Implication of Filamin A in Pulmonary Neuroendocrine Tumors aggressiveness and progression

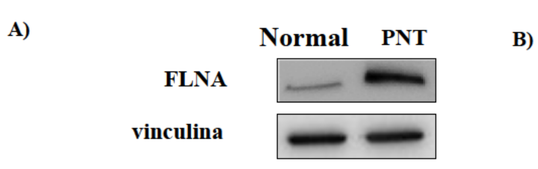
Eleonora Vitali[1], *Stefano Ferrero* [3], *Giulia Veronesi* [4], *Valeria Cambiaghi*[1], *Erika Peverelli*[2], *Giovanna Mantovani*[2], *Anna Spada*[2], *Andrea Lania* [5]

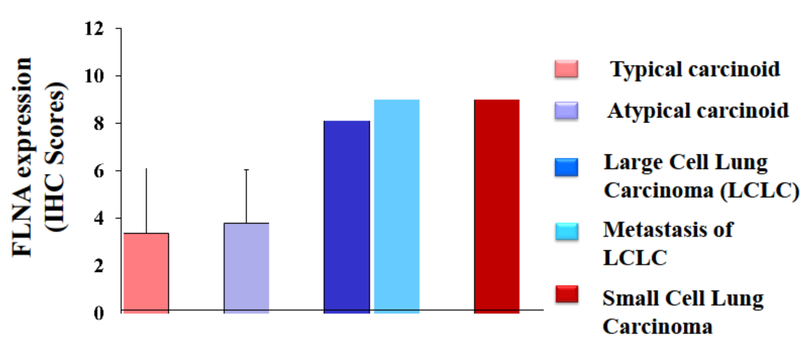
1.Laboratory of Cellular and Molecular Endocrinology, IRCCS Clinical and Research Institute Humanitas, Rozzano; 2.Endocrine Unit, Department of Clinical Sciences and Community Health, Fondazione IRCCS Ospedale Maggiore Policlinico, University of Milan; 3.Division of Pathology, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milan, Italy, 4.Division of Thoracic and General Surgery, Istituto Clinico Humanitas IRCCS, Rozzano, Italy; 5.Endocrinology Unit, Department of Biomedical Sciences, Humanitas University and Humanitas Research Hospital, Rozzano, Milan, Italy.

Pulmonary neuroendocrine tumors (PNTs) comprise different neoplasms, ranging from low grade carcinoids to the highly malignant small cell lung cancers (1). Several studies identified cytoskeleton protein Filamin A (FLNA) as determinant in cancer progression and metastasis (2-4). FLNA is a widely expressed cytoskeleton protein that acts as scaffolding molecule and is involved in different cellular events, including angiogenesis (5, 6). It has been found an interaction with Rap1, a small GTPase implicated in cell motility, and Filamin2 in microvascular smooth muscle (7), suggesting a possible role of Filamin A in mediating Rap1 effects. To date, the role of FLNA in PNTs aggressiveness and progression is still unknown.

In order to address this question, we evaluated FLNA expression in different PNTs, we studied the role of FLNA in cell proliferation, colony formation, angiogenesis, cell adhesion and migration in PNT cell line (H727 cells) and primary cultures and we focused on the possible interaction between FLNA and Rap1 GTPase, implicated in the regulation of cell mobility.

FLNA expression in lung and different PNTs

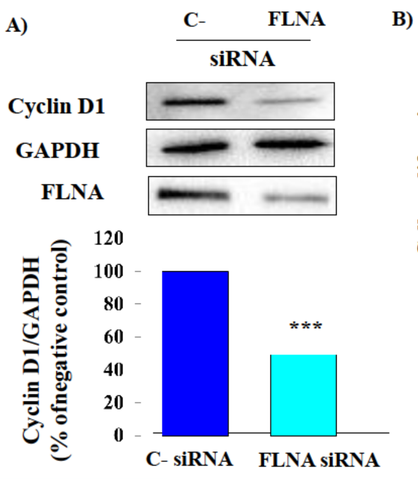


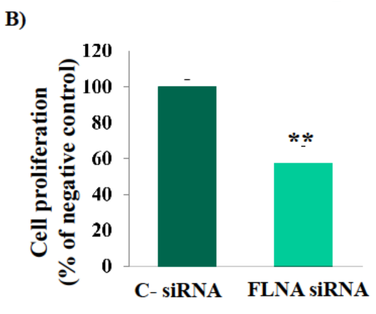


A) Immunoblot of FLNA performed on normal and PNT samples. The equal amount of protein was confirmed by stripping and reprobing with an anti-vinculin antibody.

B) Graph of FLNA expression obtained with the average of immunohistochemistry scores in different PNTs.

FLNA knockdown reduces PNTs cells proliferation

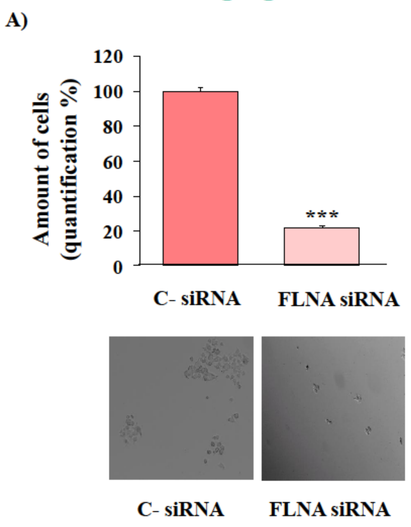


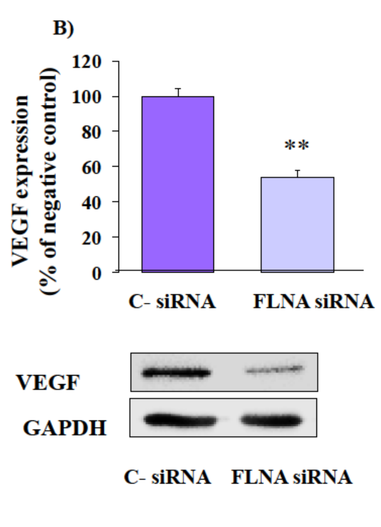


A) FLNA siRNA treated cells showed a strong decrease in cyclin D1 expression in PNTs cells. The graph shows the quantification of Cyclin D1 normalized to GAPDH. Data represent mean ± SD of three independent experiments.*** =p<0.001 vs C- siRNA

B) FLNA silencing reduced cell proliferation in PNTs. ** =p<0.01 vs C- siRNA

FLNA is involved in colony formation and angiogenesis increase in H727 cells

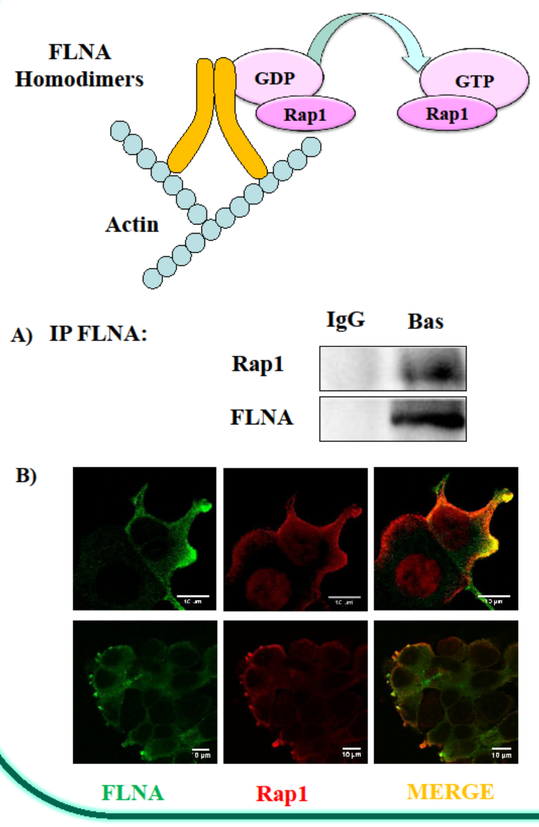




A) The graph shows that FLNA knock down reduces the amount of H727 cells in colony formation assay. Rapresentative images show the reduction of colony formation in FLNA silenced cells respect to C-siRNA cells.
Values represent mean ±SD ***=p<0.001 vs corresponding basal.

B) The graph shows quantification of VEGF expression normalized to GAPDH. Experiments were repeated at least 3 times **P<0.01 vs C- siRNA. Representative immunoblot of VEGF demonstrates that FLNA silencing reduces VEGF expression in H727 cells.

Does FLNA interact with Rap1?



Association of FLNA and Rap1 GTP-ase.

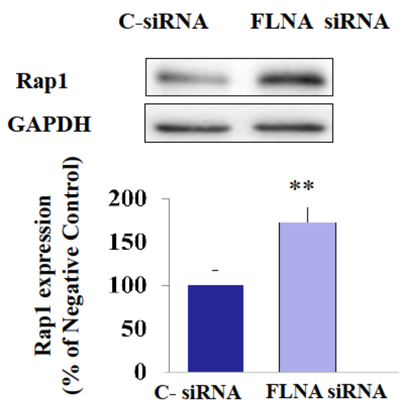
A) H727 lysates were immunoprecipitated with FLNA antibody and the presence of Rap1 together with FLNA was detected using anti-Rap1 and anti-FLNA antibodies respectively. The figure shows a representative experiment.

B) Fixed cells were analyzed by confocal microscopy. Scale bar 10 µm.

MATERIAL AND METHODS

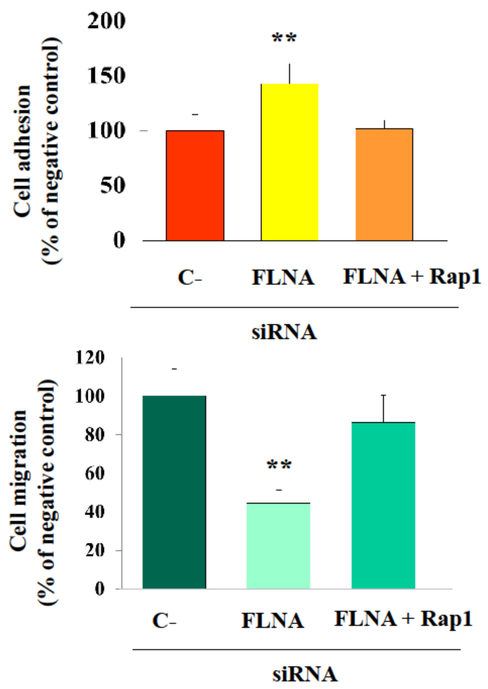
- **Silencing:** Short interfering RNA (SiRNA) were purchased from Invitrogen. H727 cells were transfected with 200 pmol of FLNA siRNA, or negative control siRNA (C- siRNA) for 72h, using Lipofectamine 2000 according to the instruction of the manufacturer.
- **Immunohistochemistry:** was performed on sections from different PNTs retrieved from the archives of Pathology Unit of IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milan. After dewaxing in Bioclear and rehydrating in ethanol, the sections were pretreated in a water bath set to 98°C in 0.01 M citrate buffer for 25 minutes. FLNA antibody (Millipore, 1:600 diluition) was used, and antigen-antibody detection was performed with the MACH1 universal polymer detection kit (Biocare Medical).
- **Western Blot Analysis:** All samples were separated on SDS-PAGE, and the proteins were detected by Western Blotting using antibodies against FLNA (AbNova), GAPDH (Ambion), Cyclin D1 (Millipore), Rap1 (Millipore), Vinculin (Cell signalling), VEGF (Abcam). The ratio of Immunoblotting signalling intensity was measured using NIH ImageJ software.
- **Colony formation:** H727 cells were transfected with FLNA siRNA and C- siRNA, after 72h cells were counted and plated in 96 wells for colorimetric quantification and 24 wells for colonies images. The number of colonies was evaluated after 7 days.
- **Immunofluorescence:** H727 cells were seeded in 24 well plates with sterile glass coverslips and transfected with control or FLNA siRNA. After 72 h, cells were fixed with 4% PFA and stained with Rap1 or FLNA antibody.
- **Cell adhesion assay:** H727 cells transfected with C-siRNA or FLNA siRNA alone or together with Rap1 siRNA were plated onto a collagen type IV-coated 48-well plate for 90 min at 37°C, as by manufacturer's protocol (Cell Biolabs INC).
- **Cell migration assay:** H727 cells transfected with C-siRNA or FLNA siRNA alone or together with Rap1 siRNA were plated in polycarbonate membrane plate (Cell biolabs INC) and placed into chemoattractant solution. After 24 hours, migratory cells were stained and quantified using a fluorometric plate reader.

FLNA down-regulated Rap1 expression



A) FLNA siRNA treated cells showed an increase in Rap1 expression in H727 cells. The graph shows the quantification of Rap1 normalized to GAPDH. Data represent mean ± SD of three independent experiments. ** =p<0.01 vs C- siRNA

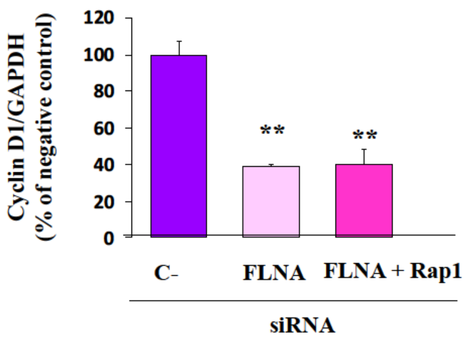
FLNA silencing promoted cell adhesion increase and cell migration decrease via Rap1

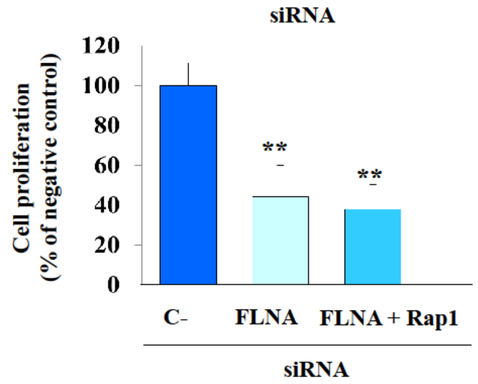


A) H727 cells transfected with C-siRNA or FLNA siRNA alone or together with Rap1 siRNA were incubated in complete medium for 90 min at 37°C. Experiments carried out in H727 cells were repeated at least 4 times. The graph shows the quantification of adherent cells.

B) H727 cells transfected with C-siRNA or FLNA siRNA alone or together with Rap1 were incubated for 24 h with serum-free medium. Migratory cells were quantified by fluorometric plate reader. Experiments were performed in triplicate four times. Values represent mean (±SD) **=p<0.01 *vs* C-siRNA

Cell proliferation inhibition induced by FLNA silencing is not mediated by Rap1





FLNA siRNA treated cells showed a strong decrease in cyclin D1 expression and cell proliferation in H727 cells not via Rap1. H727 cells were transfected with control siRNA, FLNA siRNA alone or together with Rap1.

A)The graph shows the quantification of Cyclin D1 normalized to GAPDH. Data represent mean ± SD of three independent experiments.
** =p<0.01 vs C- siRNA.

B) The graph shows BrdU incorporation during DNA synthesis. **=p<0.01 vs C-siRNA.

CONCLUSIONS

FLNA :

-is overexpressed in PNTs and increases in PNTs with high malignant grade;

-plays a crucial role in PNT cells proliferation, angiogenesis, colony formation, cell adhesion and cell migration;

-interacts with Rap1 in mediating H727 cell mobility

The involvement of FLNA in mediating PNT progression and aggressiveness, provides a potential diagnostic and therapeutic target.

REFERENCES

1. Travis WD. Pathology and diagnosis of neuroendocrine tumors: lung neuroendocrine. Thorac Surg Clin. 2014 Aug;24(3):257-266. doi: 10.1016

2. Jiang X, Yue J, Lu H, Campbell N, Yang Q, Lan S, Haffty BG, Yuan C, Shen Z. Inhibition of Filamin-A Reduces Cancer Metastatic Potential. Int J Biol Sci 2013; 9(1):67-77. doi: 10.7150/ijbs.5577

3. Savoy RM and Ghosh PM The dual role of filamin A in cancer: can't live with (too much of) it, can't live without it. Endocr Relat Cancer 201320 341-356. doi: 10.1530/ERC-13-0364

4. Yue J, Huhn S, Shen Z. Complex roles of filamin-A mediated cytoskeleton network in cancer progression. Cell Biosci. 2013 3:7. doi: 10.1186/2045-3701-3-7

5. Uramoto H, Akyürek LM, Hanagiri T. A positive relationship between filamin and VEGF in patients with lung cancer. Anticancer Research. 2010; 30:3939–3944.

6. Zheng X, Zhou AX, Rouhi P, Uramoto H, Borén J, Cao Y, Pereira T, Akyürek LM, Poellinger L. Hypoxia-induced and calpain-dependent cleavage of filamin A regulates the hypoxic response. Proc Natl Acad Sci U S A. 2014 Feb 18;111(7):2560-5. doi: 10.1073/pnas.1320815111

7. Motawea HK, Jeyaraj SC, Eid AH, Mitra S, Unger NT, Ahmed AA, Flavahan NA, Chotani MA. Cyclic AMP-Rap1A signaling mediates cell surface translocation of microvascular smooth muscle α2C-adrenoceptors through the actin-binding protein filamin-2. Am J Physiol Cell Physiol. 2013;305(8):C829-45. doi: 10.1152/ajpcell.00221.2012