig. 2d). These findings are therefore in line with the possibility that LPA₁ traffics to the nucleus in a cell density-dependent manner.

Data presented in Fig. 2e shows that the number of cells containing LPA₁ in the nucleus is markedly decreased when cells are maintained in serum for 24 hours. Delipidation of the serum or removal of serum prevented this reduction in the number of cells containing nuclear LPA₁ (Fig. 2e).

Constitutive Nuclear Localisation of LPA₁ is PTX-Sensitive--Constitutive trafficking of LPA₁ from cell membranes to the nucleus appears to be dependent upon G-protein activation. This is evident from the finding that the pre-treatment of serum deprived PC12 cells with PTX (which uncouples LPA₁ from G_{i/o}-protein) reduced the number of cells containing LPA₁ in the nucleus from 90% in control cells to ~ 20% in PTX-treated cells (n=3, Fig. 3).

Role of Rho Kinase Signalling in Regulating Nuclear Localisation of LPA₁ in PC12 cells--Based upon the cell density-dependent nuclear localisation of LPA₁, we considered whether the interaction of cells with cell matrix might have a significant role in regulating the nuclear localisation of LPA₁. Therefore, the possible involvement of an integrin-dependent cytoskeletal mechanism regulating nuclear localisation of LPA₁ in PC12 cells was investigated. We first used RGDS peptide, which disrupts interaction between integrins and secreted extracellular matrix proteins that contain RGD motifs (e.g. fibronectin). RGDS prevents focal adhesion assembly and disrupts the integrity of the cytoskeleton induced by fibronectin engagement with integrin [13]. Using this peptide, we found that the number of cells containing nuclear LPA₁ was reduced from 90% in control cells to 5% in RGDS-treated cells, whereas it remained 75-85% in RGES-treated

cells (n=3, Fig. 4a). These findings are consistent with the observation that RGDS disrupted actin polymerisation in PC12 cells (Fig. 4a), evidenced by marked changes in the organised structure of actin (red phalloidin staining in Fig. 4a). Furthermore, a higher proportion of cells contained nuclear LPA₁ when adhesion on fibronectin coated glass was compared with adherence to polylysine coated glass (Fig. 4b, % cells containing nuclear LPA₁, fibronectin coated, 75%; polylysine coated, 15%, n=3). Fibronectin engages integrins to induce integrin clustering, focal adhesion formation and actin polymerisation. In contrast, polylysine does not engage integrins and has no effect on the actin cytoskeleton. These properties are consistent with data presented in Fig. 4b, which shows that fibronectin, but not polylysine stimulates actin polymerisation in PC12 cells, evident by phalloidin red staining of the cells (see marked rearrangement of actin fibres with fibronectin compared with polylysine in Fig. 4b).

To further substantiate a role for integrin-mediated Rho activation and actomyosin contractility, we found that the pre-treatment of PC12 cells with the Rho kinase inhibitor (Y27632, 10 μ M, 4 hours) or the MLCK inhibitor (ML9, 10 μ M, 4 hours) reduced the number of cells containing nuclear LPA₁ and disrupted actin polymerisation in PC12 cells (Fig. 4c, % cells containing nuclear LPA₁, control, 85-95%; Y27632-treated (4hrs), 3-5%; ML9-treated (4hrs), 2%, n=3).

Functional Role of Nuclear LPA₁--To evaluate the function of nuclear LPA₁, we assessed the effect of LPA on the phosphorylation of nuclear proteins in PC12 cells. To designate effects to LPA₁ we used Ki16425 which selectively binds to LPA₁ [14]. The addition of LPA and/or Ki16425 to isolated nuclei present in the low-speed nuclear pellet induced the serine/threonine phosphorylation of several proteins with molecular masses of 34kDa (p34), 32 kDa (p32), 14kDa (p14) and 11kDa (p11) (Fig. 5a). Ki16425 also stimulated the serine/threonine phosphorylation of an additional protein with a molecular mass of 30kDa protein (p30), which was blocked by LPA (Fig. 5a). The addition of LPA and/or Ki16425 to isolated nuclei also stimulated the tyrosine phosphorylation of a 32kDa protein (p32) (Fig. 5b). Finally, LPA stimulated the dephosphorylation of a

60kDa phosphotyrosine containing protein (p60) and this was not mimicked or reduced by Ki16425 (Fig. 5b).

DISCUSSION

The data presented in this study demonstrates that LPA₁ is constitutively localised in the nucleus of PC12, CHO cells and HBEC. LPA stimulates an acute increase in the amount of LPA₁ in nuclear fractions of the cells and this might be a consequence of increased trafficking of LPA₁ from cell membranes. This finding extends our previous studies [8] and is in agreement with those of Gobeil et al. concerning constitutive localisation of LPA₁ in the nucleus of liver cells [7]. The results are also in line with a growing body of evidence demonstrating that a number of GPCR, e.g. endothelin, angiotensin 1, apelin R1 and bradykinin 2 receptors [15, 16] are localised in the nucleus along with attendant signalling molecules, such as GRK5 and RGS proteins [17, 18]. The trafficking of LPA₁ to the nucleus of PC12 cells appears to be dependent, in part, upon G-protein coupling, based upon results showing that the pre-treatment of these cells with PTX (which uncouples LPA₁ from G_{i/o}) dramatically reduced the number of cells containing LPA₁ in the nucleus.

The nuclear localisation of LPA₁ is also dependent upon integrin clustering. Integrin clustering induces activation of Rho and Rho kinase [19], leading to calcium sensitisation of actomyosin contractility via inhibition of a myosin light chain phosphatase. MLCK plays a critical role in initiating phosphorylation of myosin and subsequent cross-bridge formation with polymerised actin. Inhibitors of Rho kinase and MLCK reduce the number of cells containing LPA₁ in the nucleus. Moreover, fibronectin which induced actin polymerisation increased the number of cells containing LPA₁ in the nucleus while RGDS (which disrupts actin polymerisation) was inhibitory. These findings also raise the possibility that LPA may acutely regulate the trafficking of LPA₁ to the nucleus via Rho-mediated signalling. We also show here that serum treatment of cells for 24 hours decreased the number of cells containing LPA₁ in the nucleus and this was reversed by delipidation or removal of serum. One possible explanation might be that the LPA

present in serum induces a chronic desensitisation of LPA₁ trafficking to the nucleus and that delipidation removes LPA, thereby relieving this inhibitory effect.

LPA₁ trafficking from cell membranes to the nucleus may involve lipid rafts. Indeed, lipid rafts represent a major route for nuclear entry by viruses such as HIV [for review see 20] and FGF and VEGF receptor traffic from the plasma membrane to the nucleus in an agonist-dependent and -independent manner, via lipid rafts [21, 22]. LPA₁ may also be maintained in a hydrophobic environment within the nucleus, as this organelle is rich in lipid environments that are localised in areas of transcriptional activity. These lipid microenvironments contain the lipid raft components caveolin-1, cholesterol and phospholipids [23]. Indeed, Gobeil et al. [7] have demonstrated co-localisation of LPA₁ with caveolin-1 in the nucleus. Thus, caveolin-mediated endocytosis may be involved in nuclear trafficking of LPA₁. In ML9-treated cells, LPA₁ appears to 'back up' in cytoplasmic vesicular structures. These findings are consistent with the possibility that ML9 traps endocytic vesicles containing LPA₁ in the cytoplasm. This is entirely in line with the mode of action of ML9, which does not disrupt the formation of endosomes containing LPA₁, but instead inhibits actomyosin formation and thus, contractility required to transport endosomes to intracellular compartments.

We have shown here that the addition of LPA to isolated nuclei induced the serine/threonine phosphorylation of p34, p32, p14 and p11. These protein phosphorylations were insensitive to inhibition by Ki16425, which itself stimulated phosphorylation of the same proteins. The fact that both LPA and Ki16425 bind to LPA₁ and that both induce nuclear phosphorylation of the same substrates provides evidence that these events are probably mediated by LPA₁. Ki16425 also stimulated phosphorylation of a 30kDa protein (p30), which was reduced by LPA. Further studies are required to identify the mechanism by which LPA and Ki16425 stimulates nuclear protein phosphorylation. However, Ki16425 is unusual and functions as a protean agonist of cell surface LPA₁ receptor, being able to induce activation of p42/p44 MAPK on its own, but reducing the LPA-stimulated activation of this protein kinase pathway in PC12 cells [24]. In the current study, we also found that LPA stimulated the

dephosphorylation of a nuclear 60kDa tyrosine phosphorylated protein. As this was not mimicked or reduced by Ki16425, there is a possibility that this protein is regulated by a distinct nuclear LPA receptor, which does not bind Ki16425.

Additional evidence to support the concept that nuclear LPA₁ regulates protein phosphorylation has been reported by Gobeil et al. [7], who showed that LPA induced the phosphorylation of Akt in isolated liver nuclei. Moreover, LPA-induced phosphorylation of Akt was blocked by the PI3K inhibitor, wortmannin, which also reduced LPA-stimulated iNOS expression [7]. However, it will be important to identify the kinase(s) responsible for the phosphorylation to exclude direct effects of LPA and Ki16425 on this kinase.

An intranuclear role for LPA is suggested by results from others showing that induction of COX2 and iNOS is inhibited by PLA₂ inhibitors, suggesting that LPA might be formed by the action of PLA₂ on nuclear phosphatidic acid [7]. Indeed, others have shown using a human endothelial cell line (EA.hy.926) that cPLA₂ is present in the cytosol and nucleus and moves to membranes via its calcium-dependent lipid-binding domain following agonist stimulation. Nuclear localisation of cPLA₂ is dependent on cell density, with cells grown at low density containing higher levels of nuclear cPLA₂ compared with contact-inhibited high-density or serum-deprived cells [25]. Therefore, the effect of cell density on the nuclear localisation of cPLA₂ is identical to that regulating LPA₁ in PC12 cells.

In summary, the prevailing view is that LPA₁ is localized at the cell surface where it functions to transduce extracellular LPA signals into biological responses. Our findings suggest that LPA₁ is also present in the nucleus of cells where it may participate in intranuclear LPA signalling.

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Footnotes

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Figure legends

Fig. 1 Detection of nuclear LPA₁. (a, b) Serum deprived PC12 cells or (c) HBEC were immunostained with anti-LPA₁ antibody (with anti-rabbit FITC secondary antibody) and with DAPI. The photograph shows the constitutive localisation of LPA₁ in the nucleus of these cells. Western blots were probed with anti-LPA₁ antibody showing a single immunoreactive band corresponding to LPA₁ in the low speed nuclear pellet from PC12 cells; (b) Western blot probed with anti-LPA₁ antibody showing that the treatment of serum deprived PC12 cells with 5 μ M LPA for 10 min results in an increase in the amount of LPA₁ in the low speed nuclear pellet (N) and a commensurate decrease of the receptor in high-speed membrane pellet fraction (M). In (c) HBEC were treated with and without LPA (1 μ M) for the indicated times or PC12 cells treated with NGF (10ng/ml) for 5 min to demonstrate plasma membrane localisation of LPA₁. PC12 cells and HBEC were immunostained with anti-LPA₁ antibody. Also included is a Western blot probed with anti-LPA₁ antibody showing that LPA₁ was enriched in high-speed hypotonic-treated nuclear pellets (N) of HBEC. There was a low amount of LPA₁ in the subsequently obtained hypotonic/detergent high-speed supernatant fraction isolated from a nuclear pellet treated with this buffer mix (S); (d) Western blot probed with anti-LPA₁ antibody showing the effect of siRNA-mediated elimination of the LPA₁ on the amount of LPA₁ in lysates and high-speed hypotonic-treated nuclear pellets (N) from serum deprived HBEC, detected on Western blots probed with anti-LPA₁ antibody. Also shown is an immunofluorescent image of control and siRNA LPA₁-treated HBEC stained with anti-LPA₁ antibody; (e) PC12 cells were immunostained with anti-LPA₁ antibodies (with anti-rabbit FITC secondary antibody) raised against different C-terminal regions of LPA₁, and with DAPI. The photograph shows the nuclear diffuse localisation of LPA₁; (f) Confocal immunofluorescent images with anti-LPA₁ antibody-348-364 (and anti-rabbit FITC secondary antibody) showing the nuclear localisation of LPA₁ in serum deprived PC12 cells.

Fig. 2 Effect of cell density and serum on the nuclear localisation of LPA₁. PC12 cells, CHO cells and cerebellar granule neurones were immunostained with anti-LPA₁ antibody (with anti-rabbit FITC secondary antibody) and with DAPI. Cells were grown

to either low or high density. Photographs show the effect of cells density on the nuclear localisation of LPA₁ in (a) serum deprived PC12 cells; (b) serum deprived CHO cells and (c) cerebellar neurone; (d) low speed nuclear pellets (N) and high speed membrane pellets (M) from serum deprived PC12 cells grown to low and high density were subjected to Western blotting with anti-LPA₁ antibody. The Western blot shows an increase in the amount of LPA₁ in low speed nuclear (N) pellets of PC12 cells grown at low density compared with high density and the commensurate decrease of LPA₁ in high speed membrane (M) pellets from cells grown at low versus high density; (e) histogram showing the effect of serum and delipidation of serum on the number of PC12 cells containing LPA₁ in the nucleus. Cells were maintained in serum or delipidated serum or serum free conditions for 24 hours. Results are expressed as means \pm SD for n=3 different experiments (* p<005 for quiescent versus serum).

Fig. 3 Constitutive nuclear localisation of the LPA₁. Serum deprived PC12 cells were immunostained with anti-LPA₁ antibody (with anti-rabbit FITC secondary antibody). The photograph shows that the nuclear localisation of LPA₁ in serum deprived PC12 cells is reduced by pre-treating cells with PTX (100ng/ml, 24 hours), which was added at the same time as serum was removed from PC12 cells.

Fig. 4 The effect of cell matrix and Rho-dependent signalling on nuclear LPA₁ in PC12 cells. Serum deprived PC12 cells were stained for immunofluorescence using anti-LPA₁ antibody (with anti-rabbit FITC secondary antibody, green) or phalloidin red (actin). (a) Serum deprived PC12 cells were treated with RGDS (25 μ M) or RGE8 (25 μ M) for 24 hours or were grown on (b) fibronectin (10 μ g/ml) or polylysine (0.01%, v/v) coated plates or (c) were treated with and without Y27632 (10 μ M, 4 hours) or ML9 (10 μ M, 4 hours). The photographs show the effect of the various treatments on the localisation of nuclear LPA₁.

Fig. 5 Effect of Ki16425 and LPA on protein phosphorylation. Freshly prepared nuclei in low-speed nuclear pellets from serum deprived PC12 cells were pre-treated with and without Ki16425 (10 μ M, 20 min) prior to treatment with LPA (5 μ M) for 5 min. The

Western blots show the effect of Ki16425 and/or LPA on (a) nuclear protein serine/threonine phosphorylation and (b) nuclear protein tyrosine phosphorylation.

Fig. 1A

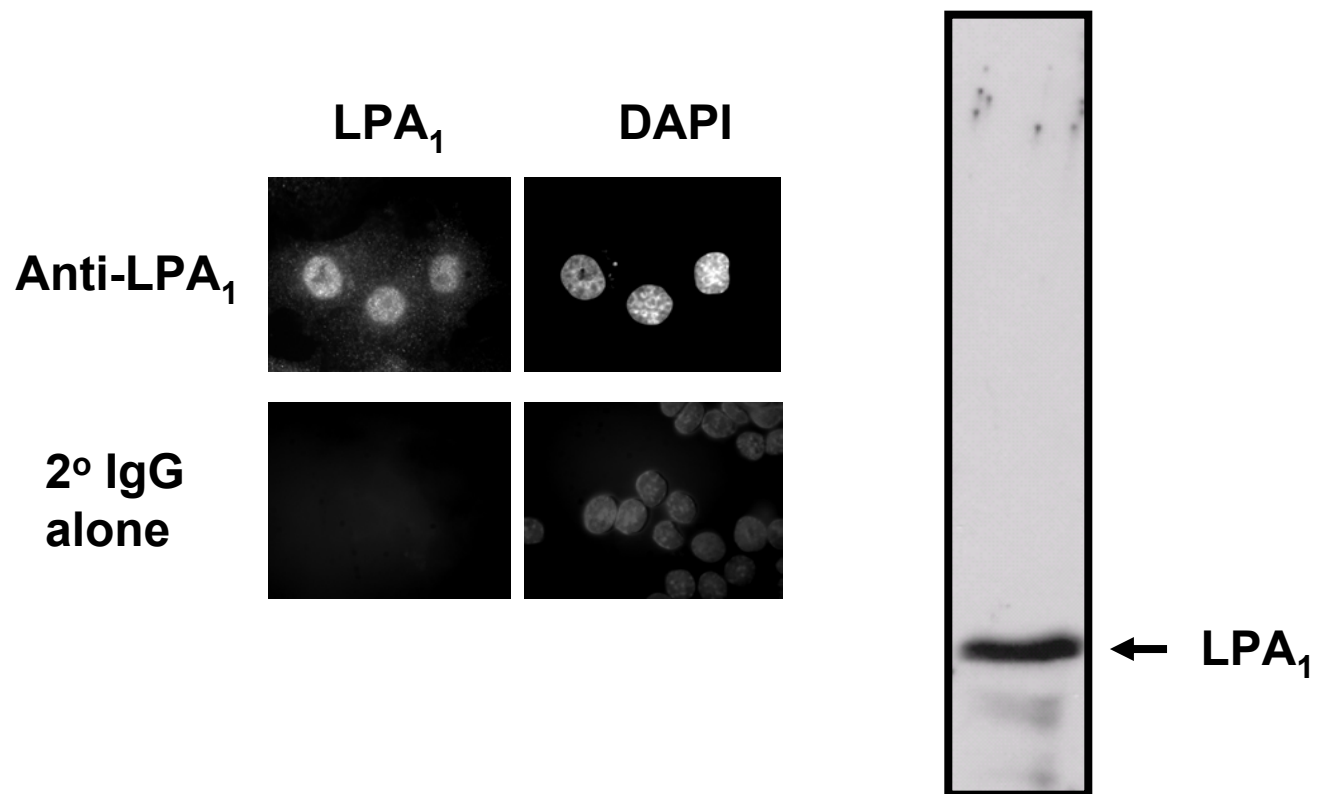


Fig. 1B

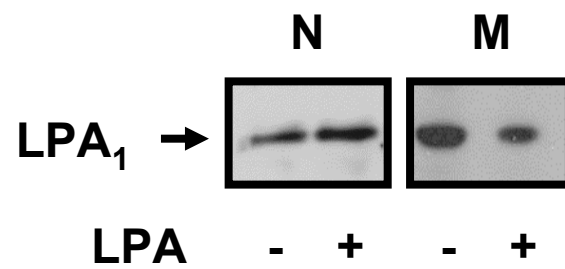


Fig. 1C

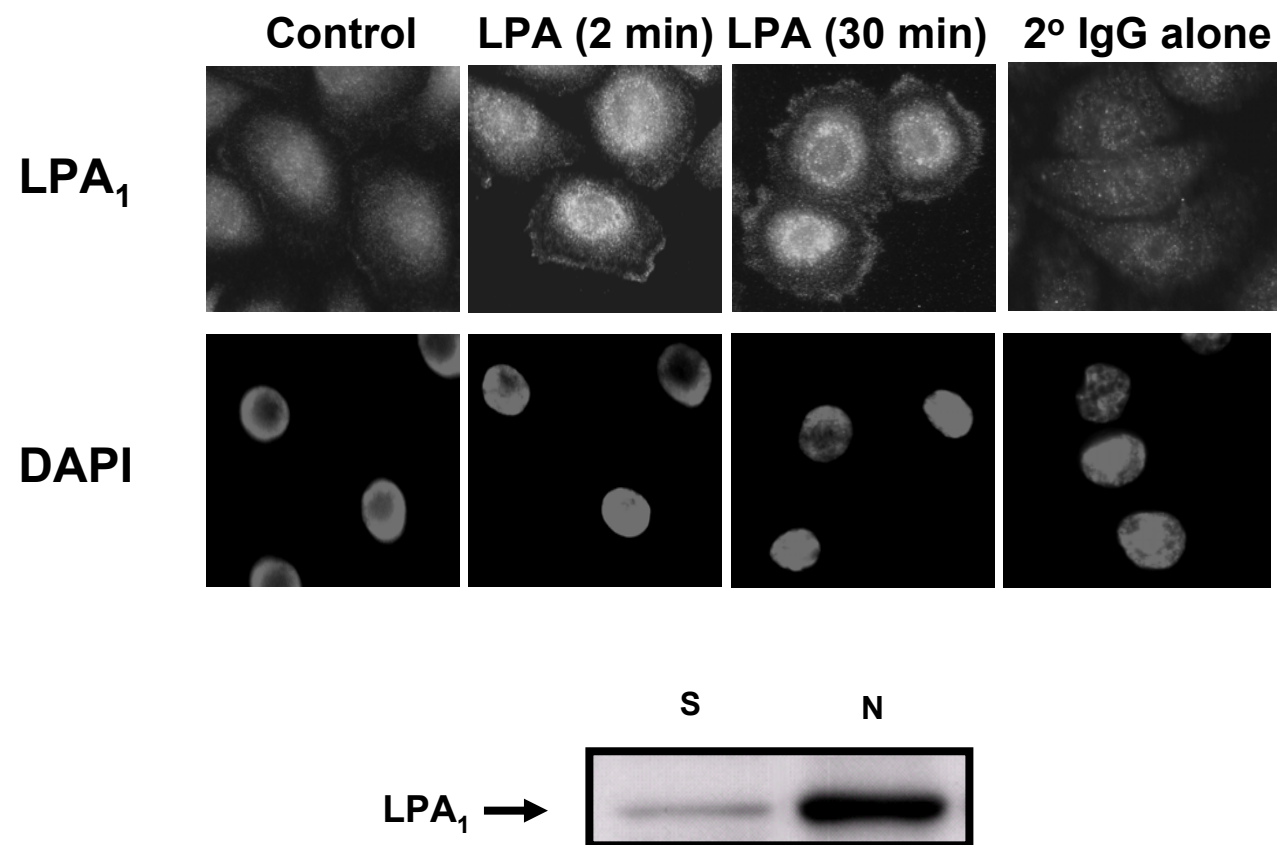


Fig. 1C continued

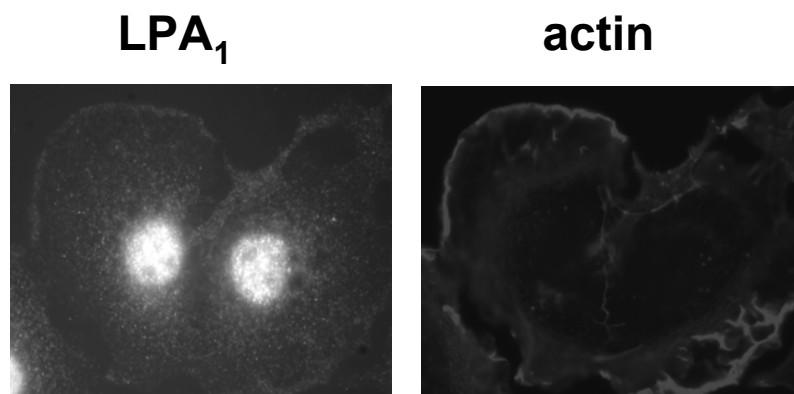


Fig. 1D

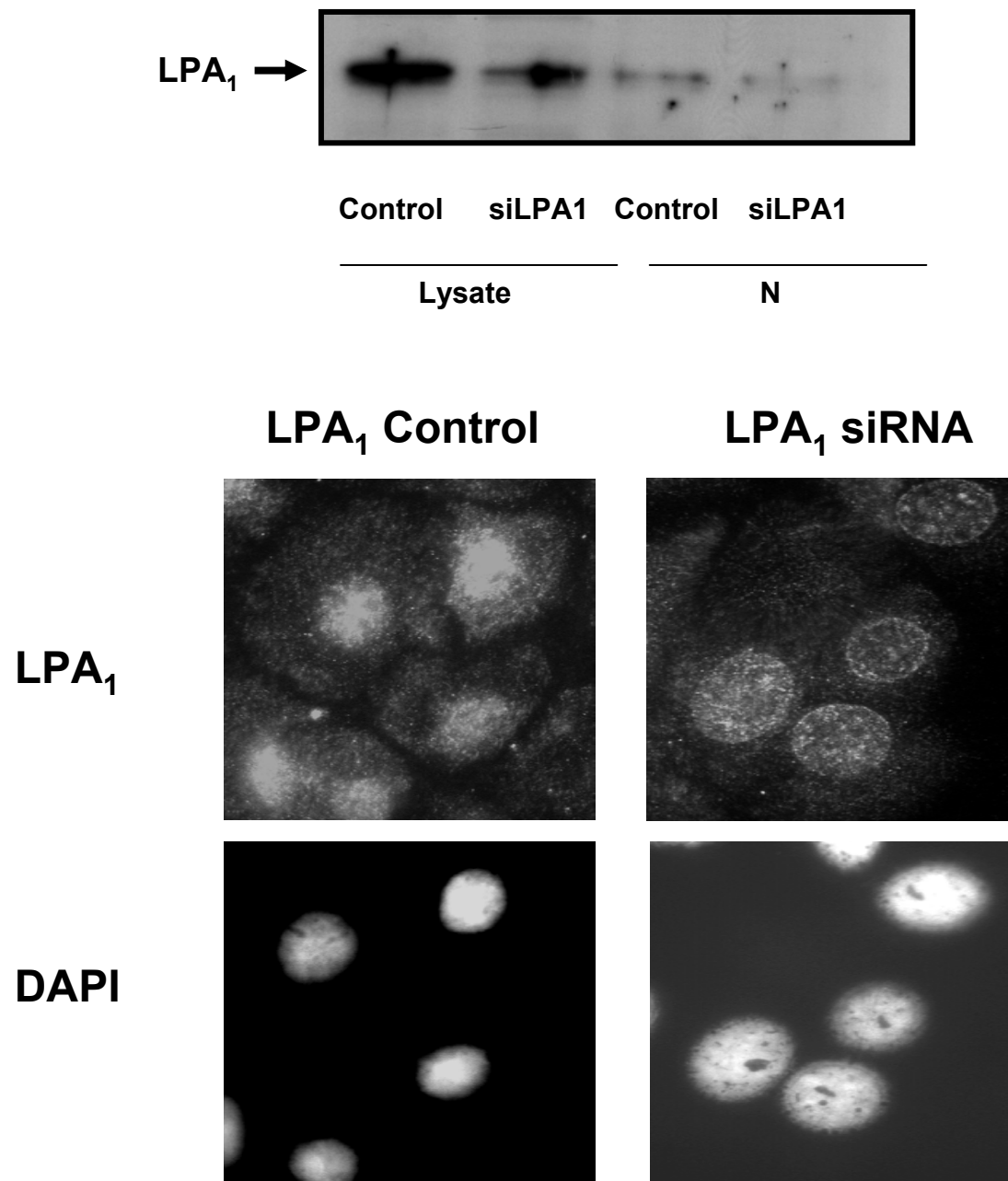


Fig. 1E

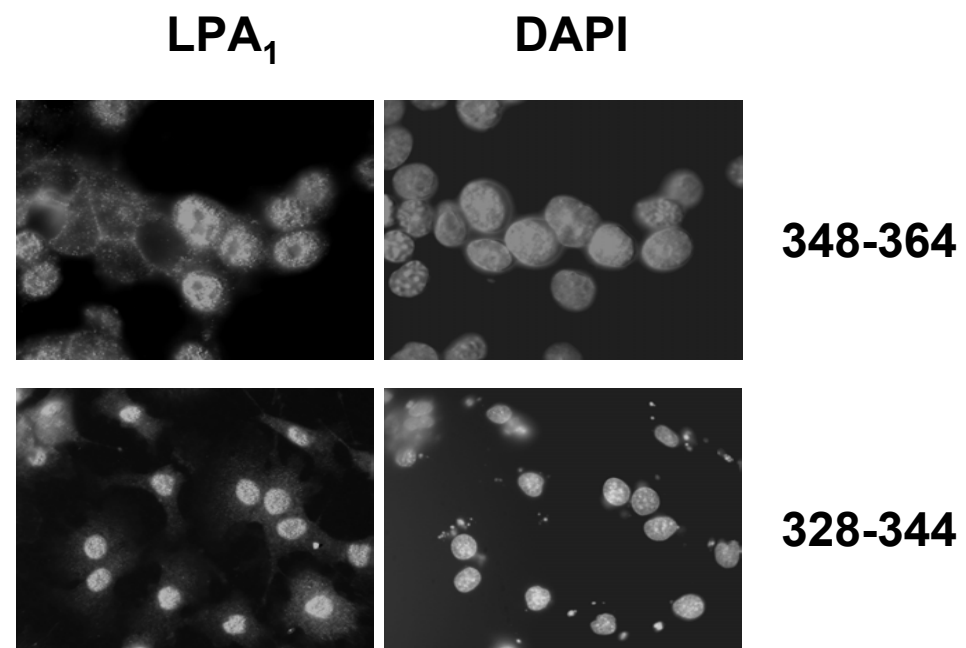


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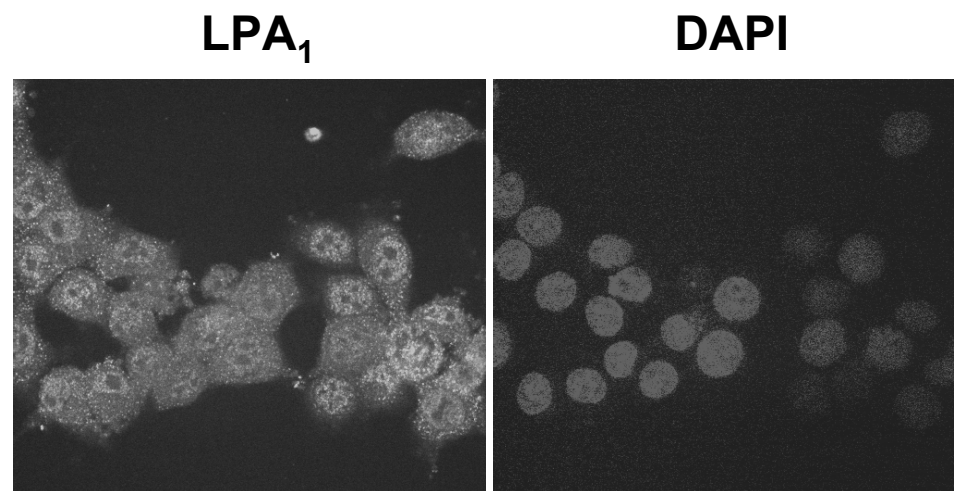


Fig. 2A

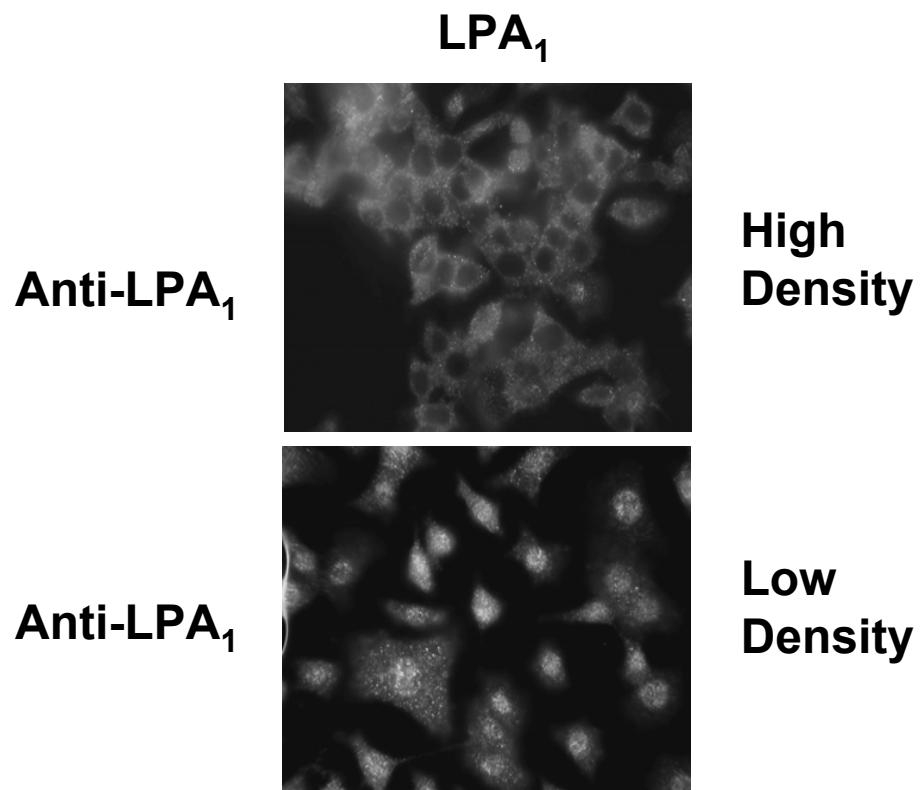


Fig. 2B

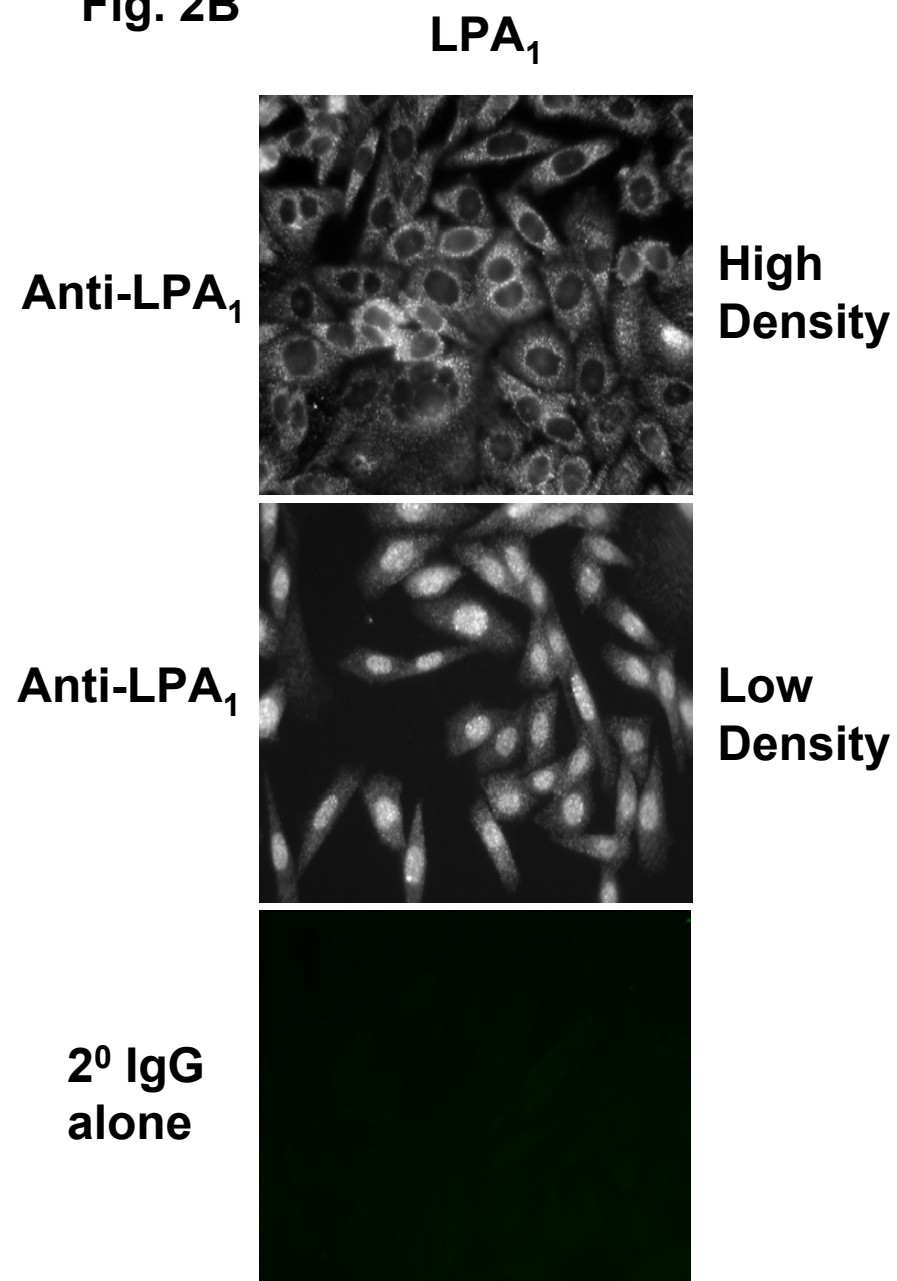


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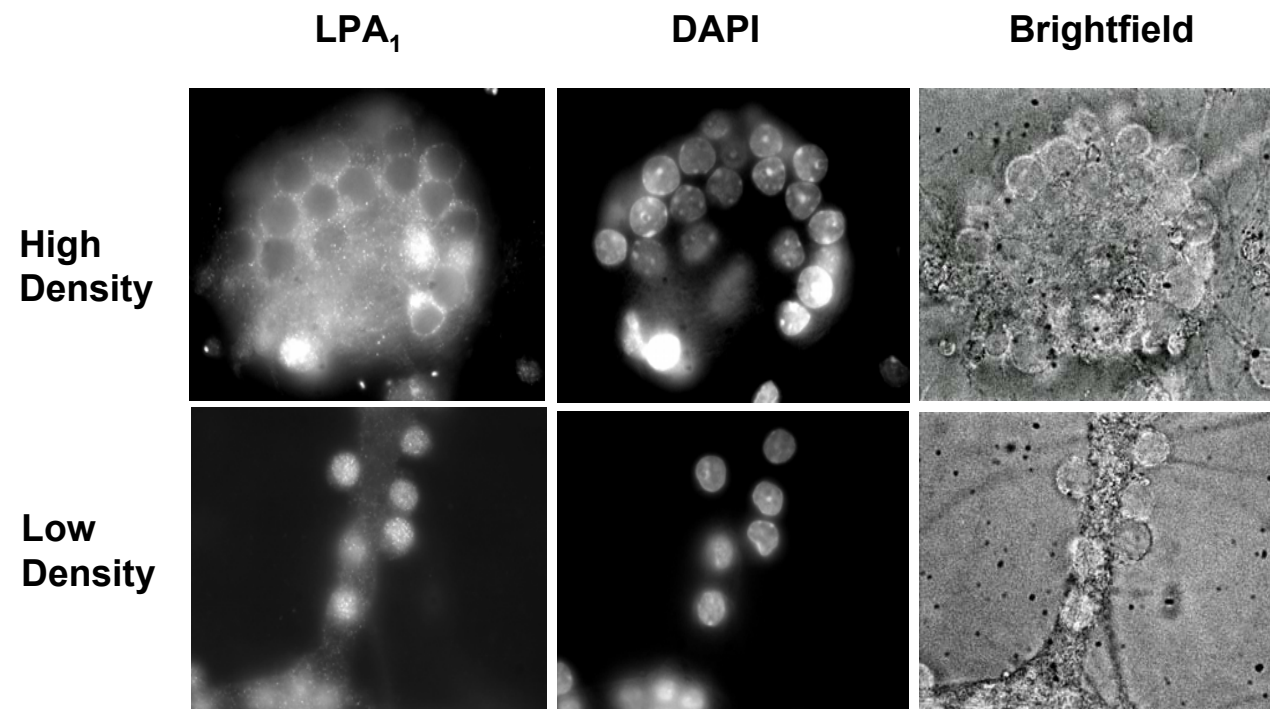


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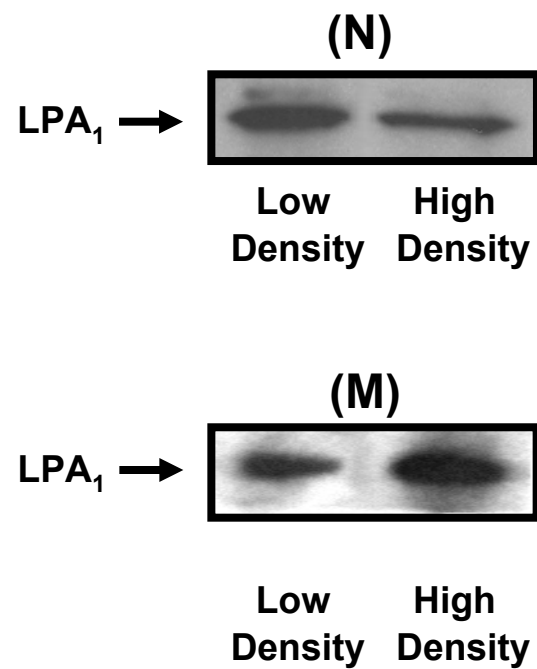


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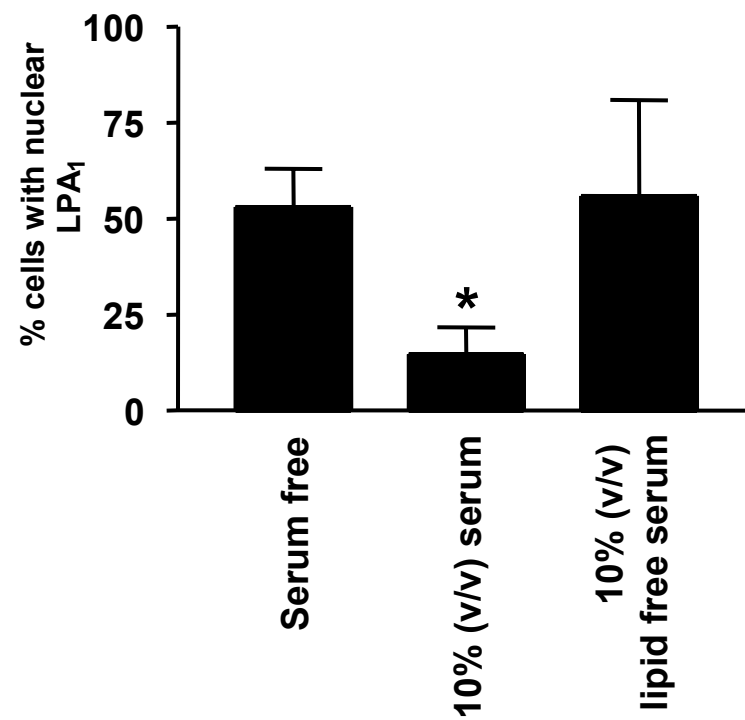
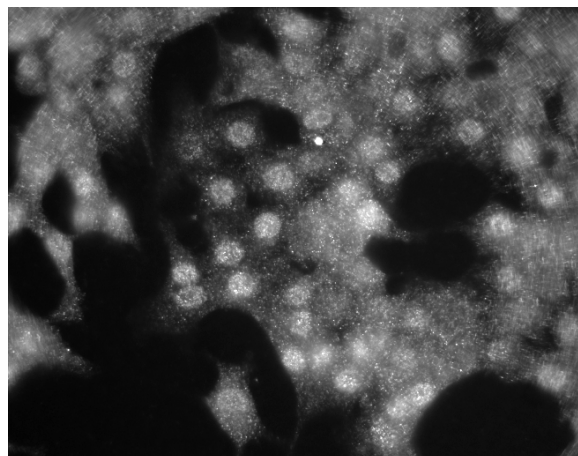


Fig. 3

Control



PTX

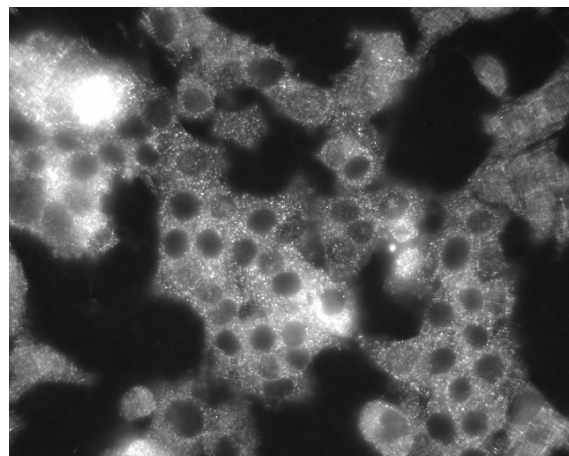


Fig. 4A

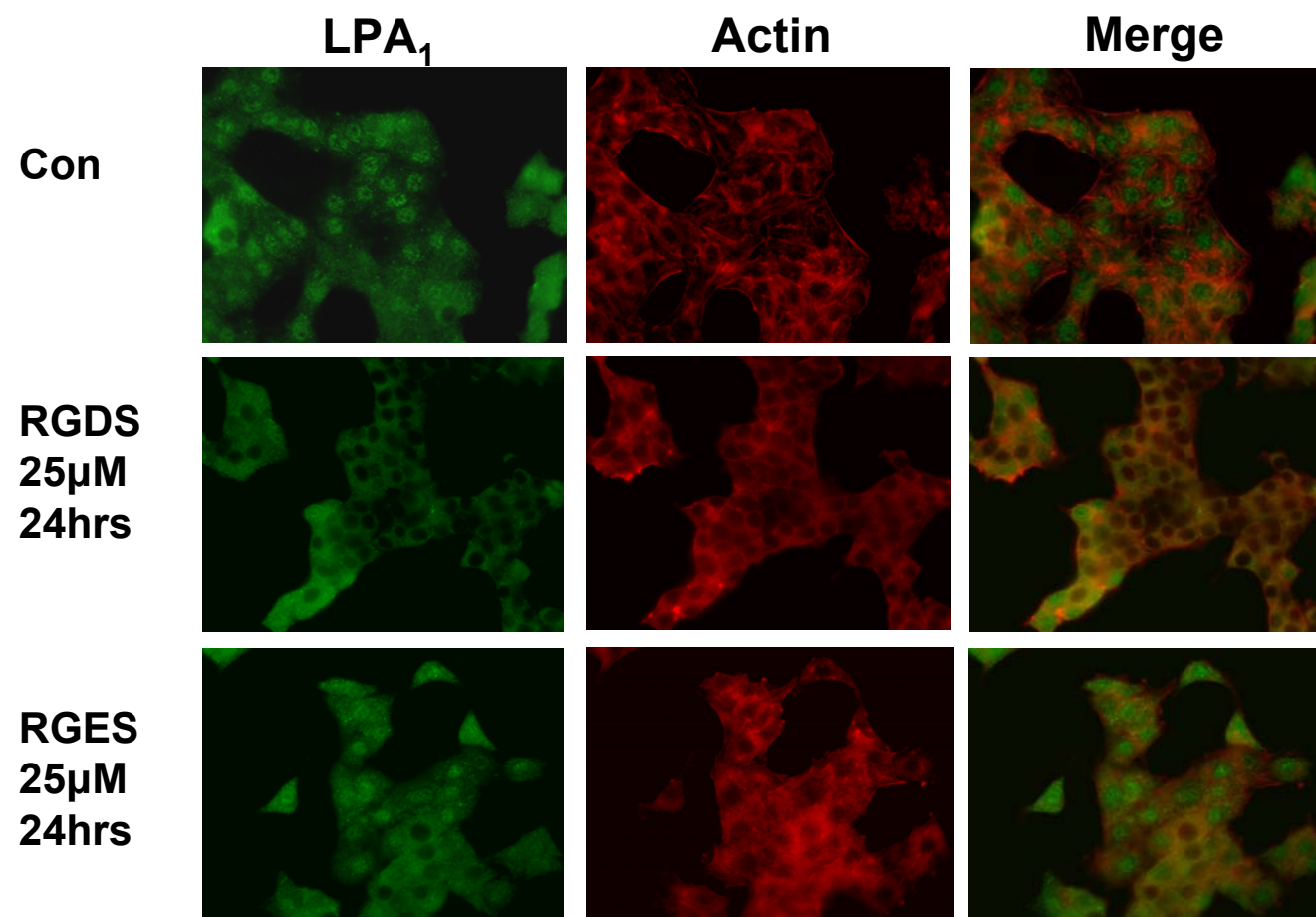


Fig. 4B

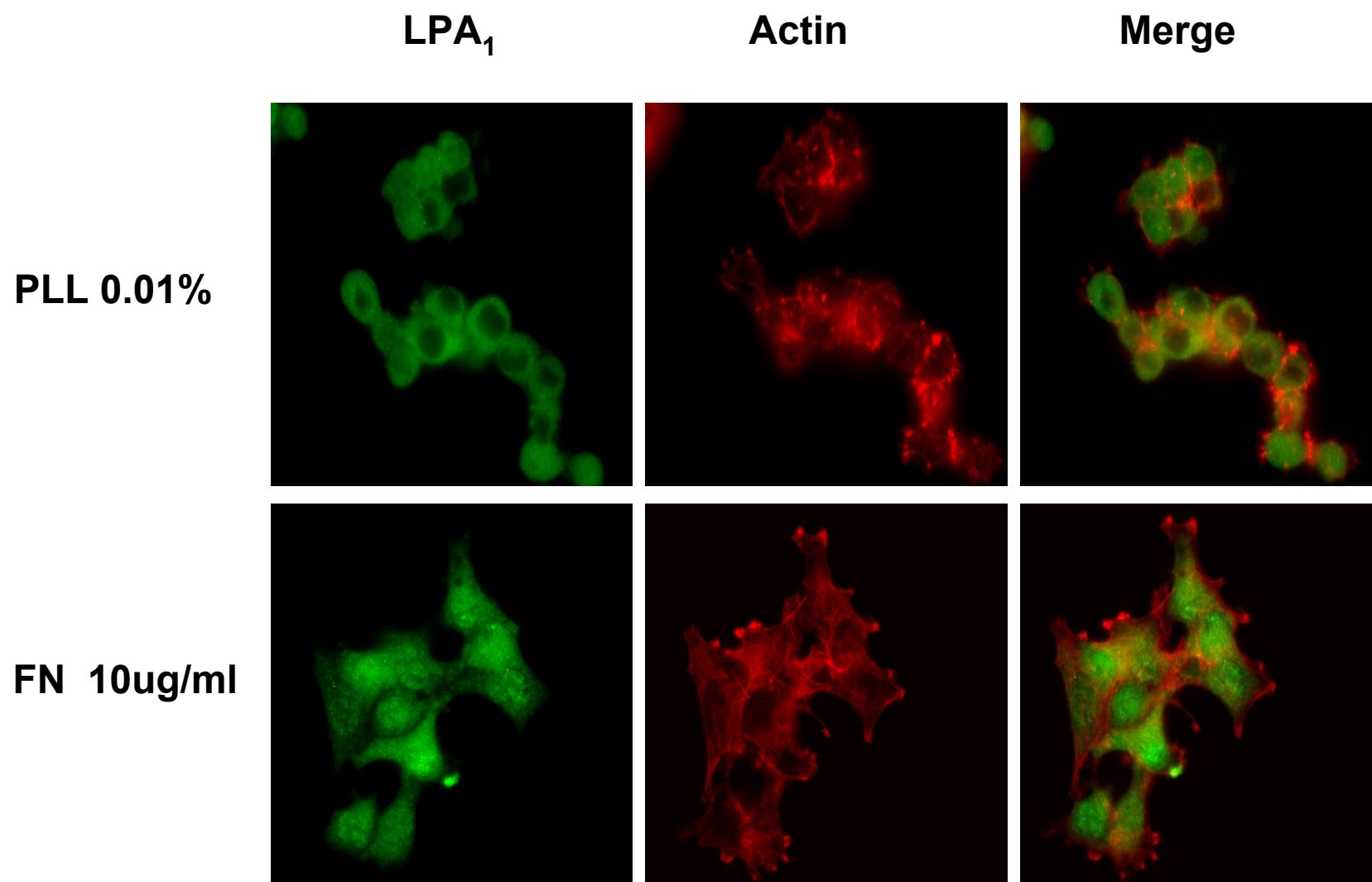


Fig. 4C

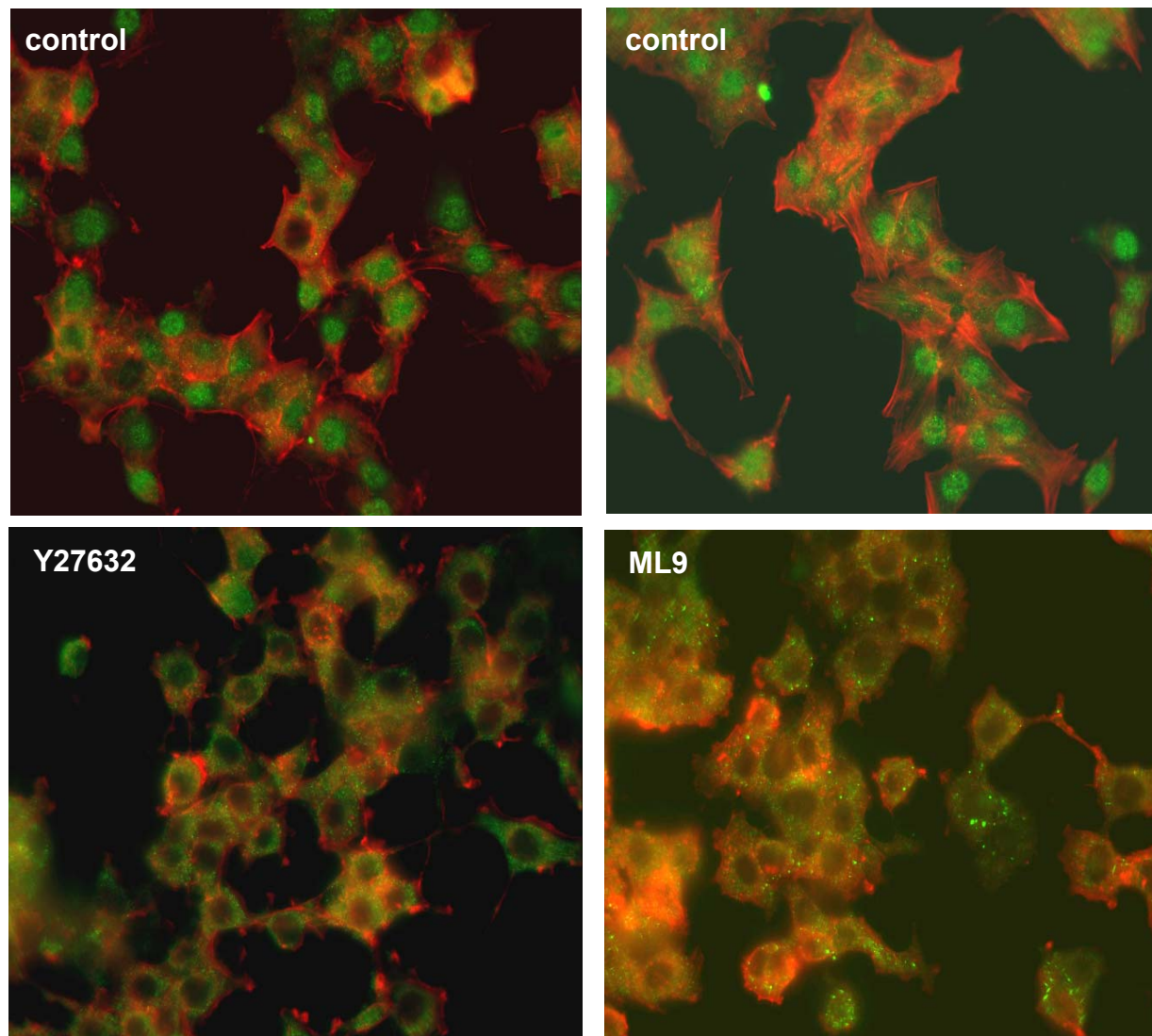
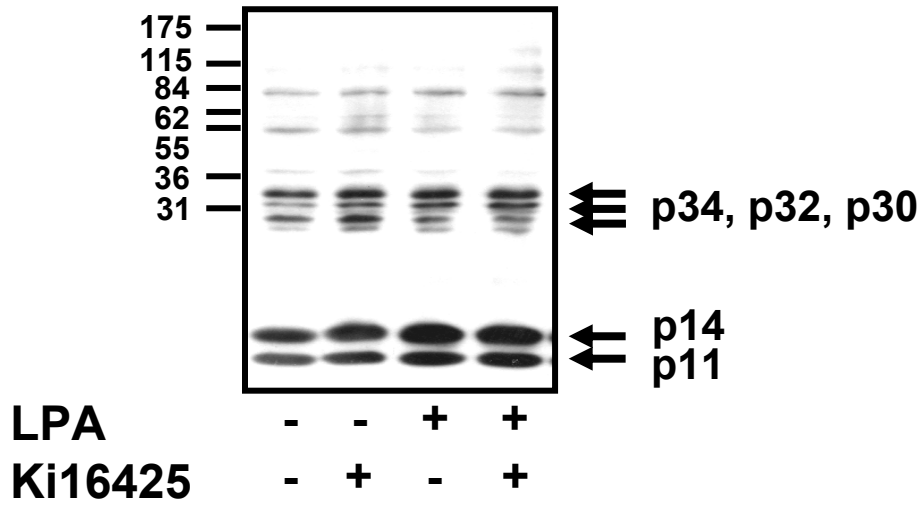


Fig. 5

A



B

