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Functional role of inositol-1,4,5-trisphosphate-3-kinase-A for motility of malignant transformed cells

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Cell migration is one of the hallmarks of metastatic disease and thus identification of migration promoting proteins is crucial for the understanding of metastasis formation. Here we show that the neuron-specific, F-actin bundling inositol-1,4,5-trisphosphate-3-kinase-A (ITPKA) is ectopically expressed in tumor cells and critically involved in migration. Down-regulation of ITPKA expression in transformed cell-lines with ectopic expression of ITPKA significantly decreased migration and the number of linear and branched cell protrusion. Conversely, up-regulation of ITPKA in tumor cell lines with low endogenous ITPKA expression increased migration and formation of cell processes. *In vitro*, ITPKA alone induced the formation of linear actin filaments, whereas ITPKA mediated formation of branched protrusions seems to result from interaction between ITPKA and the F-actin cross-linking protein filamin C. Based on these actin-modulating and migration-promoting effects of ITPKA we examined its expression in clinical samples of different tumor entities, starting with the analysis of multiple tumor tissue arrays. As in lung adenocarcinoma specimens, the highest ITPKA expression rate was found, this tumor entity was examined in more detail. ITPKA was expressed early in adenocarcinoma progression (pN0) and was largely maintained in invasive and metastatic tumor cell populations (pN1/2, lymph node metastases). Together with our result that high expression of ITPKA increases motility of tumor cells we conclude that the observed expression of ITPKA early in tumor development increases the metastatic potential of lung adenocarcinoma cells. Therefore, we suggest that ITPKA may be a promising therapeutic molecular target for anti metastatic therapy of lung cancer.

Under physiological conditions, inositol-1,4,5-trisphosphate-3-kinase-A (ITPKA, GeneAtlas, V133A gcma, BioGPS, http://biogps.gnf.org/#goto=genereport&id=3706) is only expressed in hippocampal, cortical and cerebellar neurons.[1] In these cells expression of ITPKA increases during brain development[1] and is highest in mature neurons, where the protein accumulates in dendritic spines.[2] Through conversion of inositol-1,4,5-trisphosphate ($InsP_3$) to inositol-1,3,4,5-tetrakisphosphate ($InsP_4$) ITPKA is an important regulator of $InsP_3$ induced calcium signaling in dendritic spines.[2,3] In addition to this catalytic activity, it has been shown that ITPKA has F-actin bundling activity. It bundles actin filaments by formation of homodimers and binding to F-actin, making ITPKA to an important regulator of spine morphology.[4] Beside this physiological role, we recently have found a pathological function of ITPKA in tumor cells. We revealed that particular tumor cell lines express ITPKA and demonstrated that this ectopic expression of ITPKA increased the metastatic potential of tumor cells. Because of its F-actin bundling activity, ITPKA induces the formation of filopodia- and lamellipodia-like protrusions,[5,6] which are critical for cells to migrate.[7] This non-enzymatic migration-promoting effect of ITPKA is stimulated by its enzyme activity in response to stimulation with growth factors. The product of ITPKA inhibits activity of $InsP_3$ 5′phosphatase (5PPT) leading to elongation of half-life of $InsP_3$. The resulting elongated calcium signal further increases the intracellular calcium concentration by inducing the activation of store operated calcium entry. Elevated calcium levels activate the calcium dependent F-actin severing protein gelsolin, focal adhesion kinase and myosin light-chain kinase.[8] Thereby, the enzyme activity of ITPKA enhances the non-enzymatic basal effect of ITPKA on cellular migration.

Key words: actin-bundling, adenocarcinoma, protein–protein interactions, metastatic spread, cytoskeleton
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To study this migration-promoting effect of pathological expression of ITPKA in tumor cells in more detail, the relevance of ITPKA expression in different tumor cell-lines and in clinical samples from tumor patients was examined in this study.

Material and Methods

Primary tumor cell cultures and culture conditions

For establishing the cell lines PT 4323 and LN 4323, the primary tumor tissues (PT 4323) and the lymph node metastases (LN 4323) were taken from a 47-year-old female patient who underwent surgery for advanced bronchial adenocarcinoma. The specimen was staged pT2, pN2, G3. A written consent of the patient for the removal of tissue samples for investigational purposes was obtained before surgery.

Tumors were minced with a scalpel under sterile conditions and fragments were enzymatically disaggregated with 0.5% collagenase type IV (Sigma-Aldrich, Steinheim, Germany) at 37°C on a rotary shaker. After 45 min of incubation, dissociated tissue was harvested by centrifugation (700*g* for 5 min at room temperature), the pellet was collected, washed twice in cell culture medium (RPMI, Invitrogen, NY) and resuspended in complete medium (TUM, see below). Then, the cells were plated into collagen-coated culture flasks (Becton Dickinson Labware, Bedford, MA), and cultivated at 37°C in a humidified atmosphere of 5% CO_2. The TUM medium was comprised of RPMI 1640 with Glutamax (Invitrogen, NY) supplemented with 10% of fetal calf serum (FCS), 200 U/ml of penicillin-streptomycin, 0.1 mg/ml gentamycin (Biochrom AG, Berlin, Germany), 50 nmol/ml of human transferrin (Sigma-Aldrich, Steinheim, Germany), 0.01 μg/ml of bovine insulin (Sigma-Aldrich, Steinheim, Germany), 0.01 μg/ml of recombinant human epidermal growth factor (Pepro Tech, London, UK) and 0.01 μg/ml of human basic fibroblast growth factor (Pepro Tech, London, UK). Media were changed every 4–7 days, and culture flasks were regularly checked for epithelial cells and fibroblast outgrowth. The cell line was cultured as monolayers in 25- or 75-cm^2 flasks, routinely passed by trypsinization, and maintained in complete culture medium. Cells at different culturing passages were stored in liquid nitrogen in culture medium containing 10% dimethyl sulfoxide. At this time the cells underwent 100 passages and were free of fibroblasts.

Patient tumor specimens

Specimens from 94 patients with locally advanced adenocarcinomas of the lung were included in this study. For detailed clinical information of individual samples see supplementary data.

All patients underwent thoracic surgery at the Department of Cardio-Thoracic Surgery, Medical University of Vienna, between 1999 and 2006. None of them received preoperative systemic therapy. After surgery, tissue samples were routinely fixed in 4.5% buffered formalin and embedded in paraffin for routine diagnosis. In addition, two tissue micro arrays (TMA) were used (1) The multiple tumor and 6 types of normal organ tissue array (Array II) from IMGENEX (San Diego, CA). This TMA included 72 samples with normal (uterus, cervix, muscle, skin, nerve, mesotheliom) and malignant tissues (brain, ovary, testis, thyroid, breast, lung, esophagus, stomach, small intensine, colon, rectum, uterus, uterine cervix, skin, peritoneum, retroperitoneum, lymph node, bladder, mediastinum, smooth muscle, bone). (2) The "lung cancer with matched lymph node metastasis tissue array," including six samples from small cell lung cancer, 14 samples from squamous cell carcinoma and 12 samples from adenocarcinoma of the lung. The TMA was purchased from Biomax (Rockville, MD).

Statistical analyses

Statistical analyses were performed using conditional logistic regression method, taking into account the pairwise structure of the data. Comparison of the unpaired data was carried out by means of chi-square tests. The values of $p < 0.05$ were considered as significant. Calculations were done using R (Version 2.10.1) and SPSS (Version 17.0.0). (for details see http://www.R-project.org.)

Immunohistochemistry

For all immunohistological studies formalin-fixed, paraffin-embedded samples were used. Immunohistochemistry was essentially performed as described,[6] with the following exceptions: The primary polyclonal anti-ITPKA antibody was from rabbit (14270-1-AP, Proteintech, Chicago, IL). It was diluted 1:200 and incubated with the tissue section over night at 4°C. The secondary antibody and the acetylethylcarbazol chromogen system were used according to the manufacturer's instructions (R&D Systems Rabbit-Kit, McKinley, Minneapolis). To evaluate the specificity of the anti-ITPKA antibody, paraffin-embedded samples of H1299 Xenograft mouse-tumors with overexpression or knock down of ITPKA were used for comparative immunoreactivity.

Western blot

The same protocol as described[6] was used. Expression of ITPKA was evaluated with two different antibodies: with a polyclonal antibody from goat (Sc-11206 Lot#191,Santa Cruz, CA) and with a polyclonal antibody from rabbit (14270-1-AP, Proteintech, Chicago, IL), both antibodies were used in a dilution of 1:2000. Filamin C expression was analyzed by the use of a polyclonal antibody from goat (Sc-48496 Lot#B2007, Santa Cruz, CA) in a dilution of 1:1000.

Knock down and up-regulation of ITPKA expression

Stable down-regulation of ITPKA expression was performed by using a lentiviral approach as described.[6] Overexpression of ITPKA was performed by transient transfection of cells with pEGFP-C1-ITPKA[6] and pEGFP-C1 (Clontech, Heidelberg, Germany) as control. For stable overexpression transfected cells were selected with G418 for 2 weeks.

Transwell migration assay

This experiment was performed and evaluated as described.[6]

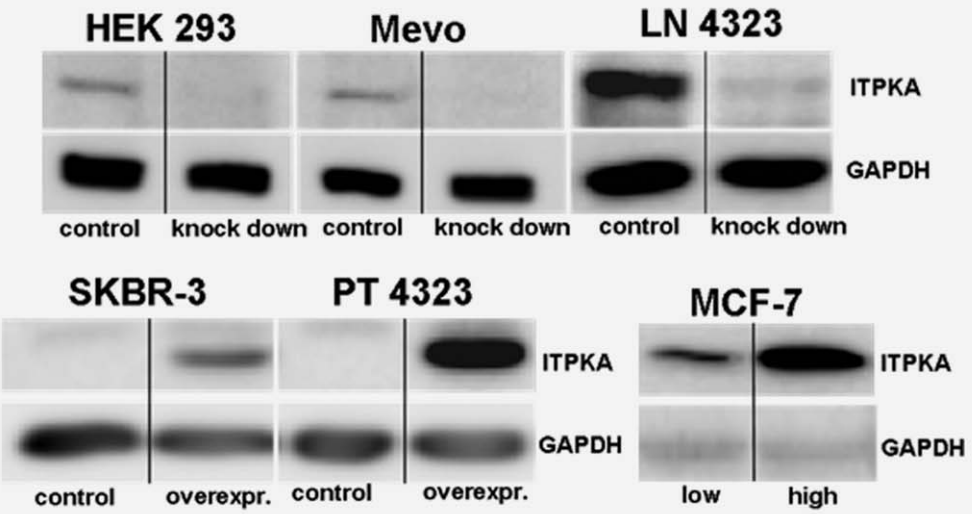

Figure 1. Modification of ITPKA expression in tumor cell lines. Upper panel: In cells with high endogenous expression of ITPKA, its expression was stably down-regulated by using a lentiviral approach. Lower panel: In human cells with low/absent ITPKA expression, an EGFP-ITPKA fusion protein or EGFP as control were stably overexpressed. In addition, the expression levels of ITPKA in MCF-7 subpopulations (right lower panel) with low or high ITPKA expression is shown. Expression levels of the modified cell lines were analysed by Western blot using an anti-ITPKA antibody. GAPDH expression served as loading control.

Bacterial expression of GST-fusion proteins

Expression and purification of GST and GST-ITPKA was performed as described.[6] The protocol for expression of GST-filamin $C^{2145\text{-}2705}$ differed in term of IPTG stimulation of bacteria. Instead of incubation for 16 hr at 4°C, stimulation occurred for 3 hr at 37°C.

Fluorescence microscopy of actin filaments

5 μM rabbit muscle actin (Cytoskeleton, Denver, USA) was incubated in assay buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP) for 10 min at room temperature to generate F-actin. Then, 10 μM test protein (GST-filamin $C^{2145\text{-}2705}$ and/or GST-ITPKA) was added and further incubated for 1 hr. Finally, 8 nM FITC-phalloidin (Sigma-Aldrich, Steinheim, Germany) was added, and incubation continued for 10 min at the dark. 5 μl of this solution was transferred onto poly-lysine-coated cover slips. Formation of actin structures was visualized by immunofluorescence microscopy.

Yeast two hybrid screen

For this screen, the full-length form of ITPKA was cloned into the pGBKT7 vector to generate the bait-vector and the experiment was performed according to the manufacturers protocol (Clontech, Heidelberg, Germany). All vectors and reagents used were a kind gift from PD Dr. Hans-Jürgen Kreienkamp (Hamburg, Germany), and originally generated by Clontech.

Immunoprecipitation

Immunoprecipitations were essentially performed as described,[5] but to avoid degradation of EGFP-ITPKA, 2 mM EGTA was added to the lysis buffer. For immunoprecipitation of filamin C the anti-filamin C antibody from goat (Sc-48496, Lot#C0909, Santa Cruz, SC) was used.

Results

ITPKA expression increases migration of malignant transformed cell lines

To reveal whether ITPKA mediated stimulation of migration observed in H1299, A549 and MDA 231 cells[6] is restricted to these cell lines or represents a more general effect, further cell lines of different origin (MCF-7, SKBR-3, breast; Mevo, melanoma; HEK 293, kidney; PT4323 and LN 4323, lung) were used for functional studies. MCF-7, SKBR-3 and Mevo are established cell lines isolated from breast tumor or skin cancer (malignant melanoma), whereas HEK 293 cells were generated by transformation of human embryonic kidney cell cultures with sheared adenovirus 5 DNA.[9] PT 4323 and LN 4323 were isolated in our laboratory from a primary lung tumor (PT) and from the corresponding lymph node metastasis (LN) of one lung cancer patient (for details see Material and Methods).

To investigate the effect of ITPKA on migration of these cell lines, ITPKA was stably down-regulated in cell lines with ectopic ITPKA expression (in LN 4323, Mevo, HEK 293) and stably overexpressed in cells with low ITPKA expression (in SKBR-3, PT 4323). In addition, we identified MCF-7 subpopulations with low and with high endogenous expression of ITPKA (Fig. 1). The modified cell lines with down-regulation of ITPKA expression were termed ITPKA knock down cells and those with recombinant ITPKA up-regulation ITPKA overexpressing cells. Migration was

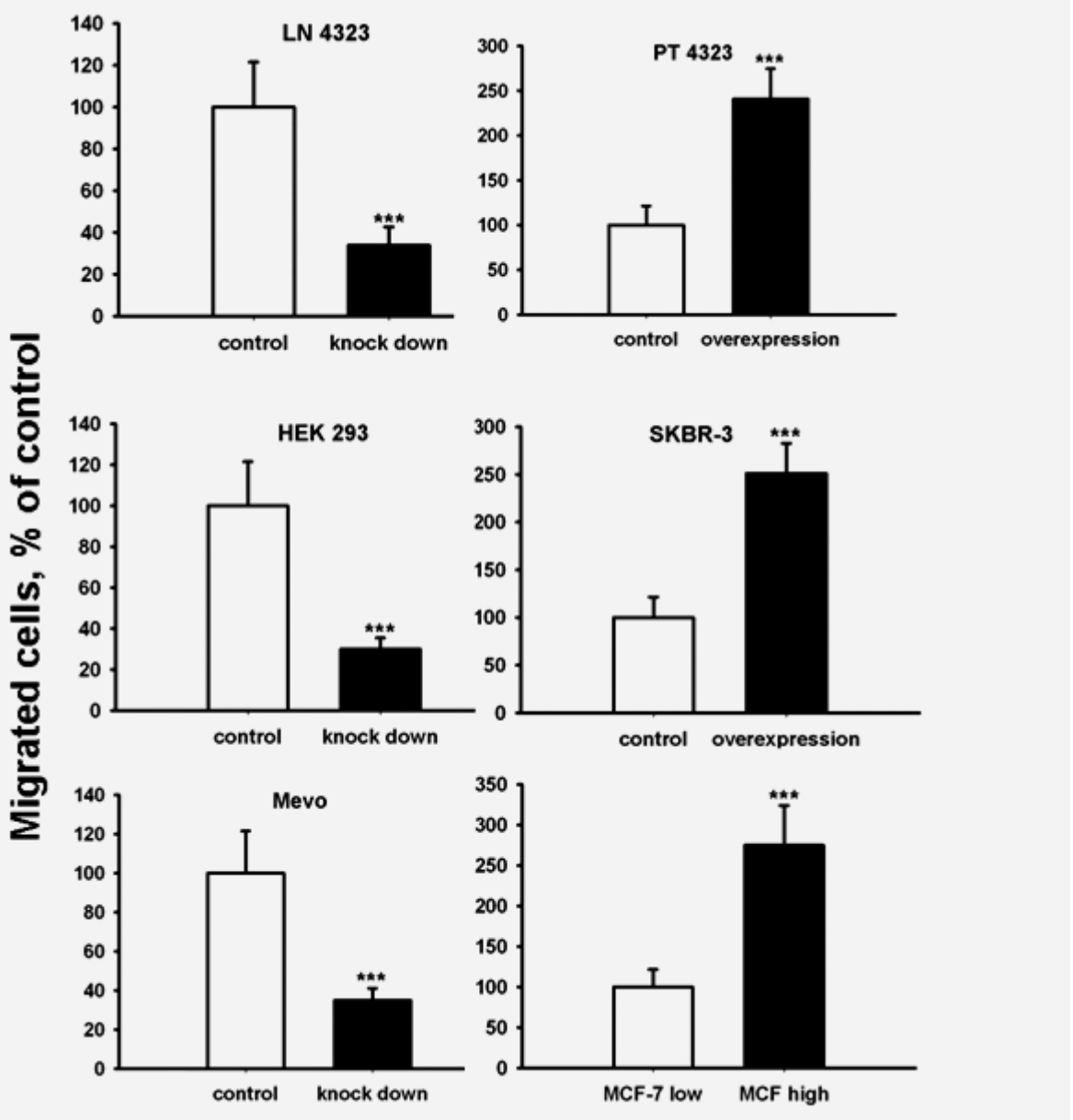

Figure 2. ITPKA expression increases migration of six different cell lines. Migration of the modified cell lines was examined by the transwell assay. The cells were seeded in Boyden chambers, and after incubation for 20 hr the transmigrated cells were fixed with paraformaldehyde and stained with DAPI. The cell number was examined by counting the DAPI stained nuclei. The mean ± SD of four independent experiments is shown. ***$p < 0.001$.

assessed by the transwell assay with cells cultivated in medium containing 10% FCS. As shown in Figure 2, ITPKA expression promotes migration of all cell lines examined. Down-regulation of ITPKA expression reduces migration of LN 4323 cells by 67%, of HEK 293 cells by 70% and of Mevo cells by 65% as compared to the respective control cell lines. Conversely, overexpression of ITPKA in PT 4323 and SKBR-3 cells increases migration by 140 and 150%, respectively. Also, the migration rate of MCF-7 cells with up-regulation of endogenous ITPKA expression was higher than that of MCF-7 cells with low ITPKA expression. In summary, these data show that expression of ITPKA increases migration in a broad range of cell lines. Obviously this effect is not solely dependent on the absolute level of ITPKA since PT 4323 cells, expressing higher levels of ITPKA than SKBR-3 cells, showed a comparable effect on migration as the latter cell line.

ITPKA expression increases the formation of cellular protrusions

In a recent study, we demonstrated that ITPKA expression growth factor independently increased migration of highly

metastatic tumor cell lines by inducing the formation of cellular protrusions.[6] To examine the effect of ITPKA expression in HEK 293, Mevo, LN 4323, PT 4323, SKBR-3 and MCF-7 cells on formation of cell processes, the morphology of the modified cell lines was examined by light microscopy (Fig. 3*a*, left panel). In addition, the actin cytoskeleton was stained with FITC-phalloidin and the cells were documented by immunofluorescence microscopy at high magnification (Supporting Information Fig. S1). All control cells with high endogenous expression of ITPKA extended cellular protrusions of different shape and size. LN 4323 cells formed large lamellipodia and many small filopodia. The lamellipodia-like protrusions of HEK 293 cells were shorter and less broad and those of Mevo cells were even more narrow, and thus do not represent the typical type of lamellipodia.[7] Despite of these differences, however, knock down of ITPKA expression strongly reduced the size of protrusion in all three cell lines. To quantify this effect, all visible cell processes from at least 200 control and 200 ITPKA knock down cells were counted by light microscopy (Fig. 3*b*). This enumeration revealed that the number of protrusions was reduced between 38% (Mevo) and 76% (LN 4323) in ITPKA knock down cells as compared to control cells. In addition, the size of cell processes differed markedly, as only control cells formed protrusion > 4 μm.

The effect of recombinant overexpression of ITPKA on formation of cell protrusions was examined in cell lines with low endogenous ITPKA expression (PT 4323, SKBR-3), among which the most drastic effect was found in PT 4323 cells (Fig. 3*a*, right panel). Whereas the control cells were round shaped and formed only small filopodia, PT 4323 cells with overexpression of ITPKA extended many large branched nearly dendrite-like protrusions, which were not visible in control cells. In SKBR-3 cells, ITPKA overexpression resulted in the formation of few lamellipodia-like cell processes, which looked very different from that observed after overexpression of ITPKA in PT 4323 cells. In addition, endogenous up-regulation of ITPKA promoted the formation of cell protrusions as well. Low ITPKA expressing MCF-7 cells only formed small filopodia, while MCF-7 cells with high ITPKA expression extended both, filopodia and large lamellipodia. A quantification of all visible protrusions (Fig. 3*b*) formed by control and ITPKA overexpressing cells revealed that cells with high expression of ITPKA formed significantly more cell processes than control cells, with an increase between 109 and 342%. Again, PT 4323 cells overexpressing ITPKA showed the most dramatic effect. Together, our data clearly demonstrate that ITPKA expression or expression increase stimulates the formation of protrusions in different malignant cell lines.

ITPKA interacts with filamin C

The data above show that ITPKA induces the formation of linear (filopodia-like) and branched protrusions (lamellipodia-like). As branched cell processes consist of a dense network of branched and/or crossed-linked actin filaments and ITPKA was identified as an F-actin bundling protein,[4,6] known to produce linear actin filaments, we assumed that interaction of ITPKA with additional accessory proteins may be necessary to induce the formation of lamellipodia-like protrusions. To identify such potential interaction partners, a yeast two hybrid screen was performed using a human neuronal cDNA library. Interestingly, this screen revealed a putative interaction of ITPKA with the F-actin cross-linking protein filamin C. Filamin C is an F-actin binding protein of 290 kD that cross-links actin filaments by formation of filamin homodimers.[10] The ITPKA interaction domain identified by our yeast two hybrid screen encompasses amino acids (aa) 2145 to 2705, which includes rod repeats and the amino acids essential for formation of filamin homodimers but lacks the actin binding domain.[11] To examine whether ITPKA may interact with filamin C in tumor cells as well, we first analyzed the protein level of filamin C in PT 4323 and LN 4323 cells by Western blot. Figure 4*a* shows that both cell lines express filamin C. As LN 4323 cells show a slightly higher level of filamin C as compared to PT 4323 cells, these cells were used for immunoprecipitation experiments. Therefore, filamin C was immunoprecipitated from protein extracts of LN 4323 cells, using anti-filamin C coupled sepharose A beads (filamin C beads) or goat IgG coupled sepharose A beads (IgG beads) as control. Analysis of ITPKA-binding to filamin C was performed by Western blotting using an anti-ITPKA antibody (Fig. 4*b*). This analysis revealed a clear signal at 52 kD, representing the molecular weight of ITPKA, confirming that in LN 4323 cells ITPKA interacts with filamin C.

As both, ITPKA and filamin C are F-actin binding proteins the observed co-immunoprecipitation of ITPKA and filamin C could be mediated via the F-actin polymer. In order to specify the type of interaction, the wild type EGFP-ITPKA fusion protein, an EGFP-ITPKA fusion protein with a point mutation that prevents actin binding (L34P[4,6]), an EGFP-ITPKA fusion protein lacking the actin binding domain (ITPKAΔABD, with deletion of aa 1-66[2]), and EGFP as control were overexpressed in LN 4323 cells. As shown in Fig. 4*c* (right panel) the fusion proteins are expressed as full length forms of molecular weights of 79 kD (EGFP-ITPKA and EGFP-ITPKA L34P) and 72 kD (EGFP-ITPKAΔABD), respectively, whereby EGFP-ITPKAΔABD showed the highest and the wt protein the lowest expression level. A potential interaction of the EGFP-ITPKA fusion protein with filamin C was examined as described above. The filamin C beads incubated with lysates from EGFP-ITPKA and from EGFP-ITPKA L34P overexpressing cells gave strong Western blot signals. This result indicates a direct interaction between ITPKA and filamin C since the non actin binding mutant ITPKA L34P also showed interaction with filamin C. Despite the strong overexpression of EGFP-ITPKAΔABD, analysis of filamin C beads incubated with lysates from EGFP-ITPKA ΔABD overexpressing cells revealed a weaker signal than that observed for the full proteins. This observation makes it likely that both, the first 66 amino acids and regions between aa 67 and 461 are involved in interaction with filamin C.

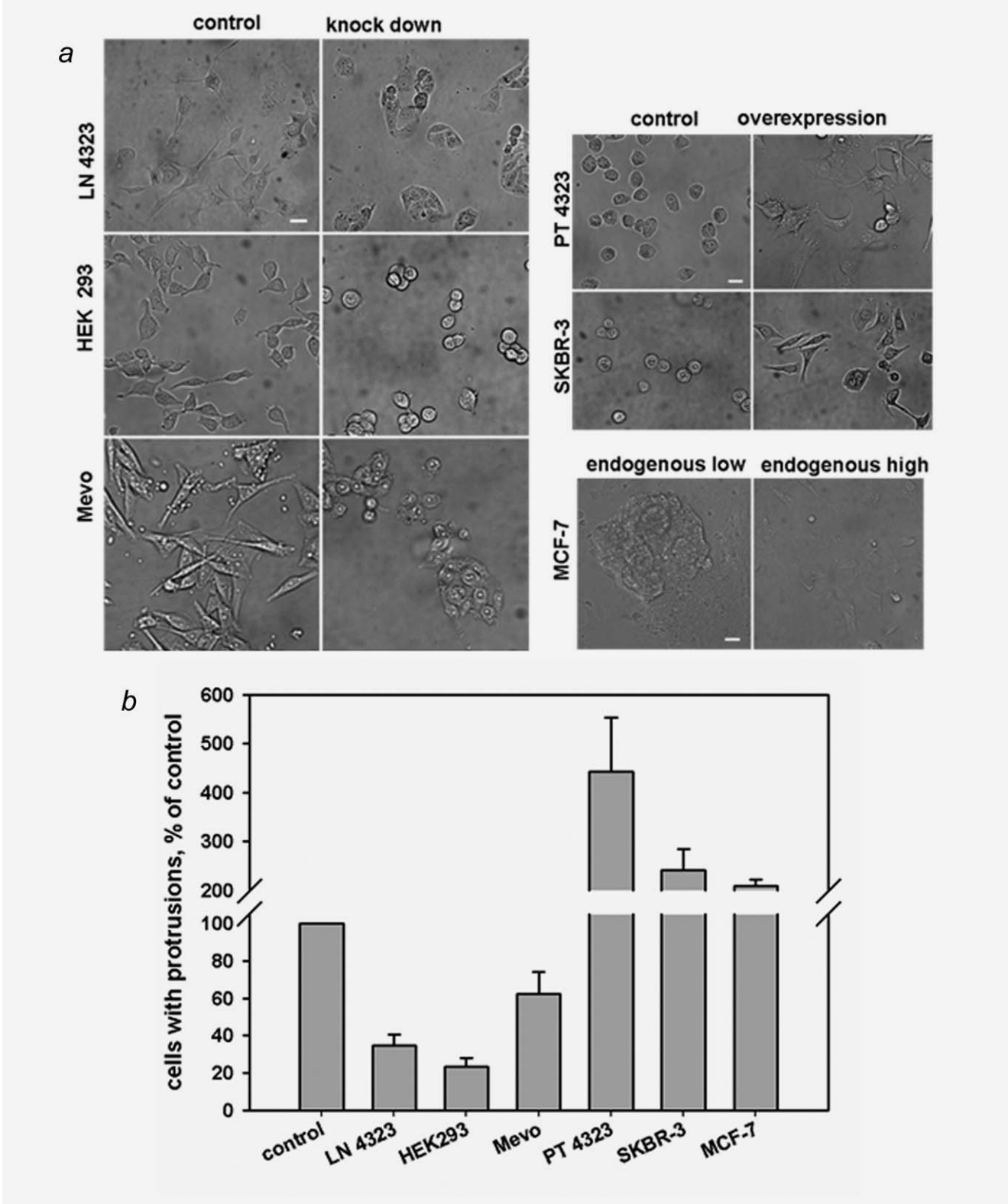

Figure 3. ITPKA increases the number of cellular protrusions. (*a*) Control cells and cells with ITPKA knock down or ITPKA overexpression were cultivated on fibronectin covered chamber slides. After incubation for 48 hr, they were covered with PBS and monitored by light microscopy. Bars, 10 μm. (*b*) Visible protrusions, independent of size from at least 200 cells per group were counted and the percentage of cells that form protrusions was calculated. For normalization the value calculated for control cells was set to 100%.

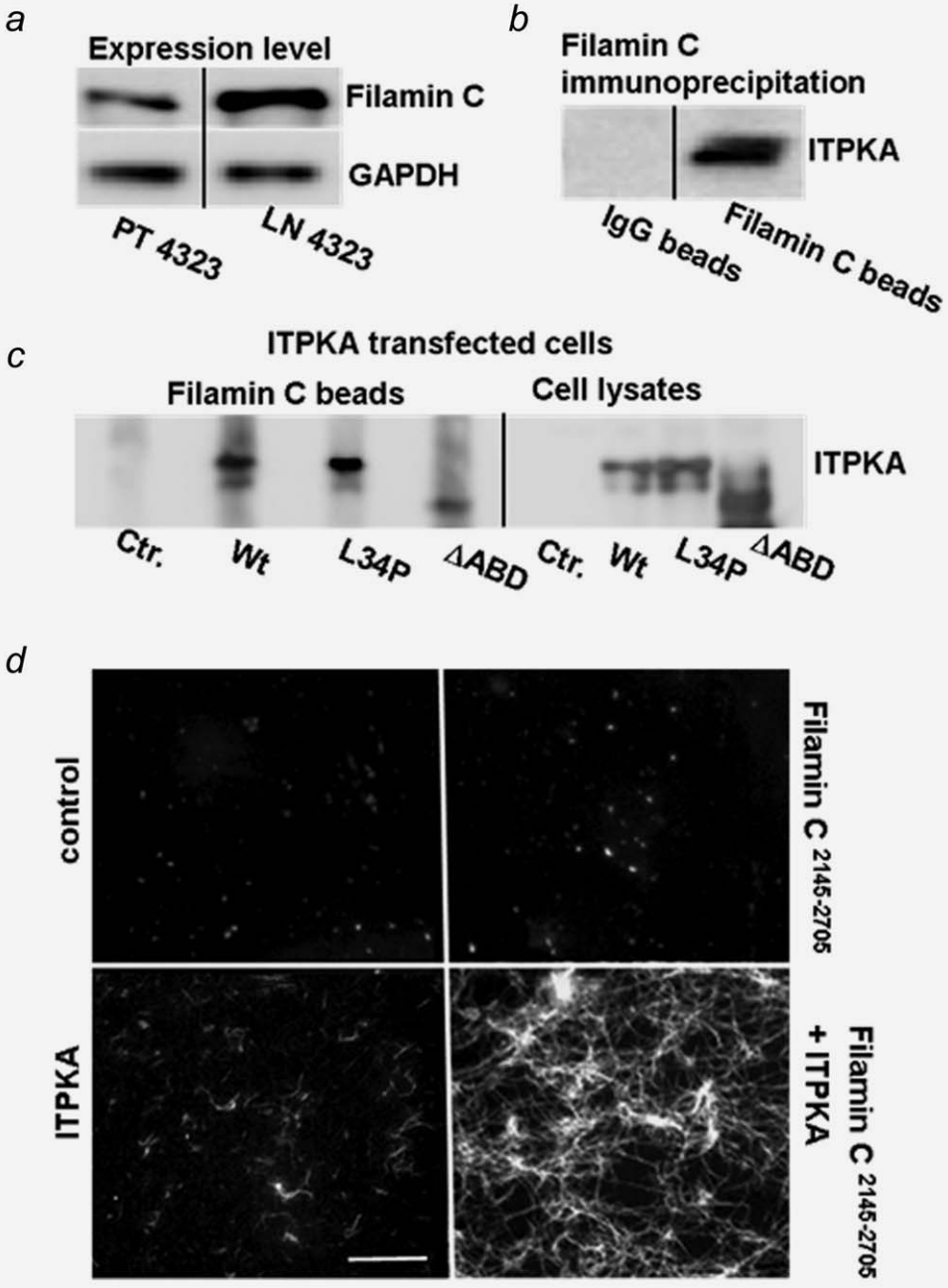

Figure 4. ITPKA interacts with the F-actin cross linking protein filamin C. *a*, Expression levels of filamin C were analyzed by western blot in PT 4323 and LN 4323 cells. GAPDH signals served as loading control (*b*) Co-immunoprecipitation of ITPKA and filamin C in LN 4323 cells. An anti-filamin C antibody was coupled to sepharose A beads, or, IgG from goat serum as control. Beads were incubated with lysates of LN 4323 cells for 16 hr, and after extensive washing subjected to SDS-PAGE. Binding of ITPKA to filamin C was analyzed by Western blot using an anti-ITPKA antibody. (c) EGFP, EGFP-ITPKA, EGFP-ITPKA L34P (point mutation that prevents actin binding) or EGFP-ITPKA□ABD (mutant lacking the actin binding domain), respectively, were transiently overexpressed in LN 4323 cells (for expression levels see "cell lysates") and the interaction of the EGFP fusion proteins with filamin C ("Filamin C beads") was analysed as described in (b). (d) F-actin and FITC-phalloidin were incubated either alone (control) or with the proteins as indicated at the micrographs. Formation of actin structures was visualized by fluorescence microscopy. The experiment was repeated twice; one micrograph per condition out of ten comparable images is shown.

Next, we examined the effect of interaction of ITPKA and the ITPKA interaction domain of filamin C (filamin $C^{aa2145\text{-}2705}$) on actin dynamics *in vitro*. Therefore, filamin $C^{aa2145\text{-}2705}$ and a recombinant full-length form of ITPKA (ITPKA) were incubated together with actin and phalloidin and the actin structures were monitored by fluorescence

Table 1. Expression of ITPKA in primary lung cancer tissues with matched lymph node metastases. Expression of ITPKA was analyzed in a lung cancer tissue micro array with matched lymph node metastases (Biomaxus) by the use of a specific antibody against ITPKA. The array includes 32 samples from the primary tumor and from the corresponding lymph node metastasis (total 64 samples). The number (no.) and percentage (%) of tumor samples is shown

	Tumor (PT)		Metasases (LN)		
	ITPKA (−), no., %	ITPKA (+), no., %	ITPKA (−), no., %	ITPKA (+), no., %	Sum
Total number of samples	13, 40.6%	19, 59.4%	1, 3.1%	31, 96.9%	32, 100%
Tumor type					
Small cell carcinoma	5, 83.4%	1, 16.6%	1, 16.6%	5, 83.4%	6, 100%
Squamous cell carcinoma	6, 42.9%	8, 57.1%	0, 0%	14, 100%	14, 100%
Adenocarcinoma	2, 16.7%	10, 83.3%	0, 0%	12, 100%	12, 100%

microscopy. As expected, we found that ITPKA alone induced linear F-actin bundles, but in combination with filamin $C^{aa2145\text{-}2705}$ a dense and highly interlinked F-actin network was visible (Fig. 4*e*). It seems that filamin $C^{aa2145\text{-}2705}$ binds and apparently cross-links ITPKA molecules, because of formation of filamin C homodimers.

In summary, our data reveal a direct interaction of ITPKA and filamin C and demonstrate that this interaction induces the formation of cross-linked actin bundles *in vitro*.

Expression of ITPKA in clinical cancer samples

The data above show that ITPKA expression significantly increases the migratory potential of different transformed cell lines by inducing the formation of cellular protrusions, indicating that ITPKA may play a functional role for growth factor independent cell motility. To investigate whether this effect observed in cultured cell lines may have a clinical significance, we investigated the occurrence of ITPKA in a series of clinical tumor samples, starting with the analysis of a multiple tumor and normal organ tissue array. To examine expression of ITPKA, the TMA was stained with a specific anti-ITPKA antibody verified by control stains in tumor cells with overexpression and knock-down of ITPKA (see Material and Methods). In specimens from normal tissues no positively stained cells were detected. Among the tumor samples tumor cells from pancreas, testis, thyroid, breast, lung, colon, liver, prostate, uterus and skin showed expression of ITPKA (Supporting Information Table S1). As in tissues from lung cancer (Supporting Information Table S1) and in cell lines derived from LN of lung cancer cells (LN 4323 (Fig. 1) and H1299[6]) expression of ITPKA was detected, the abundance of ITPKA in samples from primary tumors (PT) and the matched lymph node metastases (LN) of lung cancer patients was examined using a "lung cancer with matched lymph node metastasis tissue array." In samples derived from small cell carcinoma, one PT and five LNs (out of six) showed expression of ITPKA. Also in squamous cell and adenocarcinoma, ITPKA expression was found with higher frequency in LN then in PT. In squamous cell carcinoma, 8 PT- and 14 LN-samples (out of 14) were positively stained and in adenocarcinoma 10 PT- and 12 LN-samples (out of 12) (Table 1).

Based on the result that in lung adenocarcinoma the highest expression frequency of ITPKA was found, this tumor type was examined in closer detail. Ninety-four samples derived from the primary tumor and ten samples from healthy lung tissue of individual patients were examined using conventional histological sections with pN0 status in 25 (27%) and with pN1/2 status in 69 (73%) patients. In addition, 59 matched lymph node metastases were examined. To determine the occurrence of ITPKA expression, the percentage of cells with positive ITPKA immunostaining per tumor cell population was determined. We found expression of ITPKA in nearly all tumor samples (in 85% of PT and in 100% of LN), while in healthy lung tissues only macrophages were strongly immuno-positive for ITPKA. As among these samples the expression frequency (% of positive cells) of ITPKA was very different (ranging from 5 to 100%), only tumor cell populations with at least 50% positively stained cells were used for comparative analysis between tumor stages. Our data summarized in Table 2 reveal that 32% of the non-metastasizing (pN0) cells showed high abundance of ITPKA, while 49% of the metastasizing (pN1/2) adenocarcinoma and 56% of the matched lymph node metastases expressed high rates of ITPKA. Although the expression rate of ITPKA seems to increase from pN0 to LN (pN0 > pN1/2 > LN), these differences were not significant. Furthermore, the higher expression rates of ITPKA in low differentiated (G3/G4) relative to well differentiated (G1/G2) tumors were not significant. On the other hand a highly significant correlation ($p < 0.001$) between expression in PT and in the matched LN was found, showing that ITPKA expression is maintained during progression from PT to LN.

Discussion

Our study shows that endogenous as well as recombinant up-regulation of ITPKA expression increases migration and the number of filopodia- and lamellipodia-like cell protrusions of six transformed cell lines from different origin. As the same effect of ITPKA on migration and formation of cell processes was observed in three further lines examined in a previous study,[6] we assume that the migration-promoting and the actin-modulating effect of ITPKA is not restricted to

Table 2. Expression of ITPKA in lung adenocarcinoma tissues. 94 adenocarcinoma samples (PT, primary tumor) were stained with an anti-ITPKA antibody. Among these samples, 69 were lymph node positive (pN1/2), and 25 were lymph node negative (pN0). The matched lymph node metastases (LN) of the pN1/2 samples were available for 59 cases, 10 samples were not analyzable because of low specimen quality. Only samples with ITPKA expression frequency $\geq$ 50% positive cells were included. The number (no.) and percentage (%) of tumor samples is shown. Statistical analysis was performed by using the Qui-Quadrat-test, p values <0.05 were considered as significant

	ITPKA (−), no.; %	ITPKA (+), no.; %	Sum
Total number of samples from PT (pN0 and pN1/2)	52; 55.3%	42; 44.7%	94 (100)
Histological grade			Significance, G1/2 vs G3/4
G1/2	38; 62.3%	23; 37.7%	
G3/4	14; 42.4%	19; 57.6%	$p = 0.064$
ITPKA expression in PT depending on lymph node state			Significance, pN0 vs pN1/2
pN0	17; 68%	8; 32%	
pN1/2	35; 50.7%	34; 49.3%	$p = 0.137$
ITPKA expression in LN			Significance, pN1/2 vs LN
Number, %	26; 44%	33; 56%	$p = 1.924$

particular cell lines but seems to be a general biological principle. Because of the fact that filopodia and lamellipodia are crucial for cells to migrate,[7] we suggest that the stimulating effect of ITPKA on cell migration and ITPKA induced formation of cell processes are functionally related. The mechanism how ITPKA induces the formation of linear filopodia-like protrusions can be explained by its F-actin bundling activity[4,6] as filopodia mainly consist of linear F-actin-bundles. These thin finger-like structures are required for cells to adhere to the substrate, providing the first step for cells to migrate.[7] Consistently, up-regulation of further migration-promoting F-actin bundling proteins in tumor cells has been described (fascin,[12] actinin[13]). After adhesion to the substrate, migrating cells extend the much larger lamellipodia, which are formed by a dense network of branched and/or cross linked actin filaments.[7] Despite the fact that we did not find F-actin cross-linking activity of ITPKA, its up-regulation in tumor cells induced the formation of lamellipodia-like cell extensions. Our data show that ITPKA directly interacts with the F-actin cross-linking protein filamin C and demonstrate that *in vitro* the interaction of ITPKA and the ITPKA binding domain of filamin C induces the formation of a dense actin-network. Based on these results and the finding that in cells both proteins are bound to F-actin at the cell cortex and in cellular protrusions,[6,14] we strongly assume that ITPKA induced formation of lamellipodia-like cell protrusion results from interaction between ITPKA and filamin C. As filamin C links the actin cytoskeleton to the plasma membrane by its interaction with integrins,[10] the interaction between filamin C and ITPKA may also explain why ITPKA induced actin structures protrude the membrane.

In addition to these mechanistic data, we demonstrate here for the first time the occurrence of ITPKA in clinical samples from lung cancer patients. Our finding that ITPKA is preferentially expressed in cell lines derived from metastases (LN 4323, H1299, MDA 231) seemed to be confirmed by analysis of clinical samples from small cell lung carcinoma and squamous lung carcinoma, showing a higher expression rate in lymph node metastases (LN) than in the matched primary tumor. However, in these cases the number of sample pairs was too low to be of significance. Thus, further experiments are necessary to evaluate the role of ITPKA expression in small cell lung carcinoma and squamous lung carcinoma. Here we mainly focused on ITPKA expression analysis in adenocarcinoma as, in contrast to small cell lung carcinoma and squamous lung carcinoma, adenocarcinoma show high expression frequency of ITPKA also in primary tumor cells. Our result that even non-metastasising tumor cells express high rates of ITPKA show that adenocarcinoma cells have acquired the ability to express ITPKA early during tumor cell development. The finding that this expression is largely maintained in invading and metastasizing cells together with our data in cell cultures and in mouse models,[6] demonstrating that ITPKA significantly increases the metastatic potential of tumor cells, strongly indicates that the expression of ITPKA in early stage of adenocarcinoma progression increases the invading potential of cancer cells. This conclusion is in line with recent findings, showing that the metastatic potential can be already acquired early in tumor progression.[15–17] In particular in adenocarcinoma, which is the predominant histological type among lung cancers and the leading cause of cancer death in the world,[18] identification of the metastatic potential at early stage of tumor development is of great interest because the prognosis of adenocarcinomas depends on tumor stage. Well differentiated early stage adenocarcinoma patients have a significantly better prognosis than poorly differentiated late stage adenocarcinoma.[19] Accordingly, the overall low survival rate mainly results from late diagnosis when the primary tumor has already metastasized.[20] Screening of lung tumor samples for expression of ITPKA may therefore offer the possibility to detect the metastatic potential of early stage tumors, suggesting ITPKA as a promising molecular target for a specific therapy to decrease

metastatic spread. The fact that in normal cells ITPKA is only expressed in neurons emphasizes ITPKA as a very interesting target for tumor therapy. Corresponding lead structure searches for inhibitors of ITPKA have been already initiated.[21]

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References

1. Mailleux P, Takazawa K, Erneux C, Vanderhaeghen JJ. Distribution of the neurons containing inositol 1,4,5-trisphosphate 3-kinase and its messenger RNA in the developing rat brain. *J Comp Neurol* 1993;327:618–29.
2. Schell MJ, Erneux C, Irvine RF. Inositol 1,4,5-trisphosphate 3-kinase A associates with F-actin and dendritic spines via its N terminus. *J Biol Chem* 2001;276:37537–46.
3. Schell MJ. Inositol trisphosphate 3-kinases: focus on immune and neuronal signaling. *Cell Mol Life Sci* 2010;67:1755–78.
4. Johnson HW, Schell MJ. Neuronal IP3 3-kinase is an F-actin-bundling protein: role in dendritic targeting and regulation of spine morphology. *Mol Biol Cell* 2009;20: 5166–80.
5. Windhorst S, Blechner C, Lin HY, Elling C, Nalaskowski M, Kirchberger T, Guse AH, Mayr GW. Ins(1,4,5)P3 3-kinase-A overexpression induces cytoskeletal reorganization via a kinase-independent mechanism. *Biochem J* 2008;414:407–17.
6. Windhorst S, Fliegert R, Blechner C, Mollmann K, Hosseini Z, Gunther T, Eiben M, Chang L, Lin HY, Fanick W, Schumacher U, Brandt B, *et al.* Inositol-1,4,5-trisphosphate-3-kinase-A is a new cell motility-promoting protein that increases the metastatic potential of tumour cells by two functional activities. *J Biol Chem* 2010; 285:5541–54.
7. Le Clainche C, Carlier MF. Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol Rev* 2008;88:489–513.
8. Feldner JC, Brandt BH. Cancer cell motility--on the road from c-erbB-2 receptor steered signaling to actin reorganization. *Exp Cell Res* 2002;272: 93–108.
9. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977;36: 59–74.
10. Popowicz GM, Schleicher M, Noegel AA, Holak TA. Filamins: promiscuous organizers of the cytoskeleton. *Trends Biochem Sci* 2006;31:411–9.
11. van der Flier A, Sonnenberg A. Structural and functional aspects of filamins. *Biochim Biophys Acta* 2001;1538:99–117.
12. Yoder BJ, Tso E, Skacel M, Pettay J, Tarr S, Budd T, Tubbs RR, Adams JC, Hicks DG. The expression of fascin, an actin-bundling motility protein, correlates with hormone receptor-negative breast cancer and a more aggressive clinical course. *Clin Cancer Res* 2005;11:186–92.
13. Yamamoto S, Tsuda H, Honda K, Kita T, Takano M, Tamai S, Inazawa J, Yamada T, Matsubara O. Actinin-4 expression in ovarian cancer: a novel prognostic indicator independent of clinical stage and histological type. *Mod Pathol* 2007;20: 1278–85.
14. Washington RW, Knecht DA. Actin binding domains direct actin-binding proteins to different cytoskeletal locations. *BMC Cell Biol* 2008;9:10.
15. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003;33: 49–54.
16. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347:1999–2009.
17. Inamura K, Shimoji T, Ninomiya H, Hiramatsu M, Okui M, Satoh Y, Okumura S, Nakagawa K, Noda T, Fukayama M, Ishikawa Y. A metastatic signature in entire lung adenocarcinomas irrespective of morphological heterogeneity. *Hum Pathol* 2007;38:702–9.
18. Terasaki H, Niki T, Matsuno Y, Yamada T, Maeshima A, Asamura H, Hayabuchi N, Hirohashi S. Lung adenocarcinoma with mixed bronchioloalveolar and invasive components: clinicopathological features, subclassification by extent of invasive foci, and immunohistochemical characterization. *Am J Surg Pathol* 2003;27:937–51.
19. Mountain CF. Revisions in the International system for staging lung cancer. *Chest* 1997;111:1710–7.
20. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
21. Mayr GW, Windhorst S, Hillemeier K. Antiproliferative plant and synthetic polyphenolics are specific inhibitors of vertebrate inositol-1,4,5-trisphosphate 3-kinases and inositol polyphosphate multikinase. *J Biol Chem* 2005;280: 13229–40.