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Arf6 Promotes Cell Proliferation Via the PLD-mTORC1 and p38MAPK Pathways

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ABSTRACT

The small G-protein ADP-ribosylation factor 6 (Arf6) belongs to the Ras GTPases superfamily and is mostly known for its actin remodeling functions and involvement in the processes of plasma membrane reorganization and vesicular transport. The majority of data indicates that Arf6 contributes to cancer progression through activation of cell motility and invasion. Alternatively, we found that the expression of a wild-type or a constitutively active Arf6 does not influence tumor cell motility and invasion but instead significantly stimulates cell proliferation and activates phospholipase D (PLD). Conversely the expression of a mutant Arf6 (Arf6N48I), that is, unable to interact with PLD has no effect on proliferation but promotes motility, invasion, and matrix degradation by uPA extracellular proteinase. Studying the mechanisms of Arf6-dependent stimulation of cell proliferation, we found some signaling pathways contributing to Arf6 promitogenic activity. Namely, we showed that Arf6 in a PLD-mTORC1-dependent manner activates S6K1 kinase, a well-known regulator of mitogen-stimulated translation initiation. Furthermore, we demonstrated an Arf6-dependent phosphorylation of mTORC1 downstream targets, 4E-BP1 and ribosomal S6 protein, confirming an existence of Arf6-PLD-mTORC1-S6K1/4E-BP1 signaling pathway and also demonstrated its impact on proliferation stimulation. Next, we found that Arf6 activation potentiates Erk1/2 and p38MAP kinases phosphorylation. Surprisingly, p38 opposite to Erk1/2 significantly contributes to Arf6-dependent proliferation increase promoting S6 ribosomal protein phosphorylation at Ser235/236 residues. Therefore, we demonstrated Arf6 proliferation stimulating activity and revealed PLD-mTORC1 and p38MAP kinase as Arf6 partners mediating promitogenic activity. These results highlight a new aspect of Arf6 functioning in cancer cell biology. J. Cell. Biochem. 113: 360–371, 2012.

KEY WORDS: Arf6; mTOR; S6K1; rpS6; 4E-BP1; Erk1/2; p38; MAPK KINASES; PROLIFERATION

ADP-ribosylation factor 6 (Arf6) is a member of the ARF family within the Ras superfamily of small GTPases. Like other GTPases, it circulates between an active GTP-bound and an inactive GDP-bound forms. During the past decade series of data has been accumulated concerning Arf6 role in numerous fundamental biological processes. It is well-established that Arf6 participates in membrane trafficking including endocytosis, post-endocytic recycling and exocytosis, lipid rafts dynamics and reorganization of plasma membrane structure [Donaldson, 2003; D'Souza-Schorey and Chavrier, 2006]. Arf6 regulates actin cytoskeleton remodeling, cell spreading, and cell adhesions disassembly [Schafer et al., 2000; Palacios et al., 2001], as well as phospholipids metabolism [Honda et al., 1999; Aikawa and Martin, 2003; Lawrence and Birnbaum, 2003; Zheng and Bobich, 2004]. Over the past few years Arf6 draws more and more attention in the aspect of carcinogenesis and tumor progression via participation in growth factor receptors turnover [D'Souza-Schorey et al., 1995] and stimulation of cell migration and invasion [Hashimoto et al., 2004; Tague et al., 2004; Li et al., 2006b; Sabe et al., 2009]. Recently published data has proposed a new mechanism contributing to Arf6-dependent invasion regulation namely by directing matrix degradation through release of proteinases containing microvesicles [Cocucci et al., 2009; Muralidharan-Chari et al., 2009a]. At the same time there are only few rather contradictory data pointing on Arf6 influence on cell proliferation and mitogenic signaling mediation. [Li et al., 2009; Muralidharan-Chari et al., 2009b].

Irina Zborovskaya and Elena Tchevkina contributed equally to the work.

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While the vast majority of studies are focused on Arf6-dependent actin reorganization and membrane ruffling, promoting the acquisition of migratory phenotype mostly through activation of Rac [Santy and Casanova, 2001; Palacios and D'Souza-Schorey, 2003; Farooqui et al., 2006; Cotton et al., 2007; Hu et al., 2009] less is known about other signaling pathways and effecter proteins downstream of Arf6, which could be involved in mitogenic regulation. There are some data concerning Arf6 regulation of PIP5-kinase [Honda et al., 1999; Aikawa and Martin, 2003; Lawrence and Birnbaum, 2003] and phospholipase D (PLD) [Brown et al., 1993; Cockcroft et al., 1994; Santy and Casanova, 2001]—enzymes involved in membrane lipids modification. PLD might be one of Arf6 partners participating in mitogenic regulation as it is activated in response to various extracellular signals and catalyzes hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline, second messengers which are involved in mediation of numerous signal-transduction events and intracellular membrane trafficking. Both PLD isoforms (PLD1 and PLD2) have been implicated in mitogenic and survival signaling via PA-mediated mitogenic activities of various growth factors and hormones in several types of mammalian cells. While much has been established regarding the upstream regulation of PLD, relevant downstream PLD targets mediating its mitogenic activity are still less studied. Few studies have proposed Raf kinase as a candidate for this role, since it can interact directly with PA through PA-binding site in its C-terminus [Ghosh et al., 1996; Rizzo et al., 1999]. Such interaction can facilitate the recruitment of Raf to the plasma membrane, where it can participate in activation of mitogen-activated protein (MAP) kinase pathway.

Another target contributing to the mitogenic properties of PLD signaling is mTOR kinase which belongs to the family of phosphatidylinositol kinase like kinases (PIKK) and functions as a part of two distinct signaling complexes, mTORC1/2 (for rev. see [Zhou and Huang, 2010]). mTORC1 is rapamycin-sensitive and besides mTOR contains a positive regulatory subunit, Raptor, two negative regulators, PRAS40 and DEPTOR, and mLST8 protein. The main function of mTORC1 is regulation of cell growth, proliferation, and survival by sensing mitogen, energy, and nutrient signals [Fingar and Blenis, 2004]. Its best known downstream effectors include the ribosomal subunit S6 kinase (S6K1) and the eukaryotic initiation factor 4E binding protein (4E-BP1), both regulators of mitogen-stimulated translation initiation. Previously it has been postulated that mTOR is mainly involved in survival signaling generated by PI3K/Akt pathway [Hay, 2005; Carnero, 2010]. Later mTOR has been shown to be also directly regulated by PA, implicating this kinase as a downstream target of PLD (for rev. see [Sun and Chen, 2008; Foster, 2009]). mTOR influences both cell cycle progression and cell growth by regulation of translation, transcription, membrane traffic, and protein degradation. Since both PLD and mTOR have been implicated in survival signaling and mTOR can be regulated by PLD, mTOR is a strong candidate for a critical downstream target of mitogenic PLD activity. Recently in few studies it was shown that Arf6 could directly bind to and activate PLD, suggesting Arf6 potential participation in mitogenic signaling and regulation of proliferation [Xu et al., 2003; Liu et al., 2005; Rankovic et al., 2009].

Here, we focused on Arf6 role in aggressive cellular phenotype acquisition, particularly, in modifying cell growth properties, motility, and invasion as well as on Arf6-dependent intracellular signaling. HET-SR, a well characterized cell line of RSV-transformed Syrian hamster primary fibroblasts, was used as a convenient model for tumor progression and metastasis study since it is high tumorigenic and low metastatic in the spontaneous metastatic activity (SMA) test on immunocompetent syngeneic animals [Deichman et al., 1989; Tatosyan et al., 1996; Illan Rubio et al., 2010]. Previously, using this model system we have demonstrated SMA stimulating effect of few small GTPases and other proteins [Tchevkina et al., 2005]. Recently, Muralidharan-Chari et al. [2009b] for the first time showed Arf6 potency in metastasis stimulation. Unlike this data, we found here that Arf6 expression has no significant effect on SMA, but gives significant rise of proliferation dynamics in vitro. This effect is thought to be PLD-mTORC1-dependent, as Arf6 inability to interact with PLD as well as mTOR inhibition impaired proliferation activation. We also showed for the first time that constitutively active Arf6 stimulates phosphorylation of mTORC1 downstream effectors S6K1, S6 and 4E-BP1, giving no effect on PI3K/Akt pathway. Besides, overexpression of constitutively active Arf6 and to the less extent wild-type Arf6, in contrast to N48I mutant, potentiates Erk1/2, and p38 MAP kinases phosphorylation. Treatment with corresponding inhibitors revealed that among three candidates, mTOR, p38, and Erk1/2, the first two were essential for Arf6-dependent stimulation of proliferation.

MATERIALS AND METHODS

CELL CULTURES, PLASMIDS, AND CHEMICAL INHIBITORS

HET-SR cell line was kindly provided by Dr. G.I. Deichman (Carcinogenesis Institute, Cancer Research Center, Moscow), retrovirus packaging cell line GP-293 was purchased from Clontech. All cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS; PAA Laboratories) in 37°C and 5% CO_2 atmosphere. For inhibiting of PLD, mTOR, Erk1/2, or p38 kinases, cells were exposed for 24 h to 1% butanol-1 (Sigma), 100 nM rapamycin (Biomol), 2 μM CI-1040 (Selleck Chemicals) or 25 μM SB203580 (Upstate Biotechnology) contained medium respectively. pSRα–Arf6 vectors (Arf6WT, Arf6Q67L) were granted by Dr. P. Chavrier (Transduction du signal et oncogenèse, Institut Curie, France), pLXSN-Arf6-N48I vector was granted by Dr. V. Kanamarlapudi (Department of Physiology and Pharmacology, School of Medical Sciences, University of Bristol, Bristol, UK). Arf6 sequences were cloned into pLXSN retroviral vector by *Eco*RI and *Xho*I sites. All constructs were verified by sequencing.

PRODUCTION OF STABLE CELL LINES

GP-293 cells were cotransfected with retroviral vectors and pVSVG (Clontech) using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Forty-eight and 72 h after transfection, virus-containing medium was applied to 50% confluent HET-SR cells in the presence of 8 mcg/ml Polybrene (Sigma). Infected cells were selected in 1.2 mg/ml G-418-containing medium (Calbiochem) for 14 days.

ANALYSIS OF SPONTANEOUS METASTATIC ACTIVITY (SMA) AND TUMOR GROWTH IN VIVO

2×10^4 cells in 0.5 ml of serum-free medium were injected subcutaneously in adult (10-week old) Syrian hamsters (*Mesocricetus auratus*). Two months after injection, animals were sacrificed, tumors volume was measured and lungs were collected. Lungs were fixed in alcoholic formalin (10% of formalin and 63% of ethanol). Paraffin-embedded tissues were step-sectioned and stained with hematoxylin–eosin. Metastatic tumor nodules in the lungs were counted microscopically (72 sections per lung per hamster, 10 animals in group for each cell line study). SMA test for each cell line was performed twice. Size of subcutaneous tumors was hand measured every 5 days during 2 months and finally after dissection. The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation and the experiments were conducted in accordance with the Guidelines for Animal Experiments in N.N. Blokhin Russian Cancer Research Center.

WESTERN BLOT ANALYSIS AND ANTIBODIES

Western blot analysis was proceeded as described previously [Tchevkina et al., 2005]. Following primary antibodies were used: anti-Arf6 from Sigma, anti-phospho-Thr183 JNK1, anti-JNK1, anti-phospho-Thr180/Tyr182 p38, anti-p38, anti-β-actin, anti-phospho-Thr389 S6K1, anti-S6K1, anti-PLD from Abcam, anti-phospho-Thr202/Tyr204 ERK1/2, anti-ERK1/2, anti-phospho-Ser473 Akt, anti-Akt, anti-phospho-Thr70 4E-BP1, anti-phospho-Thr37/46 4E-BP1, anti-4E-BP1, anti-phospho-Ser235/236 rpS6, anti-phospho-Ser240/244 rpS6, anti-rpS6, anti-HA from Cell-Signaling Technology. Images of obtained blots were captured using Kodak GelLogic 2200 Imaging system and processed using Kodak Molecular Imaging Software SE version 5.0.1.27. All results are representative of three independent experiments. The band densitometries were calculated as the relative fold in band intensity compared with untreated control cells. Data were normalized by β-actin and by total expression of corresponding protein (if phosphorylation status is studied).

PREPARATION OF CONDITIONED MEDIUM

5×10^5 cells were seeded in six-well plates in full medium. Eighteen hours later the medium was replaced with 1 ml of serum-free DMEM and 24 h later the medium was collected and centrifuged 10 min at 3,000 g. The supernatant was stored at −70°C and used for zymographic analysis.

Gelatin zymography was performed using 8% SDS–PAGE gels, containing 0.2% gelatin (AppliChem). Conditioned medium samples were mixed 1:1 with zymography sample buffer (0.125 M Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 0.05% bromophenol blue; Sigma) and loaded to the gels. After electrophoresis gels were incubated for 30 min in 2.5% Triton X-100 at room temperature, 30 min in collagenase activation buffer (50 mM Tris–HCl, pH 7.4, containing 6.6 mM $CaCl_2$, 200 mM NaCl, and 0.2% Brij-35) at room temperature and 4 h in the same buffer at 37°C. After incubation, gels were stained with Coomassie Blue G-250 solution [20% EtOH, 0.08% Coomassie G-250 (Bio-Rad), 1.6% phosphoric acid, 8% ammonium sulfate] overnight. Gelatinases activity was visualized as distinct bands indicating proteolysis of the substrate.

Casein-plasminogen zymography was performed in 10% SDS–PAGE gels containing plasminogen (0.04 u/ml, Sigma) and α-casein (2 mg/ml, Fluka). Electrophoretic separation of the conditioned medium samples was performed as described for gelatin zymography. Gels were incubated for 30 min with Triton X-100 (2.5%) at room temperature, 30 min in distilled water at room temperature, and 4 h in uPA activation buffer (25 mM Tris–HCl, pH 7.4, containing 3.3 mM $CaCl_2$ and 100 mM NaCl) at 37°C. Caseinolytic bands were visualized after Coomassie Blue G-250 solution staining.

PLD-ACTIVITY ASSAY

PLD activity was measured using Amplex Red Phospholipase D assay kit (A12219, Molecular Probes, Invitrogen). 2×10^5 cells were seeded on six-well dishes. In 24 h the cell medium was removed and 2 ml of ice-cold PBS was added to the cells followed by replacement with 1× Reaction buffer. Afterwards cells were frozen and thawed three times in liquid nitrogen and detached from the dishes using a scraper. Gained solution was transferred to tubes. Protein concentrations were measured using Bredford method according to supplier's recommendations (Bio-Rad Laboratories GmbH). The total protein of 100 mcg was taken for each reaction. Further procedures were performed according to supplier's protocol. Fluorescence was measured at 590 nm wave length using excitation wavelength 530 nm using NanoDrop Fluorospectrometer ND3300 (NanoDrop Products).

PROLIFERATION ASSAY

For proliferation dynamics analysis 1×10^4 cells were seeded in triplicate on six-well plates. Proliferation analysis was conducted daily during 5 days. Cell proliferation was analyzed using cell number counting in Gorjaev's chamber. In brief, cells were removed from plates using Versene solution and cell number was counted using Gorjaev's chamber (three independent measures). Regression analysis, curve fitting, and comparison of the obtained data were done and graphs were plotted using GraphPad Prism software ver. 5.02.

IN VITRO INVASION ASSAY

Invasive ability of cells was measured with a QCM Cell Invasion Colorimetric Assay (Millipore) according to the manufacturer's protocol. Briefly, cells (2×10^4) in 0.5 ml of serum-free medium were seeded into the upper chamber with Matrigel-coated membrane. 0.75 ml of medium containing 10% FBS was added into the lower chamber. After 18 h incubating at 37°C, membranes were collected and non-invading cells were removed from the upper surface of the membrane using a cotton swab. Membranes were stained with 0.1% crystal violet, and photographed with digital camera DP71 using inverted microscope Olympus IX-51 with 10× objective.

TRANSWELL MIGRATION ASSAY

Corning Costar Transwell plates (8 μm) were pretreated according to the manufacturer's protocol. Directed motility assay was performed in uncoated chambers in similar conditions, as for in vitro invasion assay, but 1×10^4 cells were seeded in the upper chambers. After incubating (18 h at 37°C), membranes were collected and non-invaded cells were removed from the upper chamber using a cotton

swab, stained with 0.1% crystal violet, and photographed with digital camera DP71 using inverted microscope Olympus IX-51 with 10× objective.

CLONOGENICITY ASSAY

1×10^2 cells were seeded on 6-cm Petri dishes. Seven days later formed colonies were fixed with ethanol and stained with crystal violet. Pictures of Petri dishes were taken by compact camera and colony number was estimated using ImageJ software.

STATISTICAL ANALYSIS

All cell culture experiments were held in triplicate. Graph data represent the mean ± standard error calculated from indicated number of independent experiments. Differences between two groups were assessed using Mann–Whitney *U*-test. Simultaneous comparison of three or more groups was performed by using Kruskal–Wallis one-way analysis of variance followed Dunns post-test to compare with control group, when necessary. Results were analyzed and graphs built using GraphPad Prizm ver. 5.02 by GraphPad Software.

RESULTS

FUNCTIONALLY POTENT Arf6 STIMULATES IN VITRO PROLIFERATION BUT DOES NOT AFFECT CELL MIGRATION OR INVASIVENESS; BLOCKAGE OF Arf6–PLD INTERACTION CANCELS PROLIFERATION STIMULATION BUT STRENGTHENS CELL MIGRATION AND INVASIVENESS

Sequences encoding three different forms of HA-tagged Arf6: Arf6Q67L (constitutively active GTP-bound form), Arf6WT (wild-type), and Arf6N48I (wild-type Arf6 incapable of PLD activation), were stably introduced into HET-SR cells within retroviral vector pLXSN. Expression of exogenous Arf6 in cells selected on G-418 was confirmed by Western blot analysis using anti-HA and anti-Arf6 antibodies (Fig. 1A). HET-SR cells expressing empty vector pLXSN were used as the control cell line here and below. Further, we compared the most essential properties of Arf6Q67L, Arf6WT, and Arf6N48I cells, which might contribute to cell aggressiveness level.

Analysis of proliferation dynamics showed that both Arf6Q67L and Arf6WT significantly increased proliferation of HET-SR cells in comparison with control cell line ($P < 0.05$). At the same time Arf6N48I had no effect on proliferation rate, indicating that Arf6–PLD interaction is crucial to proliferation stimulation (Fig. 1B).

Next, we studied Arf6 influence on the ability of cells to grow under conditions of rare population (clonogenicity assay) to reveal its possible role in autocrine or paracrine stimulation of cell growth. We found that the number of colonies formed by all Arf6 cell derivates after seeding of 200 cells/6-cm dish was definitely higher ($P < 0.05$) than the number of colonies formed by control cells (Fig. 1C). Noteworthy, although Arf6N48I did not affect proliferation rate it gave some effect on clonogenicity, what could be a result of either less dependency from cytokines secreted by microenvironment or increased migrative activity of HET-SR-Arf6N48I cells (see below), contributing to the formation and spreading of daughter colonies. Analysis of anchorage-independent growth revealed no difference in soft agar colony formation between Arf6-expressing and control cells (data not shown).

To evaluate Arf6 impact on tumor growth dynamics in vivo, we measured primary tumor sizes, formed after subcutaneous injection of studied cells. We found no significant differences in tumor size compared to controls, although tumors formed by Arf6Q67L and Arf6WT expressing cells were more solid, more circumscribed with better defined borders (data not shown).

To study Arf6 influence on cell motility we applied serum-directed transwell migration in Boyden chambers (Fig. 1D) and wound healing assay (data not shown). We did not find statistically valid changes in motility of Arf6Q67L or Arf6WT expressing cells compared to the control cells in both tests. Surprisingly, Arf6N48I significantly promoted serum-directed transwell migration ($P < 0.05$).

The in vitro invasiveness of Arf6-expressing cells was examined using Matrigel-coated Boyden chambers (Fig. 1E). Interestingly, the results of this experiment fully agree with the data obtained from transwell migration assay, demonstrating that Arf6N48I cells had better ability to both penetrate through uncoated porous inserts and to invade through Matrigel-coated membrane. How could Arf6N48I stimulate motility and invasion? Our hypothesis is that functionally potent Arf6 interacts with PLD as its main effector and that this "partnership" leads to proliferation rise. In Arf6N48I overexpressing cells the abundance of Arf6 molecules unable to interact with PLD redirect Arf6 activity to alternative targets which normally contribute to migration and invasion but have less affinity to Arf6 than PLD.

Although an increase in cell motility may contribute in some extent to a highly invasive cell phenotype, it mostly depends on cell ability to degrade extracellular matrix. To study the mechanism of Arf6N48I-dependent stimulation of invasion we examined the activity of extracellular proteinases responsible for matrix remodeling. We analyzed activity of gelatin degrading matrix metalloproteinases (MMPs) and urokinase like plasminogen activator (uPA). The secreted proteinases activity was tested in culture medium by gelatin (for MMPs) or casein/plasminogen (for uPA) zymographies. MMP-2 was the most active gelatinase secreted by all studied cell lines. We did not reveal any difference in MMP1, MMP-2, or MMP-9 activity between studied cell lines (Fig. 1F). Comparison of uPA activity revealed its increase in condition medium from Arf6N48I expressing cells (Fig. 1G). Therefore, elevated invasiveness of Arf6N48I cells was associated with activation of uPA and correlates with an increase in motility.

Study of SMA in vivo did not reveal a statistically proved increase in amounts of histologically verified lung metastases in immunocompetent animals after subcutaneous injection of Arf6 expressing cells compared to control cells (Fig. 1H).

Arf6 ACTIVATION UPREGULATES S6K1 KINASE THROUGH Arf6–PLD–mTOR–S6K1 SIGNALING PATHWAY

Several studies support the role of Arf6 in PLD upregulation [Cockcroft et al., 1994; Boshans et al., 2000; Santy and Casanova, 2001; Xu et al., 2003; Rankovic et al., 2009]. Here, we compared the level of PLD activity in cells expressing different Arf6 variants and control cells. The graph in Figure 2A shows that although Arf6WT

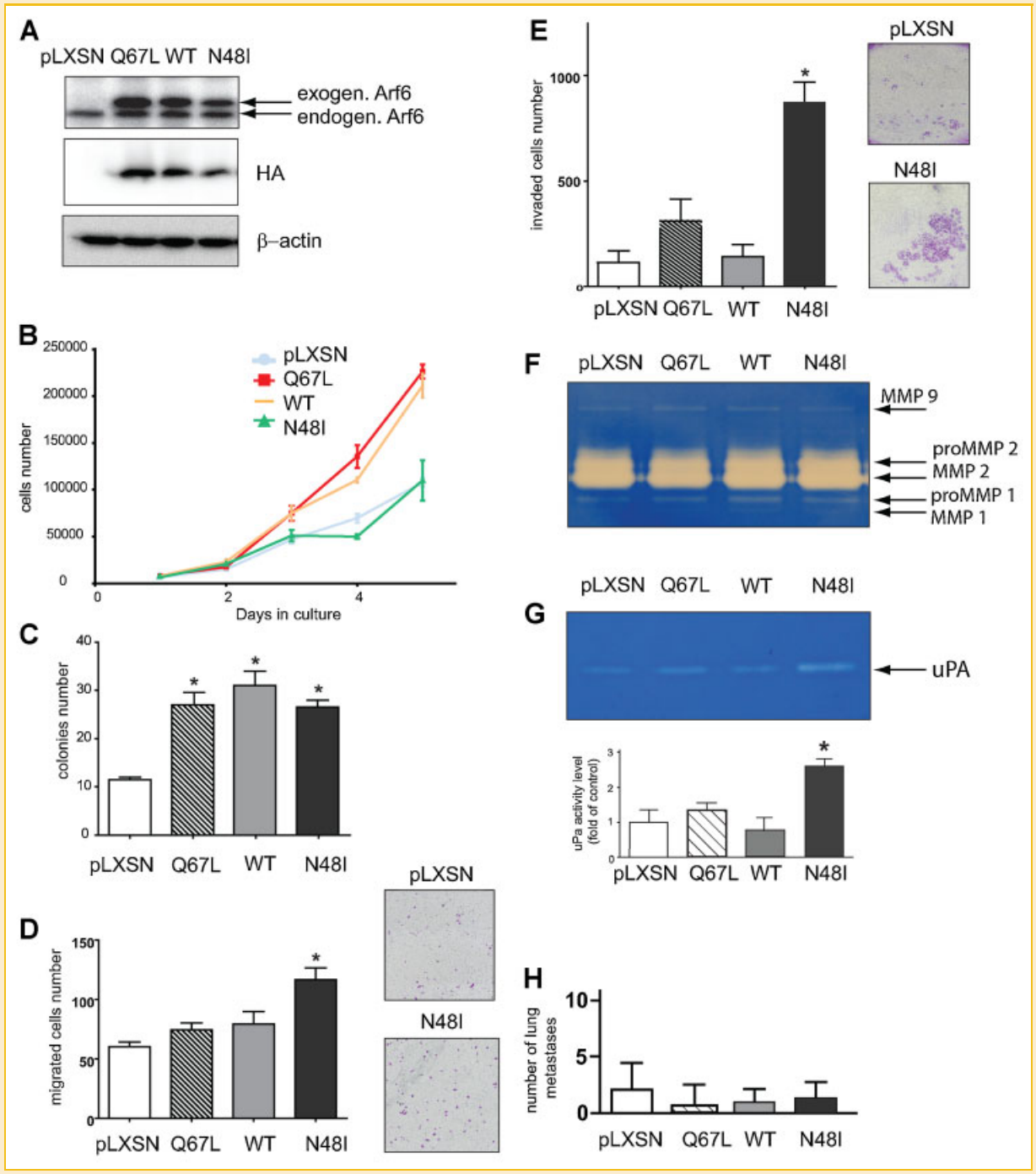

Fig. 1. Constitutively active as well as wild-type Arf6 stimulates proliferation while Arf6N48I promotes motility, invasion, and uPA proteinase activity. A: Western blot confirmation of exogenous Arf6 expression; sequences encoding HA-tagged Arf6: Arf6WT (wild-type), Arf6Q67L (constitutively active GTP-bound form), and Arf6N48I (wild-type Arf6 unable to interact with PLD), were transduced into HET-SR cells within retroviral vector pLXSN and selected on G418; whole lysates of Arf6 or empty vector expressing cells were analyzed by Western blotting using indicated antibodies. B: Arf6WT and Arf6Q67L expression promotes proliferation rate. Cell number was counted daily during 5 days using Gorjaev's chamber. Each data point on the graph represents mean ± SE of three independent measures. Arf6WT versus pLXSN: $P < 0.05$; Arf6Q67L versus pLXSN: $P < 0.05$. C: All studied Arf6 variants potentiate clonogenicity. Graphs present number of colonies formed after seeding of 1×10^2 cells, mean ± SE of three independent measures, $P < 0.05$. D: Arf6N48I promotes serum-directed transwell migration in uncoated Boyden chambers, mean ± SE, $P < 0.05$, compared with control cells. E: Arf6N48I promotes cell invasion through Matrigel-coated membrane in Boyden chambers, mean ± SE, $P < 0.05$, compared with control cells. F: Arf6 expression has no effect on gelatinase activity of secreted MMPs. Gelatine zymography was performed as described above. G: Arf6N48I expression stimulates uPA proteinase activity. Casein-plasminogen zymography was performed as described above. H: SMA analysis of Arf6 expressing cells. Graphs present amount of lung metastatic nodules per animal appearing after subcutaneous injection of described cells and xenograft tumor formation, mean ± SE of two independent experiments.

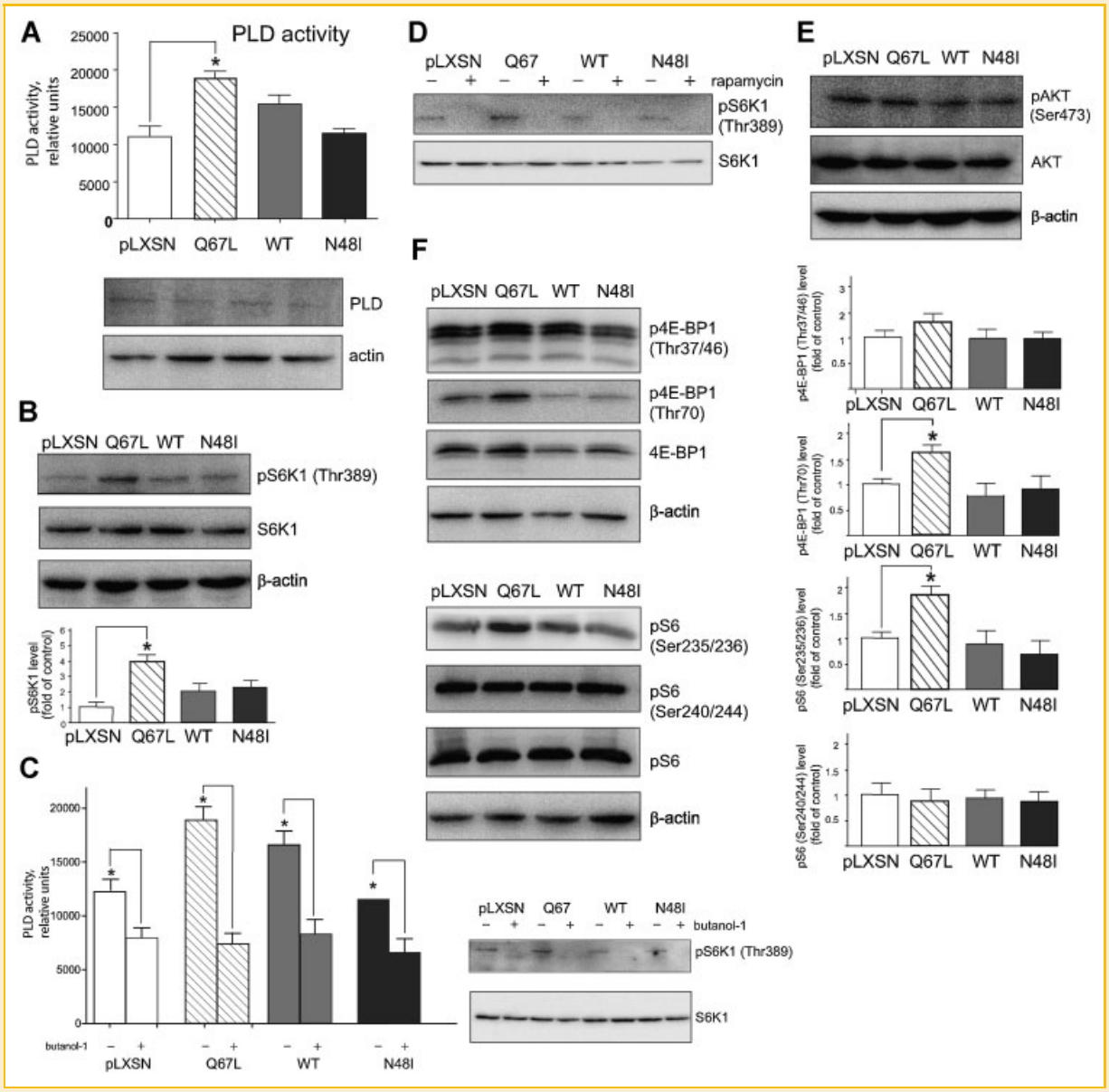

Fig. 2. Constitutively active Arf6 promotes S6K1/rpS6 and 4E-BP1 phosphorylation via Arf6-PLD-mTOR pathway. A: Arf6Q67L expression upregulates PLD activity giving no effect on PLD protein expression. PLD activity level was measured using Amplex Red Phospholipase D assay kit as described above, mean ± SE of three independent measures; PLD protein production was detected by Western blot analysis. B: Arf6Q67L expression promotes S6K1 phosphorylation. S6K1 phosphorylation and expression levels were detected by Western blot analysis of whole lysates using anti-phospho-S6K1 (Thr389) and anti-S6K1 antibodies respectively. C: Treatment with PLD inhibitor butanol-1 leads to PLD activity suppression followed by total inhibition of S6K1 phosphorylation. Cells were exposed to 1% butanol-1 for 24 h and lysed for PLD activity assay and for Western blot analysis with indicated above antibodies. D: Treatment with mTOR inhibitor rapamycin fully suppressed S6K1 phosphorylation. Cells were exposed to 100 nM rapamycin during 24 h and cell extracts were analyzed by Western blot analysis using indicated above antibodies. E: Arf6 expression gives no effect on Akt expression and phosphorylation level. Akt phosphorylation and expression levels were detected by Western blot analysis of whole lysates using anti-phospho-Akt (Ser473) and anti-Akt antibodies respectively. F: Arf6 promotes 4E-BP1 Thr70 and rpS6 Ser235/236 phosphorylation. 4E-BP1 and rpS6 phosphorylation status was examined by Western blot analysis of whole cell extracts using anti-phospho-4EBP1 (Thr37/46 or Thr70) and anti-phospho-rpS6 (Ser235/236 or Ser240/244) antibodies, respectively. Level of 4E-BP1 and rpS6 protein expression was detected in parallel. The bar graphs represent means ± SE of three independent measures. *Significant difference from corresponding controls ($P < 0.05$).

leads to PLD activity rise to some extent, statistically significant increase was observed only for cells expressing constitutively active Arf6. The level of PLD protein production was the same in all cell lines. We suggested that PLD activation could contribute to promitogenic effect of Ar6 and proposed mTOR kinase and its direct effector, kinase S6K1, as PLD downstream partners. To check this possibility we tested the level of S6K1 Thr389 phosphorylation in Arf6-expressing and control cells (Fig. 2B). Western blot analysis demonstrated a fourfold increase of phospho-S6K1 in Arf6Q67L expressing cells compared to the control. In contrast, in Arf6N48I or Arf6WT cells we did not find elevation of S6K1 phosphorylation, confirming the correlation between activation of PLD and

upregulation of S6K1. To prove the role of PLD and mTOR in S6K1 activation we treated all cell lines with selective PLD inhibitor, butanol-1, or with mTOR inhibitor, rapamycin, respectively, and measured PLD activity and level of pS6K1 phosphorylation in treated cells. As shown in Figure 2C,D, inhibition of PLD as well as mTOR activity suppression gave the same effect of crucial inhibition of S6K1 phosphorylation, indicating that S6K1 acts downstream from PLD and mTOR. As the majority of published data point out mTOR prosurvival role regulated by PI3K-Akt-mTOR signaling cascade, we examined the possible participation of this pathway in Arf6-dependent S6K1 upregulation. We compared Akt Ser473 phosphorylation level in Arf6-expressing and control cells and found that level of phospho-Akt as well as total Akt expression was roughly the same in all studied cell lines (Fig. 2E). This result confirms that Arf6 upregulates S6K1 independently from PI3K-Akt signaling.

For further study of Arf6-mTOR pathway upregulation we tested the phosphorylation status of mTORC1 downstream targets, 4E-BP1 and ribosomal S6 protein rpS6 (S6K1 effector). 4E-BP1 acts to repress cap-dependent translation as its binding to eukaryotic translation initiation factor 4E (eIF4E) prevents the formation of complex between eIF4E and eIF4G. 4E-BP1 undergoes phosphorylation at seven sites including Thr37, Thr46, Ser65, Thr70, which are linked to mTOR signaling. Two N-terminal threonins (Thr37 and Thr46) are required for phosphorylation of Thr70, which in turn is required for phosphorylation of Ser65. Phosphorylation of Thr70 appears to be of major importance in bringing about the release of 4E-BP1 from eIF4E, although the role of Thr70 and Ser65 phosphorylation is still controversial (for rev. see [Averous and Proud, 2006; Mamane et al., 2006]). While both 4E-BP1 and S6K1 regulate protein synthesis, 4E-BP1 appears to be mainly involved in cell proliferation, while S6K1 kinase in mammalian cells is pointed as a key player in control of cell growth (cell size) and proliferation [Dowling et al., 2010]. Ribosomal protein S6, a protein of the 40S ribosomal subunit, is one of the main targets of activated S6K1 which promotes translation initiation. At least five sites of phosphorylation exist in rpS6 C-terminus, although physiological role of rpS6 phosphorylation is quite unclear. Particularly, four serine residues, Ser235/236 and Ser240/244, are thought to be phosphorylated by ribosomal kinases (RSKs) and by S6K1, although data on which kinase is responsible for each site phosphorylation is rather controversial. Thus, Ser235/236 phosphorylation, which was found to correlate with assembly of the translation preinitiation complex and increased cap-dependent translation, seems more likely to be phosphorylated by RSK kinases family, while S6K1 is considered to phosphorylate every site [Steelman et al., 2011].

Here, we examined Thr37/46 and Thr70 phosphorylation level of 4E-BP1 as well as Ser235/236 and Ser240/244 phosphorylation level for rpS6. We found high enough basic phosphorylation level of both proteins in parental HET-SR cells. Expression of Arf6Q67L slightly potentiated the level of 4EB-P1 phosphorylation at Thr37/46 but significantly facilitated the level of phospho-Thr70 (Fig. 2F). rpS6 phosphorylation of Ser240/244 site was steady-state in all studied cells, while phosphorylation of Ser235/236 was noticeably increased in Arf6Q67L expressed cells.

Therefore, we conclude that Arf6 upregulates mTOR signaling in PLD-dependent manner, promoting activation of S6K1-rpS6 and 4E-BP1 phosphorylation.

Arf6 ACTIVATES MAP KINASES Erk1/2 AND p38 AND THIS ACTIVATION DEPENDS ON Arf6–PLD INTERACTION

According to some earlier publications, one of the strongest candidates for mediating Arf6 impact on cell proliferation could be well-known mitogen activator kinase Erk1/2, although data concerning Arf6 effect on Erk1/2 are rather discrepant. Data obtained on melanoma LOX cell line indicate that Arf6 promotes activation of MEK/ERK signaling in human [Tague et al., 2004; Hoover et al., 2005; Muralidharan-Chari et al., 2009b]. At the same time, study performed on glioma cells demonstrates that Arf6 suppression by siRNA gives no effect on EGF-stimulated Erk1/2 activity [Hu et al., 2009]. Besides, evidence obtained also on glioma cell model indicates that Arf6 is in turn regulated by Erk1/2 [Li et al., 2009]. To clarify the possibility of Arf6-dependent Erk1/2 activation we compared the Erk1/2 Tyr202/204 phosphorylation level in all studied cell lines and found its dramatic increase in both Arf6Q67L and Arf6WT cells (Fig. 3A). Thus Arf6Q67L gave 3.5-fold and Arf6WT showed threefold increase of Erk1/2 phosphorylation. In contrast, Arf6N48I cells showed even lower Erk1/2 phosphorylation status compared to the control cell line, substantiating that Arf6–PLD interaction is necessary for Erk1/2 activation.

Despite the data discrepancy concerning Arf6 effect on Erk1/2 activation, Arf6 influence on other key MAP kinases had not been studied before. We examined expression of p38 and JNK, main mitogen-activated kinases from parallel MAPK signaling. Study of JNK kinase did not reveal significant alterations either in expression or in Tyr183 phosphorylation status (Fig. 3B). Analysis of activating p38 Thr180/Tyr182 phosphorylation found its significant (fourfold) elevation in Arf6Q67L as well as in Arf6WT cells (twofold) compared to the control in distinction from Arf6N48I cells, which demonstrated less than control cells level of pp38 (Fig. 3C).

These data indicate that Arf6 overexpression activates Erk1/2 and p38 MAP kinases. In both cases constitutive activation of Arf6 gave slight additional effect on Erk1/2 and p38 upregulation compared to that of Arf6WT, while interaction with PLD was essential.

BOTH mTORC1–S6K1 AND p38MAP KINASE PATHWAYS CONTRIBUTE TO Arf6-DEPENDENT INCREASE OF PROLIFERATION AND UPREGULATION OF S6 RIBOSOMAL PROTEIN PHOSPHORYLATION

As we found three potential pathways, which could be involved in Arf6-dependent increase of proliferation namely, mTOR, through S6 and 4E-BP1 effectors, and MAP kinases Erk1/2 and p38, we further evaluated the significance of each candidate. For this purpose we compared proliferation dynamics of Arf6Q67L cells before and after treatment with specific inhibitors, such as rapamycin for mTOR suppression, CI-1040 and SB203580 for downregulation of MAP kinases Erk1/2 and p38 respectively. Effects on Erk1/2, p38 and S6K1 inhibition were confirmed by Western blotting (Fig. 3D). Graphs presented on Figure 3D show that suppression of mTORC1-S6K1 and to the less extent p38 resulted in statistical significant

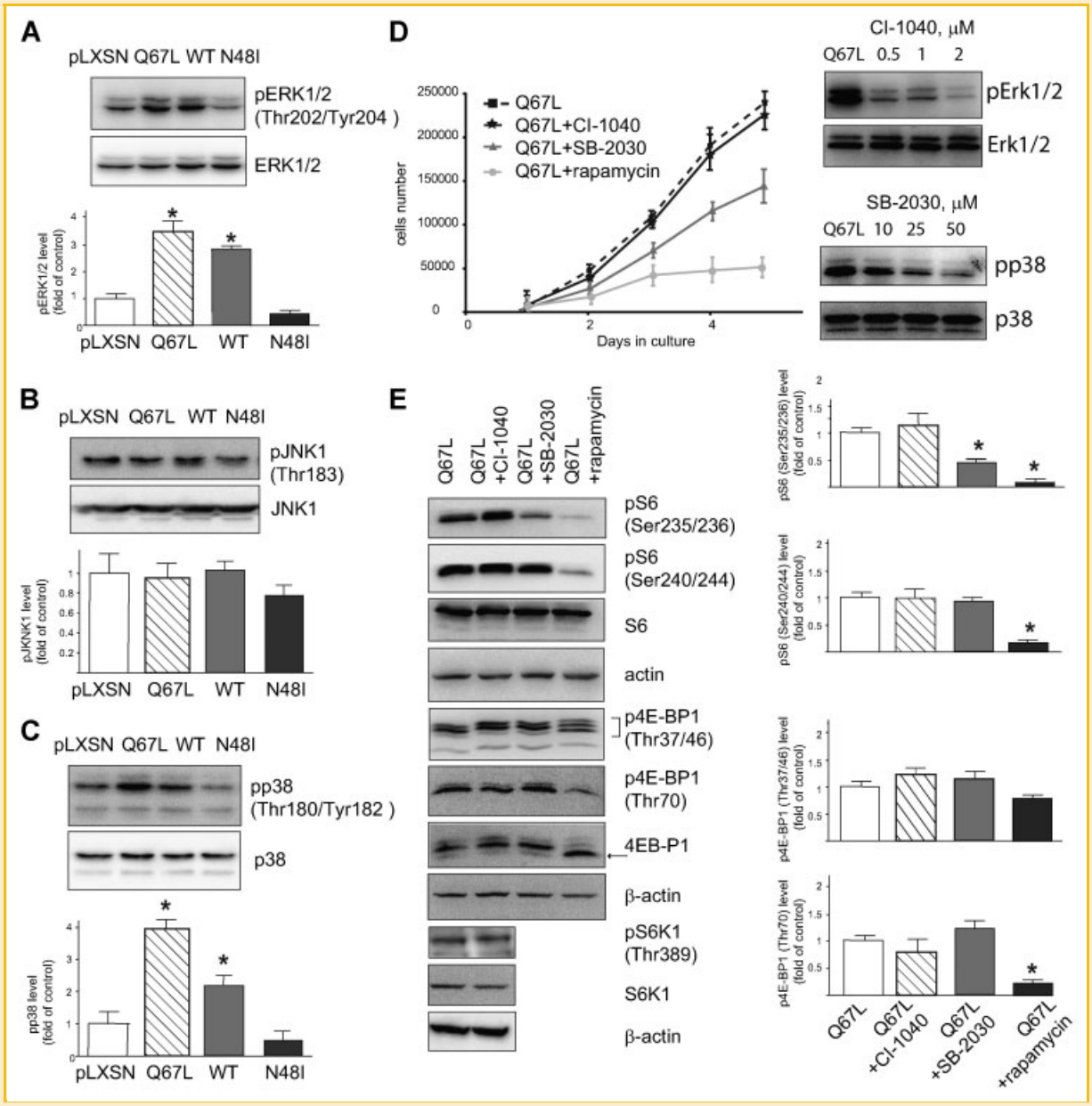

Fig. 3. Arf6 stimulates Erk1/2 and p38 MAP kinases phosphorylation in PLD-dependent manner; both mTORC1-S6K1 pathway stimulation and upregulation of p38 MAP kinase contribute to Arf6-dependent increase of proliferation, while Erk1/2 activation is insignificant. A: Arf6Q67L and Arf6WT opposite to Arf6N48I promote Erk1/2 phosphorylation. Activating Erk1/2 phosphorylation and expression levels were detected by Western blot analysis of whole cell extracts using anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-Erk1/2 antibodies, respectively. B: Arf6 has no influence on Jnk phosphorylation status. Activating Jnk phosphorylation and expression levels were detected by Western blot analysis of whole cell extracts using anti-phospho-JNK1 (Thr183) or anti-Jnk antibodies, respectively. C: Arf6Q67L and Arf6WT in contrast to Arf6N48I promote p38 phosphorylation level. Activating p38 phosphorylation and expression levels were detected by Western blot analysis of whole lysate using anti-phospho-p38 Thr180/Tyr182 and anti-p38 antibodies respectively. D: Arf6-dependent proliferation rise depends on both mTORC1-S6K pathway and p38 kinase activity. Proliferation dynamics of Arf6Q67L expressing cells treated with CI-1040 Erk1/2 inhibitor, SB-2030 p38 inhibitor, or rapamycin was compared with that of untreated Arf6Q67L cells. Optimal working concentrations were determined as 2 μM for CI-1040 and 25 μM for SB-2030 (since concentration 50 μM had a toxic effect on cells), for confirmation of 100 nM rapamycin treatment efficiency on S6K inhibition (see Fig. 2D). Number of cells was counted daily during 5 days using Gorjaev's chamber. E: Erk1/2 inhibition gave no suppressive effect on rpS6 or 4E-BP1 phosphorylation status, while mTOR-S6K1 inhibition led to significant decrease of S6 phospho-Ser235/236 as well as on S6 phospho-Ser240/244 levels. p38 inhibition suppressed rpS6 phosphorylation at Ser235/236 site, giving no significant effect on phospho-Ser240/244 level. Each data point on the graph represents mean ± SE of three independent measures. Arf6Q67L cells treated with CI-1040, SB-2030, and rapamycin inhibitors versus Arf6Q67L untreated cells ($P < 0.05$). The bar graphs represent means ± SE of three independent measures. *Significant difference from corresponding controls ($P < 0.05$).

decrease in Arf6Q67L proliferation, while Erk1/2 inhibition unexpectedly gave no effect on proliferation dynamics. Thus, we conclude that both mTORC1 complex and p38 MAP kinase contribute to Arf6-dependent promotion of proliferation.

It has been established that Erk1/2 via activation of RSK family of RSKs could converge in a few ways to the mTORC1 pathway to promote mTORC1 signaling: firstly, by RSK-mediated phosphorylation of Tsc2 which inactivates the suppressive effect of the Tsc1/2 complex on small GTPase Rheb; secondly, by RSK-dependent Raptor phosphorylation and thirdly, by RSK-dependent phosphorylation or rpS6 (for rev. see [Anjum and Blenis, 2008]). To study the possible cross-talk between Arf6-dependent mTORC1 and Erk pathways upregulation, we tested the influence of Erk1/2 inhibition on phosphorylation of mTORC1 downstream effectors. For this purpose Arf6Q67L cells were exposed to selective MEK-Erk inhibitor, CI-1040, and levels of S6K1Thr389 and 4E-BP1Thr70 phosphorylation were compared. As shown on the Figure 3E, Erk1/2 suppression did not decrease phosphorylation level of both mTORC1 effectors. These data indicate that Arf6 upregulates mTORC1 independently from Erk1/2.

Another question that should be answered is how Arf6-dependent p38 kinase activation could promote cell proliferation. As was mentioned above, RSK impinge mTORC1 signaling involved in translation regulation through phosphorylation of S6 ribosomal protein and, what is especially important in our case, RSKs are thought to phosphorylate S6 predominantly at Ser235 and Ser236 sites. Accordantly to canonical pathway, RSKs are directly activated by Erk1/2 or PDK1 kinases. At the same time, recent data indicate that p38 can also upregulate RSKs through phosphorylation of MAPK-activated kinases MK2 or MK3 [Zaru et al., 2007].

Subsequently, in our model both Arf6 stimulated kinases, Erk1/2 and p38, could promote RSK to phosphorylate S6 ribosomal protein independently from mTORC1 signaling. Besides, S6 could be also phosphorylated by S6K1 via mTORC1 signaling. To clarify which pathway is responsible for S6 activation we examined the effect of mTORC1-S6K1, Erk1/2 and p38 suppression (by chemical inhibitors rapamycin, CI-1040 and SB-2030, respectively) on S6 phosphorylation status. In parallel 4E-BP1 phosphorylation status in response to treatment with above mentioned inhibitors was also tested (Fig. 3E). Noteworthy, the results showed that Erk1/2 inhibition gave no suppressive effect on S6 Ser 235/236 or Ser 240/244 phosphorylation, as well as on 4E-BP1 phosphorylation status. As expected, mTOR-S6K1 inhibition led to significant decrease of phospho-Ser235/236 as well as on phospho-Ser240/244 level of S6 (10- and 5-fold, respectively). Intriguingly, p38 inhibition drastically suppressed (2.5-fold) rpS6 phosphorylation at Ser235/236 site which is supposed to be phosphorylated by RSKs, although level of phospho-Ser240/244 remained steady-state. These data suggest that Arf6-dependent S6 activation could be a result of two pathways impacts: RSK-p38 signaling, which mostly contributes to Ser235/236 phosphorylation increase, and mTORC1-S6K1 pathway, which is responsible for both Ser235/236 and Ser240/244 phosphorylation. As concerning 4E-BP1, its activation is independent from p38 or Erk1/2 signaling and is mediated by mTORC1-S6K1 pathway demonstrating fourfold decrease of Thr70 phosphorylation after rapamycin treatment.

Therefore, activated Arf6 (Arf6Q67L) upregulates PLD activity to promote mTOR and its both downstream effectors involved in translation regulation, that is, 4E-BP1 and S6K1. This pathway at least partially participates in S6 phosphorylation. Besides, Arf6Q67L and less pronounced Arf6WT upregulate p38, which also strongly contributes to S6 activation promoting its phosphorylation on Ser235/236. These results strongly agree with the data on proliferation analysis, as Arf6Q67L and to the less extent Arf6WT stimulate proliferation rise and this effect depends mostly on mTORC1-S6K1 and less on p38 kinase activity. In summary, we conclude that Arf6 facilitates HET-SR cells proliferation through PLD-mTORC1 and p38 signaling pathways. In addition, activated and wild-type Arf6 potentiate Erk1/2 kinase, although Erk1/2 upregulation does not contribute to stimulation of proliferation.

DISCUSSION

Several data confirm Arf6 promigrative and proinvasive functions as of most importance in its role in carcinogenesis [Palacios et al., 2001; Santy and Casanova, 2001; Sabe, 2003; Hashimoto et al., 2004; Tague et al., 2004; Li et al., 2006a; Cotton et al., 2007; Hu et al., 2009; Muralidharan-Chari et al., 2009b]. One study gives direct evidence of Arf6-dependent in vivo metastasis stimulation [Muralidharan-Chari et al., 2009b]. The goal of this study was to study the Arf6 influence on cluster of properties involved in tumor progression and acquisition of highly aggressive cell phenotype, including mentioned above motility, invasiveness and metastatic potential. The chosen HET-SR cell line is characterized by high level of tumorigenicity and low level of lung-directed metastasis after subcutaneous injection in immunocompetent syngeneic animals [Deichman et al., 1989; Tatosyan et al., 1996; Illan Rubio et al., 2010]. Pointed features make this model especially attractive for studying influence of different proteins on SMA. We have shown previously metastasis stimulating activity of some other G-proteins, such as Ha-Ras, RalA [Tchevkina et al., 2005], and RalB (Rybko et al., 2011, in press) using this cell line. However, we did not reveal significant effect of Arf6 on metastatic activity of HET-SR cells. Moreover, in contrast to the majority of previously published studies, we found that neither overexpression of wild-type Arf6 nor constitutive activation stimulated migrative or invasive capacity of studied cells. Instead, we demonstrated, that both wild-type and constitutively active Arf6 promoted proliferative activity in vitro. Interestingly, expression of mutant Arf6 unable to interact with PLD (Arf6N48I) did not change the proliferation dynamic, confirming that Arf6 promitogenic action is PLD-dependent.

It should be mentioned, that all published data on Arf6-dependent migration and invasion stimulation have been obtained on cell lines of ectodermal origin, that is, glioma [Li et al., 2006a; Hu et al., 2009], melanoma [Tague et al., 2004; Hoover et al., 2005; Muralidharan-Chari et al., 2009a,b], and few other epithelial cell lines Palacios et al., 2001; Santy and Casanova, 2001; Cotton et al., 2007], particularly breast cancer [Hashimoto et al., 2004; Sabe et al., 2008, 2009]. Here, we studied primary fibroblasts which differ from above in the character of movement. Moreover, studied cells are characterized by originally high motility level in transwell

migration assay as well as of high level of proteinases secretion and matrix degradation. Surprisingly, we found that expression of Arf6N48I mutant, which is unable to interact with PLD, stimulates both serum directed migration and invasion and elevates activity of the secreted proteinase uPA. Arf6N48I expressing cells demonstrated no increase in wound healing, suggesting that better migration through uncoated Boyden chambers could be a result of increased chemotactic activity. Therefore in the described model expression of wild-type Arf6 as well as constitutively active Arf6 gives no strengthening effect on migration and invasion but significantly stimulates proliferation and this stimulation is PLD-dependent. Consequently, introduction of Arf6N48I is not followed by proliferation increase as it does not activate PLD. We hypothesize that in this case the pool of overexpressed Arf6N48I molecules titrates the rest of Arf6 effectors (e.g., Rac1), sequestering them from endogenous Arf6, and thus redirecting Arf6 activity from PLD-dependent stimulation of proliferation to PLD-independent stimulation of motility and invasion. It should be also mentioned that the level of Arf6N48I expression was comparable to that of endogenous Arf6 in control cells (Fig. 1A) and thus seemed to be not enough to demonstrate dominant negative effect on endogenous PLD signaling in Arf6N48I expressing cells.

Therefore, we suggest two possible Arf6 downstream pathways—PLD-dependent, which leads to the proliferation stimulation, and PLD-independent stimulating motility and invasion. Importantly, cells with activated PLD-independent pathway (Arf6N48I) preserve the steady-state level of metastasis, confirming that invasion does not always determines cell metastatic potential.

Studying the mechanisms of Arf6-dependent cell proliferation stimulation we found few signaling pathways contributing to Arf6 promitogenic activity. Firstly, we showed that Arf6 activates S6K1 kinase, well-known regulator of mitogen-stimulated translation initiation. This activation is PLD and mTOR-dependent, as suppression of PLD or mTOR by their selective inhibitors butanol-1 and rapamycin, respectively, abrogates the effect of S6K1 activation. Therefore, we first demonstrated existence of the Arf6 → PLD → mTOR → S6K1 signaling pathway. Interestingly, S6K1 activation as well as PLD upregulation takes place only in case of constitutively active Arf6. Further examining mTORC1-S6K1 downstream effectors we found significant increase of 4E-BP1 and ribosomal S6 proteins phosphorylation in Arf6 expressing cells. Effect of 4E-BP1 phosphorylation rise was strongly expressed for Thr70 and less pronounced for Thr37/46 sites. Notably, rapamycin treatment canceled Thr70 phosphorylation giving very slight effect on phospho-Thr37/46 level. These results evidence that Thr37/46, unlike Thr70 phosphorylation, is thought to be independent from mTORC1. Therefore, Arf6 promotes 4E-BP1 Thr70 phosphorylation and this finding further confirms the Arf6-PLD-mTOR-4E-BP1 signaling pathway. Arf6-dependent S6 phosphorylation was also found to be site-specific. Thus, we found increase of phospho-Ser235/236 in Arf6Q67L and to the less extent in Arf6WT expressing cells, while the level of phospho-Ser240/244 was steady-state in all cell lines including control cells. Rapamycin treatment of Arf6Q67L expressing cells resulted in crucial decrease of proliferation rate, pointing on the significance of mTORC1 signaling in Arf6-dependent stimulation of proliferation.

Next, we showed that Arf6 activates MAP kinase Erk1/2, well-known mitogenic stimulator. Data concerning Arf6 influence on this protein are rather contradictive. Thus, few studies published by one group of authors indicate that Arf6 promotes activation of MEK/ERK signaling in human melanoma LOX cell line [Tague et al., 2004; Hoover et al., 2005; Muralidharan-Chari et al., 2009a,b]. At the same time, study on glioma cells demonstrates that Arf6 suppression by siRNA gives no effect on EGF-stimulated Erk1/2 activity Hu et al., 2009]. Besides, evidence also obtained on glioma cell model indicates that Arf6 is in turn regulated by Erk1/2 [Li et al., 2009]. Here, we found that Erk1/2 phosphorylation unlike S6K1 was elevated in both constitutively active Arf6 and wild-type Arf6 expressing cells. Noteworthy, incapability of Arf6–PLD interaction canceled Erk1/2 activation pointing on the essential role of Arf6–PLD partnership in this process. To check the possibility of Erk1/2 impact in Arf6-dependent upregulation of mTORC1 signaling, which could be accomplished through Erk1/2-and RSK-mediated suppression of Tsc1/2, Raptor, or S6 phosphorylation, we studied the effect of Erk1/2 inhibition (CI-1040 treatment) on phosphorylation of mTORC1 downstream targets and found no changes in phosphorylation status of S6K1, S6, and 4E-BP1. We also, surprisingly, found no effect of Erk1/2 downregulation on proliferation dynamics of Arf6Q67L expressing cells. Therefore, we conclude that Arf6-dependent Erk1/2 stimulation was insignificant for both mTORC1 signaling and proliferation.

As distinguished from Erk1/2, there are no up to date published data concerning Arf6 influence on other mitogen-activated kinases. Here, we studied p38 and Jnk, two key MAP kinases from parallel pathways. We found Arf6-dependent stimulation of p38 phosphorylation, and showed that this process also needs Arf6–PLD interaction. Noticeably, p38 unlike Erk1/2 significantly contributes to Arf6-dependent promotion of proliferation. Searching for the possible mechanisms of p38 influence on proliferation, we suggested that this MAP kinase could stimulate S6 ribosomal protein. It was shown previously that p38 could upregulate RSKs through activation of MAPK-activated kinases MK2 or MK3 [Zaru et al., 2007]. Other data indicate that p38 is also needed for the amino acid-induced phosphorylation of S6K1 (at Thr421/Ser424 positions, which is distinct from Thr389 site, specific for mTOR-dependent phosphorylation) and for that of S6 ribosomal protein [Casas-Terradellas et al., 2008]. Intriguingly, in presented model Arf6Q67L and to the less extent Arf6WT, stimulated S6 phosphorylation at Ser235/236 (site that is thought to be predominantly phosphorylated by RSKs) and gives no effect on phospho-Ser240/244. Moreover, suppression of p38 in Arf6Q67L expressing cells decreased phospho-Ser235/236 level of S6 and had no effect on Ser240/244 phosphorylation. Therefore, p38 could potentiate S6 either directly or through RSKs stimulation. Besides, activated RSKs could themselves additionally promote proliferation upregulating numerous transcriptional factors through other pathways. In sum, these data indicate that p38 greatly contributes to S6 phosphorylation as well as to Arf6-dependent proliferation rise.

Therefore, we show that Arf6 promote cell proliferation and activates at least three promitogenic signaling pathways, that is, PLD-mTORC1-S6K1, p38, and Erk1/2. Surprisingly, only two first pathways participate in proliferation stimulation, while Erk1/2 was

indifferent. We found that both partnerships mediate Arf6 promitogenic action promoting cap-dependent translation through regulation of ribosomal protein S6 and 4E-BP1 components of translation initiation machinery.

These results highlight Arf6 involvement in translation regulation through mTORC1 and p38 MAP kinase activation and give new evidence confirming Arf6 significant role in tumor cell biology.

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