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The flightless I protein interacts with RNA-binding proteins and is involved in the genome-wide mRNA post-transcriptional regulation in lung carcinoma cells

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Abstract. The flightless I protein (FLII) belongs to the gelsolin family. Its function has been associated with actin remodeling, embryonic development, wound repair, and more recently with cancer. The structure of FLII is characterized by the N-terminal leucine-rich repeats (LRR) and C-terminal gesolin related repeated units that are both protein-protein interaction domains, suggesting that FLII may exert its function by interaction with other proteins. Therefore, systematic study of protein interactions of FLII in cells is important for the understanding of FLII functions. In this study, we found that FLII was downregulated in lung carcinoma cell lines H1299 and A549 as compared with normal HBE (human bronchial epithelial) cell line. The investigation of FLII interactome in H1299 cells revealed that 74 of the total 132 putative FLII interactors are involved in RNA post-transcriptional modification and trafficking. Furthermore, by using high-throughput transcriptome and translatome sequencing combined with cell fractionation, we showed that the overexpression or knockdown of FLII impacts on the overall nuclear export, and translation of mRNAs. IPA analysis revealed that the majority of these target mRNAs encode the proteins whose functions are reminiscent of those previously reported for FLII, suggesting that the post-transcriptional regulation of mRNA might be a major mechanism of action for FLII.

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Abbreviations: FLII, flightless I; LRR, leucine-rich repeats; HBE, human bronchial epithelial; IPA, ingenuity pathway analysis; NSCLC, non-small cell lung cancer; RNC, ribosome-nascent chain complex; NPC, nuclear pore complex; rpkM, reads per kilobase per million reads; EEF2, eukaryotic elongation factor 2

Key words: flightless I, interactome, lung cancer, RNA-binding protein, RNA post-transcriptional regulation

Introduction

Lung cancer has become the most common cause of cancer-related mortality and a serious public health concern worldwide. In China, the lung cancer-related mortality rate has been multiplied by 4.6 in the past 3 decades. It will reach the alarming 1 million per year by 2025, as estimated by WHO (1). Among all lung cancers, 85% are NSCLC (non-small cell lung cancer). At the moment of diagnosis, most of NSCLC patients present with locally advanced or metastatic disease, when the surgical intervention is no longer feasible despite its high success rate if applied earlier. For these patients, systemic chemotherapy with concurrent radiotherapy remains a necessary option for cure. For this reason, the identification of the proteins playing key roles in the physiopathology of NSCLC that could be potential targets for the chemotherapy reveals to be important and emergent.

The flightless I protein (FLII) belongs to the gelsolin family of actin severing proteins. At the time of its discovery, *Drosophila melanogaster* FLII was shown to play an important role in the embryonic development. The following studies on mammalian FLII demonstrated that this protein is also involved in the regulation of wound repair, skin barrier development, the recovery after blistering and regulation of immune response (2-9). Recently, studies have also demonstrated that FLII is involved in colorectal cancer, hepatocellular and prostate cancer (10-13). Notably, a role of tumor suppressor in prostate cancer has been attributed to this protein (14).

Despite the previous attempts of identifying the FLII interactome in the cells using yeast-two hybrid system (15,16), the systemic picture of protein interaction network of FLII still remains elusive. In this study, by combining co-immunoprecipitation and mass spectrometry analysis, we have identified 132 putative FLII interactors in lung adenocarcinoma cancer H1299 cells, and found that more than a half of them are proteins involved in RNA post-transcriptional regulation and protein biosynthesis. By combining cell fractionation,

mRNA-seq and translating mRNA sequencing (RNC-seq), we evaluated the function of FLII on the transcription, nuclear export and translation of mRNAs. We demonstrated that FLII affects the overall nuclear export and translation of mRNAs.

Materials and methods

Antibodies. Rabbit polyclonal anti-FLII (sc-30046) antibody, mouse polyclonal anti-HNRNPQ (sc-56703) antibody, goat polyclonal anti-TIAL1 (sc-1749) antibody mouse monoclonal anti-actin (sc-47778), mouse monoclonal anti-NUP88 (sc-136009) antibody, peroxidase-conjugated AffiniPure goat anti-Mouse IgG (H+L), peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L), rabbit anti-goat IgG (H+L)-HRP (sc-2768) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. Human H1299 and HBE cells (Cell Resource Center, Institute of Life Science Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand island, NY, USA) supplemented with 10% fetal bovine serum (PAA Laboratories, Weike Biochemical Reagent, Shanghai, China), 1% penicillin/streptomycin (Genom, Hangzhou, China) at 37˚C in a humidified atmosphere containing 5% CO_2.

Plasmid constructions. The pot-b7-FLII plasmid containing the cDNA of the human FLII was obtained from Yingrun Biotechnologies Inc. (Changsha, China). The cDNA fragment encoding FLII was PCR-amplified and inserted into pCMV-N-FLAG-vector using the EcoRV and SpeI restriction sites. A primer pair (upstream: 5'-ATTGATATCATGGAGGCCACC GGGGTGCTG-3' and downstream: 5'-ATAACTAGTTTAG GCCAGGGCCTTGCAGAA-3') was designed based on NCBI GenBank NP_002009.1. All the plasmids were accuratly confirmed by DNA sequencing.

Transfections and immunoprecipitation of FLII. Cells were transfected with either empty pCMV-N-Flag or pCMV-N-Flag-FLII vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, cells were lysed using EBC lysis buffer. Total soluble extract (1 mg) was incubated with anti-Flag antibody overnight at 4˚C. Protein A/G-Sepharose was added and incubated for 4 h at 4˚C. After washing, the precipitated proteins were eluted and separated using SDS-PAGE. The gel was stained with Silver nitrate after migration, and gel lanes were subsequently cut, destained, reduced, alkylated and digested with gold-trypsin at 37˚C overnight. The tryptic peptides were extracted, and the peptide mixtures were concentrated by SpeedVac centrifuge to dryness and re-dissolved with 2% ACN in 0.1% formic acid before LC-MS/MS analysis. For the confirmation of the presence of Flag-FLII in the immune-complexes, the immunoprecipitates were washed with immunoprecipitation assay buffer three times, and subjected to western blot analysis using anti-Flag antibody.

MS analysis. The peptide mixtures were analyzed by reverse-phase liquid chromatography coupled with LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) as previously described with minor modification (17). Briefly, the peptide mixtures were firstly loaded on a C18 reverse-phase column (100-mm i.d., 10-cm long, 5-mm resin from Michrom Bioresources, Auburn, CA, USA) using an auto sampler. The peptide mixtures were eluted with 0-40% gradient buffer solution (Buffer A, 0.1% formic acid, and 5% ACN; Buffer B, 0.1% formic acid and 95% ACN) over 180 min. The eluate was then analyzed online in the LTQ-Orbitrap mass spectrometer operated in a data-dependent mode with capillary temperature of 2001 C and spray voltage of 1.80 kV. A full MS scan with m/z 350-1800 was carried out in the Orbitrap at resolution r5100,000 at m/z 400, and followed by five MS2 scans in the LTQ with Dynamic Exclusion setting: 2 repeat counts with repeat duration of 30 sec and exclusion duration of 90 sec. MS3 was further performed if an ion had a neutral loss of -98.00, -58.00, -49.00, -38.67,-32.67 or -24.50 Da in the MS2 and the ion was one of the top five most intense ions in the MS2. Conditions with 35% normalized collision energy, activation q of 0.25 and activation time of 30 msec were applied for MS2 and MS3 acquisitions.

Stable FLII knockdown in HBE cells. Lentiviral shRNA vectors (GenePharma, Shanghai, China) targeting human FLII were utilized for stable knockdown in HBE cells. Procedures were conducted according to the manufacturer's protocol. The FLII siRNA sense sequence (5'-GGGCTAGACATCTACG TAT-3') and Scramble siRNA sense sequence (5'-TTCTCC GAACGTGTCACGTTTC-3') were used to design the shRNA that were inserted into PGLV3/H1/GFP+ pur Vector under H1 promoter. Cells resistant to puromycin (1.5 μg/ml) were selected and passaged for further study.

Cell fractionation. Six million human H1299 or HBE cells were resuspended in 500 μl ice cold Extraction Buffer (10 mM Tris-HCl, 10 mMKCl, 5 mM $MgCl_2$, protease and phosphatase inhibitors, RNase inhibitor, VRC) and incubated on ice for 10 min, 500 μl 0.6% Triton/Extraction Buffer was then added, followed by 10 sec of vortex, and incubation on ice for further 10 min, then vortex for 10 sec again. Lysate was then subjected to vertical centrifugation for 5 min at 600 x g speed at RT. Supernatant corresponding to the cytoplasmic fraction was transferred into a new tube. The pellet corresponding to the cell nucleus was subsequently resuspended in 1 ml 0.6% Triton Extraction Buffer by pipetting 10 times, incubated on ice for 5 min, followed by vertical centrifugation for 5 min at 600 x g speed at RT, The supernatant corresponded to nuclear extract was then collected. This experiment was repeated thrice, and the cytoplasmic or nuclear extract resulted from the three experiments were pooled together, and served for the RNA extraction using Trizol reagent (Invitrogen).

Ribosome-nascent chain complex (RNC) extraction. The RNC extraction was performed as described by Esposito *et al* (18) with modifications. In brief, cells were pre-treated with 100 mg/ml cycloheximide for 15 min, followed by pre-chilled phosphate buffered saline washes and addition of 2 ml cell lysis buffer [1% Triton X-100 in ribosome buffer (RB buffer) 20 mM HEPES-KOH (pH 7.4), 15 mM $MgCl_2$, 200 mMKCl, 100 mg/ml cycloheximide and 2 mM dithiothreitol]. After

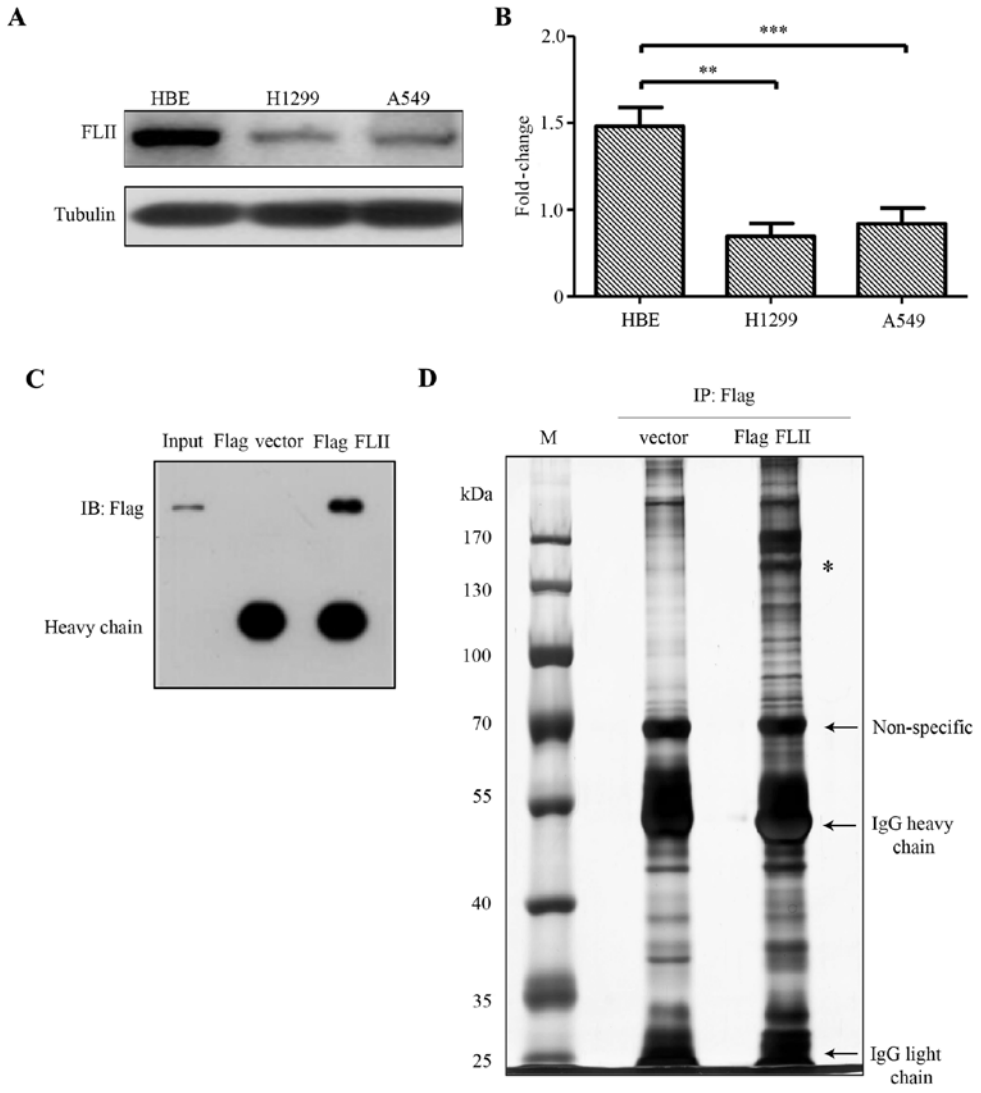

Figure 1. Identification of FLII interactome in H1299 lung carcinoma cells. (A) FLII expression in HBE, H1299 and A549 cells determined by western blotting. (B) Quantification of the FLII protein bands shown in (A) by using ImageJ. Data are from five independent experiments; Error bars indicate s.d. $^{**}P<0.05$, $^{***}P<0.01$, by Student's t-test. (C) H1299 cells transfected with the empty Flag vector or the Flag-tagged FLII was subjected to immunoprecipitation with anti-Flag antibody. The presence of the immunoprecipitated FLII was revealed by western blotting using the anti-Flag antibody with 1/10 of the immunoprecipitates. (D) The remaining immunoprecipitates from (C) were run in SDS-PAGE and colored by silver staining. The protein band possibly corresponds to FLII is denoted by the asterisks. The heavy and light chains of the anti-Flag antibody, together with the 70 kDa non-specific protein band are also indicated.

30-min ice-bath, cell lysates were scraped and transferred to pre-chilled 1.5 ml tubes. Cell debris was removed by centrifuging at 13, 200 x g for 10 min at 4˚C. Supernatants were transferred on the surface of 20 ml of sucrose buffer (30% sucrose in RB buffer). RNCs were pelleted after ultra-centrifugation at 185,000 x g for 5 h at 4˚C. RNC-RNA was purified using TRIzol method.

Next-generation sequencing and sequence analysis. Total RNA and RNC-RNA of human H1299 and HBE cells were extracted as previously described (18). Equal amounts of total mRNA or RNC-mRNA isolated from three independent cultures were pooled and reverse-transcribed into cDNA. The yielded cDNA library was then constructed and subjected to RNA-seq analysis using Illumina HiSeq™ 2000. The sequencing data sets are deposited in Gene Expression Omnibus database under the accession number of GSE92979. Reads were mapped to human mRNA reference sequence (RefSeq) for GRCh37/hg19 in UCSC genome browser (downloaded from http://hgdownload.cse.ucsc.edu/downloads, accessed January 21, 2013) using FANSe2 mapping algorithm with the options -L78 -S8 -I0 -E9 -B1. The reads mapped to splice variants of one gene were summed. The mRNA abundance was normalized using both rpkM (reads per kilobase per million reads). Differential expression was evaluated using edgeR since it outperforms the other mainstream differential expression models (19). Genes with >10 mapped reads were considered as quantified genes. The cytoplasm/nucleus distribution of each given mRNA has been quantified in all experimental groups of cells, and the variation of such distribution between the FLII knocked down HBE or the FLII overexpressing H1299 cells and their respective control cells were calculated. Where Flag-FLII and Flag-Vector refer to H1299 cells transfected with Flag-FLII and Flag vector; shRNA-FLII and shRNA-Ctrl refer to HBE cells expressing the shRNA against FLII and the control shRNA. 'C' for cytoplasmic, and 'N' for nuclear.

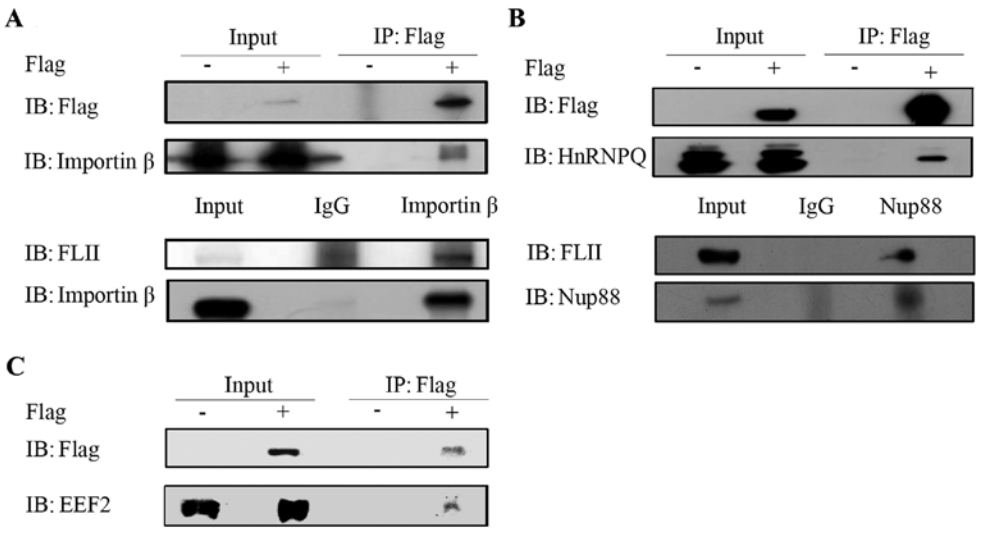

Figure 2. Validation of interactions between FLII and the representative interacting partners. (A) H1299 cells transfected with the Flag-tagged FLII was subjected to immunoprecipitation (IP) with Flag antibody and the interaction of endogenous importin β and overexpressed FLII was determined by western blotting (WB) with indicated antibodies (upper panels). The interaction of endogenous importin β and FLII was confirmed by co-IP with the negative control IgG and importin β antibodies (lower panels). (B) Co-immunoprecipitation of Flag-FLII with the endogenous hnRNPQ (upper panels) in H1299 cells transfected with Flag-FLII, and co-immunoprecipitation of endogenous FLII and Nup88 in H1299 cells (lower panels) by using appropriate antibodies as indicated. (C) H1299 cells were transfected with the Flag-tagged FLII then subjected to co-immunoprecipitation (IP) with Flag antibody for the interaction between endogenous EEF2 and overexpressed FLII. WB were performed with the immunoprecipitates using the indicated antibodies.

Reverse transcription and PCR. Total RNA or RNC-RNA, isolated from both H1299 and HBE cells, were reverse transcribed to cDNA with poly-dT primer using Reverse Transcriptase XL (AMV) (Takara, Foster City, CA, USA), by following the manufacturer's instructions. The quantitative real-time PCR (qPCR) was then performed with gene-specific primers and the SsoFast™ EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) on a Bio-Rad MiniOpticon real-time PCR system (Bio-Rad) by following the manufacturer's instructions. The specificity of the primers was verified by both in Silico Computation (NCBI Primer-BLAST) and melting curve measurement after the qPCR amplification.

Computational analyses. Protein interaction network was built by using STRING-DB (string-db.org) with default settings (all interaction sources selected, 'medium confidence (0.400)' for minimum required interaction score, and 'query protein only' for max number of interactors to show). Ingenuity pathway analysis (IPA) was performed as described previously with minor modifications. Briefly, target proteins (DEPs) were uploaded into www.ingenuity.com (Ingenuity Systems, Inc., Redwood City, CA, USA). Core analyses were performed to identify top canonical pathways, bioprocesses and effects on functions.

Cell migration and invasion. *In vitro* migration and invasion assays were performed using Boyden chambers as previously described with minor modifications (20). In brief, for migration assays, cells transfected with pCMV-N-Flag-FLII or pCMV-N-Flag plasmid were resuspended in serum-free medium and seeded in the upper transwell chamber (8.0 mM pore size, Corning), and medium supplemented with 10% FCS was added to the bottom chamber, and cultured under regular conditions for 6 h, cells on the upper surface of filters were then removed and those on the under-surface were stained with 5% crystal violet. Images were captured from each membrane and the number of migrated cells was counted under a microscope. For invasion assays, similar transwell chambers coated with Matrigel (8.0 mM pore size, Chemicon) were used to analyze the invasive potential regulated by FLII.

Statistics. The Spearman correlation coefficients were calculated to determine bivariate relationships. The regression, data distribution and standard deviations were calculated by using MATLAB R2012a software package (MathWorks, Natick, MA, USA). Data are shown as mean ± standard deviation. Statistical difference was accepted at $P<0.001$.

Results

FLII is downregulated in lung carcinoma cells. In order to assess the possible role of FLII in the physiopathology of NSCLC, we firstly examined its expression pattern in two lung adenocarcinoma epithelial cell lines, namely H1299 and A549 cell lines. Of note, we observed that FLII was significantly downregulated in A549 and H1299 lung carcinoma cells, as compared with the human bronchial epithelial (HBE) cells (Fig. 1A and B). This result is consistent with the recent study reporting that FLII is a tumor suppressor (14).

Identification of FLII interactome in H1299 lung carcinoma cells. We then tried to identify the possible interactors of FLII in H1299 cells. Immunoprecipitation experiment was carried out using anti-Flag antibody in Flag-FLII transfected H1299 cells as described in Materials and methods. The immunoprecipitation of Flag-FLII was then confirmed by western blotting using an anti-Flag antibody with one tenth of the immunoprecipitates from the Flag-FLII transfected and the empty Flag vector transfected cells (Fig. 1C). The remaining immunoprecipitates were run on an SDS-PAGE gel that was

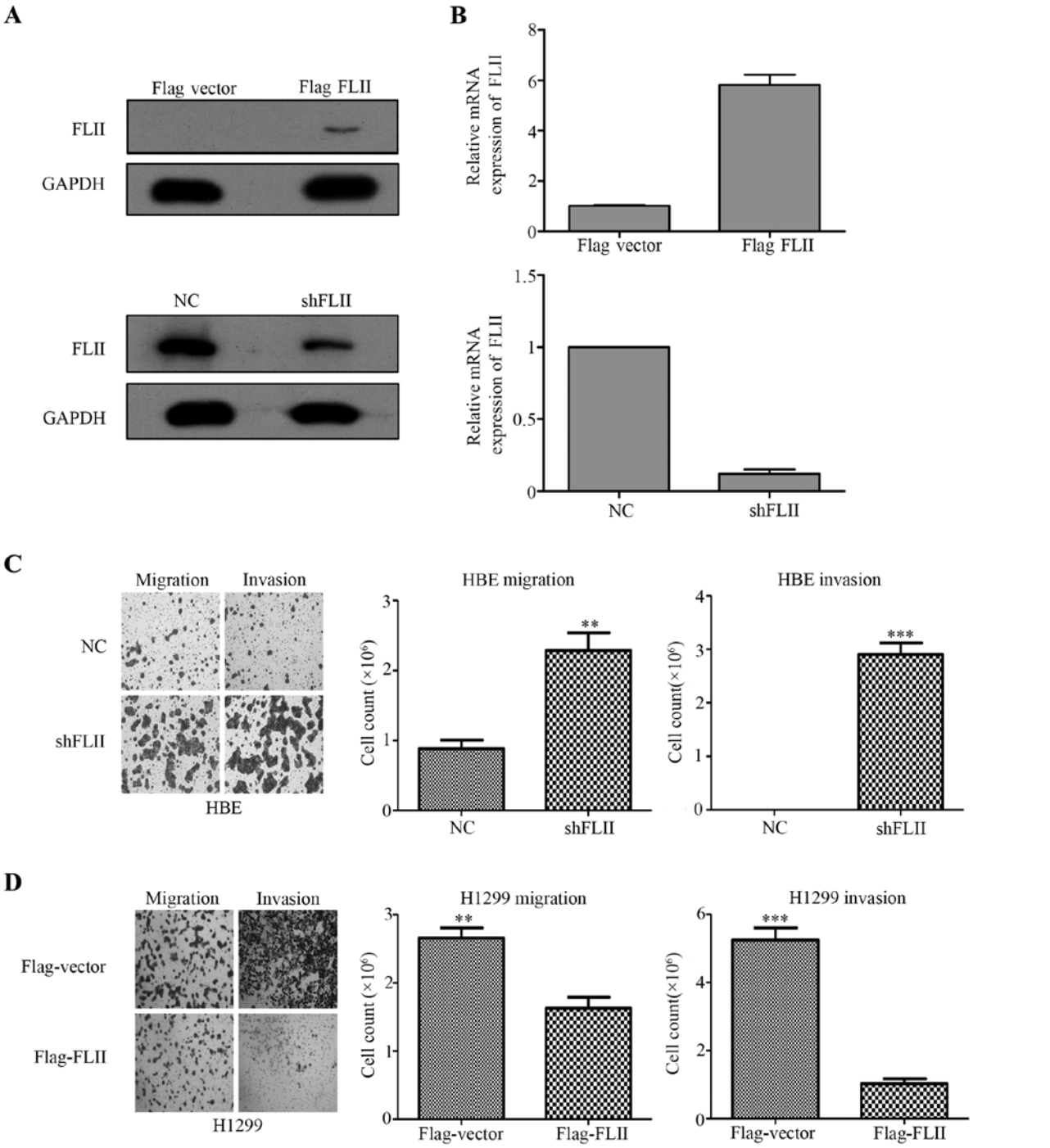

Figure 3. Effectiveness of FLII overexpression or knockdown in the corresponding HBE or H1299 cells. (A) H1299 cells transfected with Flag-FLII and HBE cells with stable lentiviral-mediated FLII knockdown were analyzed by western blotting with anti-Flag (upper panels) or anti-FLII antibody (lower panels). H1299 cells transfected by the empty Flag vector, or HBE cells infected by a control virus was respectively used as negative controls. (B) mRNA levels of FLII in Flag-FLII overexpressing H1299 and FLII knocked down HBE cells are determined by qRT-PCR with appropriate primers. Data are expressed by setting the relative value of the control cells to 1. Results shown represent the mean value of three independent experiments. (C) Boyden's chamber assays were performed to assess the impact of FLII knockdown on the migrating and invasive abilities of HBE cells as described. The migrated cells (images shown in the left panels) were counted, and the results represent the mean values of three independent experiments. Error bars indicate s.d. $^{**}P<0.05$, $^{***}P<0.01$, by Student's t-test. (D) Analyses similar to (C) were performed with Flag-FLII overexpressing H1299 cells.

subsequently silver-stained (Fig. 1D). The protein bands of both Flag-FLII transfected and the negative control lanes were extracted for tryptic in-gel digestion. Digested peptides were then subjected to LC-MS/MS analysis for protein identification. Proteins (263) were identified in Flag-FLII transfected lane. One hundred and thirty of them were also found in the negative control lane, thus considered to be unspecific and excluded from the candidates for further analyses. Among the 133 remaining proteins, we found FLII protein with 15 unique peptides specifically counted, further confirming the effectiveness of the immunoprecipitation. The other 132 proteins were considered to be the putative FLII-interacting partners.

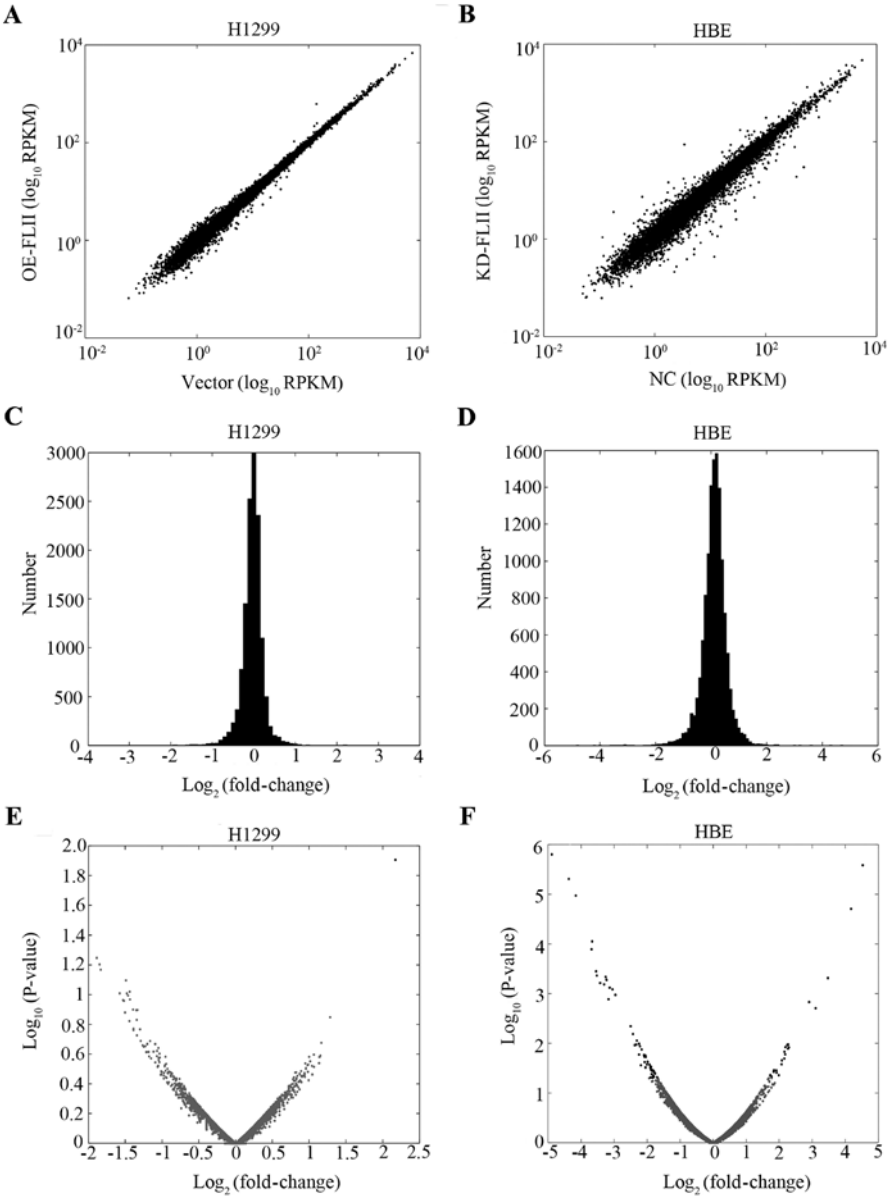

Figure 4. Assessment of FLII functions in RNA transcription by high throughput sequencing. The variation of total mRNA levels in FLII-overexpressing H1299 cells (A), or FLII knocked down HBE cells (B), and the corresponding distributions in fold changes (C and D). (E and F) EdgeR analysis for the differential mRNA levels caused by FLII-overexpression or FLII knockdown in the corresponding cells as indicated. Significantly altered genes are denoted by the black dots while the others are gray dots.

Validation of interactions between FLII and the representative interacting partner. In order to confirm the interactions of FLII with its putative partners identified by mass spectrometry, we carried out experiments to confirm the co-immunoprecipitation of FLII with its several representative partners using corresponding specific antibodies, as shown in Fig. 2 for Importin β (karyopherin β), Nup88, Syncrip and EEF2. Due to the variable quality or suitability of the antibodies for co-immunoprecipitation assays, the experiments were performed either with endogenous or overexpressed Flag-tagged FLII as indicated when appropriate. Fig. 2A shows the interaction of the overexpressed FLII with Importin β using the anti-Flag antibody in Flag-FLII transfected H1299 cells (upper panel) and the interaction between endogenous FLII and Importin β in H1299 cells (lower panel). The interaction of FLII with Syncrip and Nup88 has been confirmed, respectively, by using the anti-Flag antibody in Flag-FLII transfected H1299 cells (Fig. 2B, upper panel) and by using the Nup88 antibody in H1299 cells (Fig. 2B, lower panel). As shown in Fig. 2C, we confirmed the interaction between FLII and EEF2 in Flag-FLII transfected H1299 cells. Among these confirmed representative partners, Importin β and Nup88 are, respectively, important nuclear transport receptor and component of the nuclear pore complex (NPC) involved in the nucleocytoplasmic transport. Particularly, both of them have been reported to be involved in the nuclear export of mRNA, protein and 60S ribosomal complex (21-23). Syncrip is an RNA-binding protein involved in RNA metabolism, such as RNA stability, splicing, and translational control (24-27). EEF2 (eukaryotic elongation factor 2), as its name indicates, is an essential factor for protein synthesis, promoting the GTP-dependent translocation of the nascent

Table I. Flightless I-interacting proteins associated with RNA post-transcriptional regulation.

Protein IDs	Protein names	Gene names	Function
IPI00893918	Valyl-tRNA synthetase	VARS	Protein translation
IPI00001639	Karyopherin subunit β-1	KPNB1	Protein import into nucleus
IPI00001738	Nuclear pore complex protein Nup88	NUP88	RNA translocation
IPI00877174	2'-5'-oligoadenylate synthase 3	OAS3	RNA binding
IPI00003704	RNA-binding protein 4	RBM4	RNA binding
IPI00644708	Nucleolysin TIAR	TIAL1	RNA binding
IPI00005675	NF-κ-B-repressing factor	NKRF	RNA binding
IPI00218187	Serine/threonine-protein phosphatase PP1-γ catalytic subunit	PPP1CC	RNA binding
IPI00006025	Squamous cell carcinoma antigen recognized by T-cells 3	SART3	mRNA splicing
IPI00337397	Nuclear pore complex protein Nup98	NUP98	mRNA export from nucleus
IPI00007818	Cleavage and polyadenylation specificity factor subunit 3	CPSF3	mRNA export from nucleus
IPI00179713	Insulin-like growth factor 2 mRNA-binding protein 2	IMP2	mRNA transport
IPI00010200	Probable ATP-dependent RNA helicase YTHDC2	YTHDC2	RNA processing
IPI00010700	Large proline-rich protein BAT2	BAT2	RNA binding
IPI00873899	ATP-binding cassette sub-family F member 1	ABCF1	Protein translation
IPI00013877	Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3	RNA splicing, RNA processing
IPI00014474	A-kinase anchor protein 8	AKAP8	RNA binding
IPI00015952	Eukaryotic translation initiation factor 4γ	EIF4G2	Translational initiation
IPI00016910	Eukaryotic translation initiation factor 3 subunit C	EIF3C	Translational initiation
IPI00017669	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	BAF57	RNA binding
IPI00018120	28S ribosomal protein S29	DAP3	RNA binding
IPI00018140	Heterogeneous nuclear ribonucleoprotein Q	HNRPQ (Syncrip)	RNA splicing, RNA processing, RNA binding
IPI00018522	Protein arginine N-methyltransferase 1	PRMT1	RNA binding
IPI00019380	Nuclear cap-binding protein subunit 1	NCBP1	mRNA export from nucleus, RNA splicing, RNA binding
IPI00021435	26S protease regulatory subunit 7	PSMC2	Regulation of mRNA stability
IPI00926977	26S protease regulatory subunit S10B	PSMC6	Regulation of mRNA stability
IPI00025491	Eukaryotic initiation factor 4A-I	EIF4A1	RNA binding, translational initiation
IPI00026665	Glutaminyl-tRNA synthetase variant	QARS	RNA binding, protein translation
IPI00026969	SEC23-interacting protein	SEC23IP	RNA binding
IPI00027107	Elongation factor Tu	TUFM	Translational elongation, RNA binding
IPI00027415	Probable ATP-dependent RNA helicase DHX36	DHX36	RNA binding
IPI00847793	Dermcidin isoform 2	DCD	RNA binding
IPI00043407	Trinucleotide repeat-containing gene 6C protein	TNRC6C	Translation regulation, RNA binding
IPI00384265	Constitutive coactivator of PPAR-γ-like protein 1	FAM120A	RNA binding
IPI00073779	28S ribosomal protein S35	MRPS35	RNA binding
IPI00100151	5'-3' exoribonuclease 2	XRN2	RNA processing, RNA binding
IPI00106567	WD repeat-containing protein 33	WDR33	mRNA processing, RNA binding
IPI00141318	Cytoskeleton-associated protein 4	CKAP4	RNA binding
IPI00163084	Pre-mRNA-splicing factor SYF1	XAB2	mRNA processing, mRNA splicing
IPI00165434	YLP motif-containing protein 1	YLPM1	RNA binding
IPI00168885	Putative ATP-dependent RNA helicase DHX57	DHX57	RNA binding, RNA processing
IPI00171127	Ubiquitin-associated protein 2	UBAP2	RNA binding

Table I. Continued.

Protein IDs	Protein names	Gene names	Function
IPI00646361	Nuclear pore complex protein Nup214	NUP214	mRNA export from nucleus, regulation of mRNA stability
IPI00220609	Nucleoporin SEH1-like	SEH1L	mRNA export from nucleus
IPI00186290	Elongation factor 2	EEF2	RNA binding
IPI00217413	ATP-dependent RNA helicase DHX29	DHX29	RNA binding, RNA processing
IPI00217466	Histone H1c	HIST1H1D	RNA binding
IPI00375144	Serrate RNA effector molecule homolog	SRRT	mRNA splicing, RNA binding
IPI00396171	Microtubule-associated protein 4	MAP4	RNA binding
IPI00941899	Pyruvate kinase isozymes M1/M2	PKM2	RNA binding
IPI00549248	Nucleophosmin	NPM1	RNA binding, regulation of translation
IPI00220834	ATP-dependent DNA helicase 2 subunit 2	XRCC5	RNA binding
IPI00221106	Splicing factor 3B subunit 2	SF3B2	mRNA processing, mRNA splicing, RNA binding
IPI00291939	Structural maintenance of chromosomes protein 1A	SMC1A	RNA binding
IPI00910194	Nuclear pore complex protein Nup153	NUP153	mRNA export from nucleus
IPI00294242	28S ribosomal protein S31	MRPS31	RNA binding
IPI00294536	Serine-threonine kinase receptor-associated protein	STRAP	mRNA processing, mRNA splicing, RNA binding
IPI00296337	DNA-dependent protein kinase catalytic subunit	PRKDC	RNA binding
IPI00646058	Scaffold attachment factor B	SAFB	regulation of mRNA processing, RNA binding
IPI00304692	Heterogeneous nuclear ribonucleoprotein G	RBMX	mRNA processing, mRNA splicing
IPI00304925	Heat shock 70 kDa protein 1	HSPA1A	regulation of mRNA stability
IPI00853598	Protein SEC13 homolog	SEC13	mRNA transport
IPI00382470	Heat shock protein HSP 90-α	HSP90AA1	RNA binding
IPI00940033	Trinucleotide repeat-containing gene 6B protein	TNRC6B	Translation regulation
IPI00396435	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15	mRNA processing, mRNA splicing
IPI00396485	Elongation factor 1-α1	EEF1A1	translational elongation
IPI00418497	Mitochondrial import inner membrane translocase subunit TIM50	TIMM50	RNA binding
IPI00453473	Histone H4	HIST1H4A	RNA binding
IPI00455134	Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3	mRNA processing, mRNA splicing, RNA binding, mRNA transport
IPI00644127	Isoleucyl-tRNA synthetase	IARS	protein translation
IPI00647635	PERQ amino acid-rich with GYF domain-containing protein 2	GIGYF2	RNA binding, regulation of translation
IPI00784224	Zinc finger RNA-binding protein	ZFR	RNA binding
IPI00783872	Caprin-1	CAPRIN1	RNA binding
IPI00871240	Protein SCAF8	RBM16	mRNA processing, mRNA splicing

peptide from the A-site to the P-site of ribosome during the translational process (28).

Construction and functional enrichment analysis of the protein interaction network involving FLII and its interactors. The biological functions of all putative FLII binders were then categorized by consulting the genecards databases, we found that more than a half (74 out of the 132 total) of the putative FLII interactors are associated with RNA post-transcriptional modifications, RNA and protein nucleocytoplasmic transport and protein biosynthesis (Table I). To further assess the relevance of this result and to identify the biological processes and signaling pathways involving FLII and its interacting proteins, we used STRING program to build a protein interaction network comprising these putative interactors (not shown due to the impossibility of exporting a figure which could meet the

Table II. Functional enrichments in Flightless I-interacting network.

Pathway ID	Pathway description	Count in gene set	False discovery rate
Biological process (GO)			
GO:0010467	Gene expression	61	7.44e-10
GO:0006807	Nitrogen compound metabolic process	68	1.16e-07
GO:0034641	Cellular nitrogen compound metabolic process	65	1.57e-07
GO:0010033	Response to organic substance	41	9.68e-07
GO:0043170	Macromolecule metabolic process	77	9.68e-07
Molecular function (GO)			
GO:0044822	poly(A) RNA binding	54	9.17e-30
GO:0003723	RNA binding	57	4.45e-27
GO:0003676	Nucleic acid binding	56	1.66e-08
GO:0000166	Nucleotide binding	42	6.87e-08
GO:0036094	Small molecule binding	43	4.56e-07
Cellular component (GO)			
GO:0031981	Nuclear lumen	68	1.16e-18
GO:0005654	Nucleoplasm	62	1.89e-18
GO:0044428	Nuclear part	70	1.89e-18
GO:0070013	Intracellular organelle lumen	72	9.78e-12
GO:0005634	Nucleus	84	2.02e-12
KEGG pathways			
03013	RNA transport	12	8.61e-08
03040	Spliceosome	8	0.000217
04141	Protein processing in endoplasmic reticulum	7	0.00885
03015	mRNA surveillance pathway	5	0.0177
PFAM protein domains			
PF00076	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	7	0.0306
PF03144	Elongation factor Tu domain 2	3	0.0466

publication standard). Among the total 133 proteins analyzed (nodes), 115 are connected with at least one other partner by at least one kind of interactions (edges). An important number of nodes are connected with its partner(s) by multiple edges. For the 133 nodes analyzed, the number of edges was 305, significantly superior to the expected value that is 164, resulting in an average node degree of 4.59, a clustering coefficient of 0.539, and a PPI enrichment P-value of 0. These network statistics clearly demonstrate that this protein interaction network has significantly more interactions than expected, and is therefore statistically reliable and functionally relevant. The functional enrichments in this network were determined according to biological process, molecular function, cellular component, KEGG pathways and PFAM protein domains (Table II). As expected, the results clearly indicate that the interactome of FLII is associated with RNA post-transcriptional regulations and functions, with the top ranked biological process being the gene expression, and the top molecular functions being the RNA/nucleic acid binding, supported by the top ranked KEGG pathways as RNA transport, spliceosome, protein processing and mRNA surveillance (Table II). These results, which depict a comprehensive interactome of FLII in H1299 cells, suggest strongly that FLII might play a role in the post-transcriptional regulation and function of RNA through interactions with the RNA-binding proteins and nucleoporins.

Assessment of FLII functions in RNA trafficking and translation by high throughput sequencing. The nature of the FLII binders identified by us strongly pointed to an involvement of FLII in RNA post-transcriptional modification and trafficking in lung adenocarcinoma H1299 cells, we strived to verify this hypothesis. We have stably knocked down FLII in HBE cells by using a lentiviral shRNA vector, and in parallel, overexpressed Flag-FLII in H1299 cells by transient transfection. The effectiveness of the knockdown and overexpression of FLII respectively in HBE and H1299 cells was confirmed by both western blotting using anti-FLII and anti-Flag antibodies (Fig. 4A), and by qPCR (Fig. 3B). Furthermore, Boyden chamber assays were performed to confirm the functional impact of the alteration of FLII expression in these cells. As shown in Fig. 3C and D, FLII knockdown in HBE cells considerably stimulated their migration and invasion, whereas FLII overexpression showed inhibition ability.

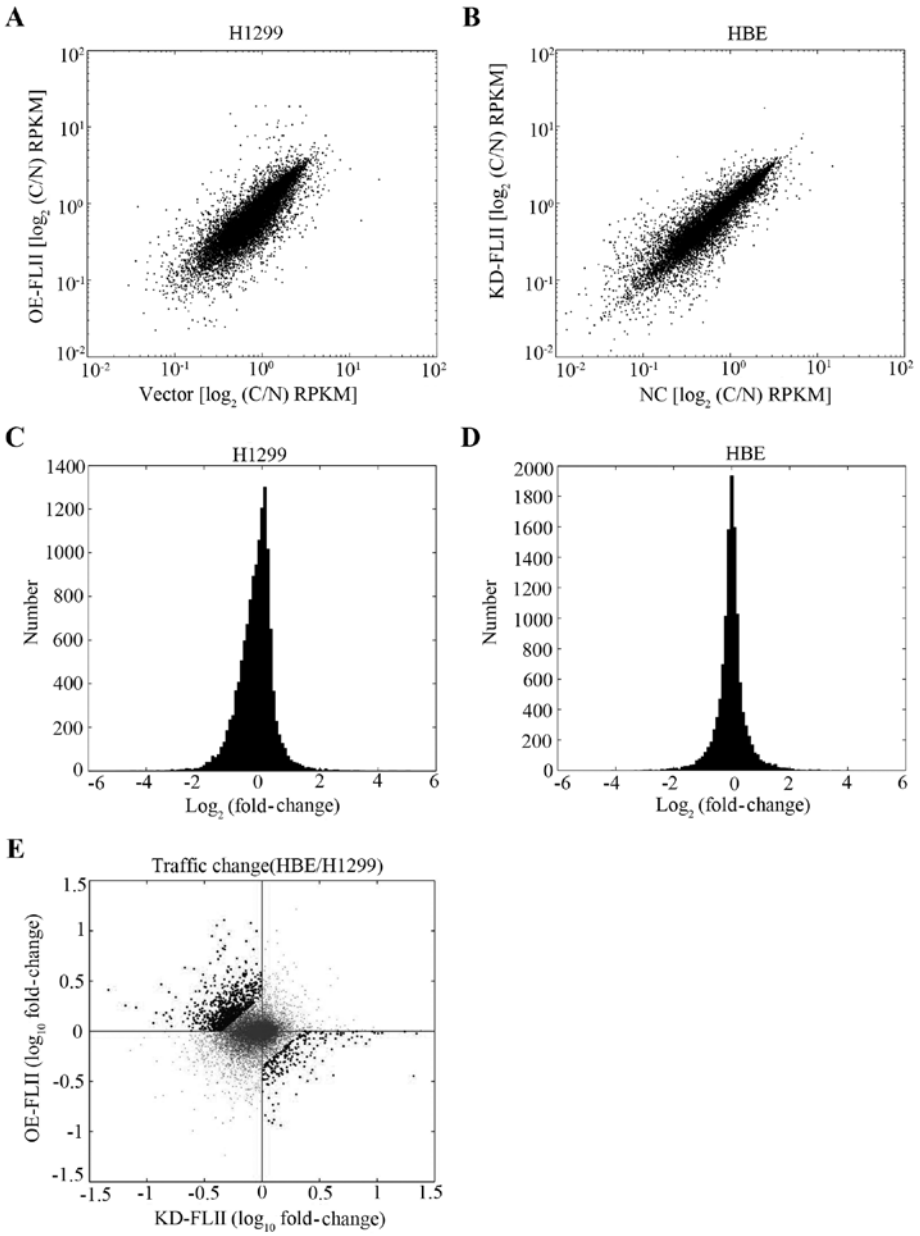

Figure 5. Assessment of FLII functions in nucleocytoplasmic trafficking of mRNA by high throughput sequencing. The variation of the cytoplasm/nucleus ratio of each mRNA (represented by a corresponding gray dot in the diagram) in FLII-overexpressing H1299 (A) or FLII-knocked down HBE cells (B). The cytoplasm/nucleus ratio of each mRNA in FLII-overexpressing or FLII-knocked down cells were plotted versus their respective controls (Vector or NC, as indicated). (C and D) The distribution in fold changes of the FLII regulated mRNA in the corresponding cells used in (A and B). (E) Changes of mRNA cytoplasm/nucleus ratio caused by FLII-overexpressing in H1299 cells versus those caused by FLII- knocked down in HBE cells. The black dots in the second and fourth quadrants represent the mRNA whose cytoplasm/nucleus distribution ratios are regulated by FLII.

High throughput sequencing was then used to assess the role of FLII in the overall biosynthesis and metabolism of the mRNA. We checked the effect of FLII knockdown and overexpression on the overall transcription of the genes. Total mRNA for both FLII knocked down HBE cells and H1299 cells overexpressing FLII, together with their corresponding control cells (respectively the empty vector-transfected and control virus-infected cells), were extracted and reverse transcribed into cDNA. High throughput sequencing was then performed to determine the mRNA species and levels in the various cells. Approximately 14,000 genes were quantified. As shown in Fig. 4, no significant effect of FLII knockdown or overexpression could be observed on the level of total mRNA levels in HBE or H1299 cells, suggesting that FLII does not affect the overall transcription of genes.

We then evaluated the impact of FLII knockdown or overexpression on the nucleocytoplasmic distribution of mRNA to investigate the possible role of FLII in the nucleocytoplasmic trafficking of mRNA. Cell fractionation experiments were performed with FLII knocked down HBE cells, FLII overexpressing H1299 cells, and the corresponding control cells described above, and the cytoplasmic and nuclear mRNA of each type of cells were separately extracted and subjected to high-throughput sequencing. The variations of the cytoplasmic and nuclear mRNA levels in FLII knockdown or overexpression cells versus their control cells for each gene

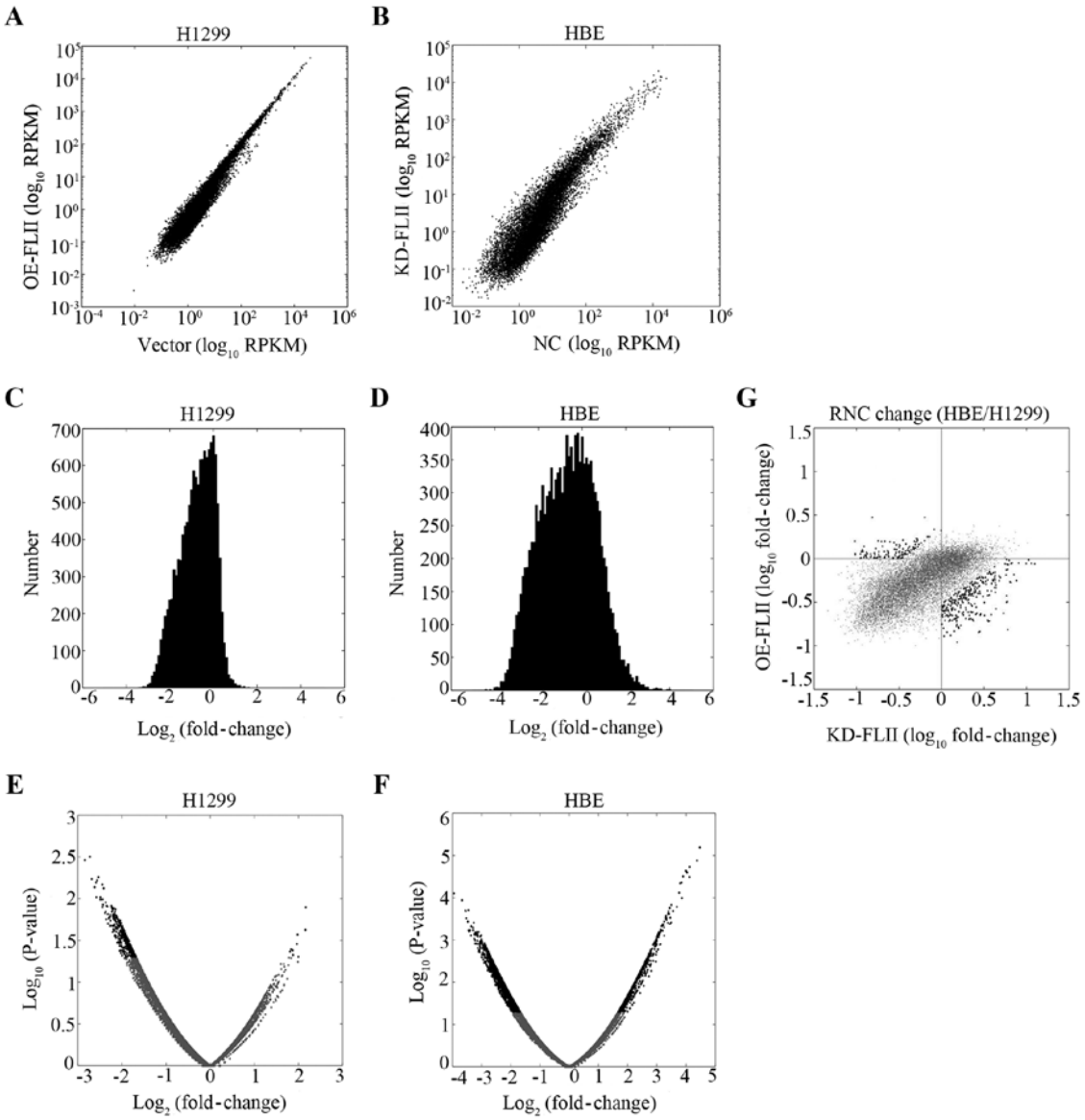

Figure 6. Assessment of FLII function in translation by high throughput sequencing. The variation of RNC-bound mRNA levels in FLII-overexpressing H1299 cells (A), or FLII knocked down HBE cells (B), and the corresponding distributions in fold changes (C and D). (E and F) EdgeR analysis for the differential RNC mRNA levels caused by FLII-overexpression in H1299 cells or FLII knockdown HBE cells. (G) Changes of RNC mRNA levels caused by FLII-overexpressing in H1299 cells versus those caused by FLII knockdown in HBE cells. The black dots in the second and fourth quadrants represent the RNC-bound mRNA whose levels are regulated by FLII. EdgeR analysis for the differential RNC-mRNA levels caused by FLII-overexpression (G), or FLII knockdown (F). Significantly altered genes are denoted by the black dots.

were analyzed as described in Materials and methods. Our results showed that both FLII overexpression in H1299 cells and its knockdown in HBE cells significantly affected the general nuclear-cytoplasmic distribution of mRNA (Fig. 6A-D). These two sets of data were then challenged against each other to compare the quantitative variation of mRNA for each single gene in the two experimental conditions (Fig. 5E). Among all genes whose nucleocytoplasmic distribution was affected by FLII overexpression or FLII knockdown, we considered as relevant those on which FLII knockdown and overexpression significantly exerted opposite effects, that is, either their level was significantly increased by FLII knockdown and decreased by FLII overexpression, or vice versa (Fig. 5E, black spots in the second and fourth quadrants). By this method, we eliminated the bias introduced by the transfection and infection systems we used in this study.

In order to examine the role of FLII on the translational process of mRNA, ribosome-nascent chain complex-associated mRNAs (RNC-mRNAs) from the same cells used for the studies described above were extracted and similarly quantified. Translatome sequencing has quantified over 12,000 genes in translating mRNA. Contrary to the results of the total mRNA levels, we found that FLII knockdown and overexpression significantly affected the level of RNC-mRNAs. Similarly as described above, we have compared the effect of FLII knockdown and overexpression on each gene analyzed and selected as potential FLII target genes those on which FLII knockdown and overexpression significantly exerted opposite effects (Fig. 6E, black spots in

Table III. Functional categorization of Flightless I-regulated genes.

Top diseases and bio-functions

Name	P-value	# Molecules
Diseases and disorders		
Dermatological diseases and conditions	2.28E-02-7.41E-08	55
Organismal injury and abnormalities	2.68E-02-7.41E-08	337
Cancer	2.68E-02-3.48E-06	329
Reproductive system disease	2.28E-02-3.48E-06	142
Endocrine system disorders	2.28E-02-5.20E-06	112
Molecular and cellular functions		
Cellular movement	2.45E-02-1.42E-08	80
Cellular growth and proliferation	2.73E-02-3.30E-07	112
Cell-to-cell signaling and interaction	2.85E-02-8.30E-06	66
Cell morphology	1.94E-02-2.46E-05	39
Cellular development	2.73E-02-2.46E-05	99
Physiological system development and function		
Cardiovascular system development and function	2.71E-02-2.06E-06	33
Organismal development	2.71E-02-2.06E-06	49
Tissue development	2.73E-02-2.46E-05	56
Connective tissue development and function	2.71E-02-1.15E-04	22
Nervous system development and function	2.73E-02-1.09E-03	12

Top networks

ID	Associated network functions	Score
1	Cellular movement, cellular growth and proliferation, cell morphology	40
2	Cellular development, cellular growth and proliferation, cellular function and maintenance	38
3	Cellular movement, hematological system development and function, immune cell trafficking	34
4	Cellular movement, organismal injury and abnormalities, reproductive system disease	24
5	Cancer, organismal injury and abnormalities, reproductive system disease	19

Top canonical pathways

Name	P-value	Overlap
IL-12 signaling and production in macrophages	1.08E-04	7.6%, 11/144
VDR/RXR activation	1.14E-04	10.4%, 8/77
Axonal guidance signaling	3.68E-04	4.5%, 20/441
Production of nitric oxide and reactive oxygen species in macrophages	1.26E-03	5.7%, 11/192
LPS/IL-1 mediated inhibition of RXR function	2.37E-03	5.3%, 11/208

Top upstream regulators

Upstream regulator	P-value of overlap	False discovery rate
ESR1	4.94E-06	
Estrogen receptor	6.22E-05	
PDGF BB	7.15E-05	Activated
TNF	9.44E-05	
SMARCA4	1.49E-04	Activated

Table III. Continued.

Top regulator effect networks			
ID	Regulators	Diseases and functions	Consistency score
1	NF-κB (complex)	Differentiation of cells (+1 more)	3.13
2	TGFB1	Cell movement	-6.935
3	NF-κB (complex)	Proliferation of cells	-7.906
4	ERBB2	Invasion of tumor cell lines	-12.522
5	PDGF BB	Cell proliferation of tumor cell lines	-15.497

the second and fourth quadrants). As indicated, for 4% of the genes analyzed, the transcription was upregulated by FLII overexpression and downregulated by its knockdown, whereas for another 9.5%, the transcription was inversely regulated by FLII. These genes were then submitted to IPA program to assess their possible involvement in various biological processes and signaling pathways. Table III shows the top ranked diseases and biological functions, top networks and canonical pathways, upstream regulators, and the top regulator effect networks. As shown, the top ranked molecular and cellular functions are cellular movement and cellular growth and proliferation, and the top diseases and disorders associated with FLII regulated genes are dermatological diseases and conditions, organismal injury and abnormalities, and cancer. The top canonical pathways revealed to be the IL-12 signaling and production in macrophages and the VDR/RXR activation, whereas the top upstream regulator is estrogen receptor 1, and the top regulator effector network is NF-κB. Taken together, these results suggest that FLII might be involved in the determination of the cell fate, the regulation of development, the injury repair, the immune response and cancer by controlling the mRNA metabolism and trafficking of several groups of genes.

Discussion

Growing evidence indicates that FLII plays important roles in multiple cellular processes (8,9,29). The characteristic feature of FLII being composed by two protein-protein interaction domains suggests that protein interactions might be key elements of the mechanisms governing its versatile functions. However, little has been done with success in the past to specifically and systematically identify the interacting partners of this protein. Two yeast two-hybrid screenings, respectively reported by Liu and Yin in 1998 (15), and by Fong and de Couet in 1999 (16), have led to the identification of three homologous proteins as the interactors of FLII LRR domain, referred to as LRRFIP1 and LRRFIP2 (LRR FLII interacting protein 1 and 2) and FLAP (FLII associating protein). Unfortunately, neither study was able to depict, at the systemic level, the whole interactome of FLII, because all [4 clones, (15)] or most [12 from the 18, (16)] of the positive clones from these two screenings were derived from the genes encoding these three proteins. Besides, a few other proteins have also been independently reported to bind FLII in various contexts and with diverse functions, such as actin, BAF53, CARM1, ChREBP and Rac1 (10,15,30-34). In this study, by combining immunoprecipitation and mass spectrometry analysis, we successfully identified 132 FLII interacting proteins and constructed the first putative interaction network of FLII in the cell. Interestingly, our data displayed striking directivity, in that more than a half of these candidate interactors are associated with RNA post-transcriptional regulation such as splicing, maturation, trafficking and protein synthesis (Table II). These results strongly suggested an undiscovered function of FLII in the regulation of RNA metabolism.

The target genes of FLII that we identified by using the RNC-bound RNA sequencing and IPA analysis encode mainly the proteins involved in the cellular processes such as cellular movement, cellular growth and proliferation, and the development at cellular, tissue, and organismal levels. They are also associated with the diseases and disorders such as dermatological diseases and conditions, organismal injury and abnormalities, and cancer. Moreover, the top ranked canonical pathways identified are IL-12 signaling and production in macrophages and VDR/RXR activation. These physiological and pathological processes and functions are very reminiscent of the reported functions of FLII, that are embryonic development, the regulation of wound repair, skin barrier development, the recovery after blistering and regulation of immune response, and more recently cancer (2-13). This confirmed, on one hand, the reliability of our approaches and the authenticity of our results, and on the other hand, suggested a novel mechanism of action of FLII, that is the overall post-transcriptional regulation of mRNA.

We find that FLII knockdown or overexpression affects at the same time the nucleus/cytoplasm ratio of mRNA and the RNC-bound mRNA amounts and species. The result of FLII interactome suggest that FLII might bind to both the proteins involved in the mRNA post-transcriptional modifications and translation, and those involved in mRNA trafficking, such as the nucleoporins. However, it remains to be determined whether FLII is involved in both the trafficking and the translational regulation of mRNA, or just play a role in mRNA trafficking which might impact on the subsequent translational process. No known RNA-binding domain has been found on FLII protein, which suggests that its function in the regulation of RNA trafficking or/and post-transcriptional regulation might be mediated by its interactions with numerous proteins involved in these biological processes. FLII might take part in the protein complexes associated with mRNA, and exerts its functions through direct and indirect

interactions with various partners regulating one or several steps of mRNA post-transcriptional modification, trafficking and metabolism. For example, its interaction with Nup88 and Nup214 might be involved in the nuclear export of mRNP complexes since Nup214-Nup88 nucleoporin subcomplex has been shown to play an important role in the nuclear export of the mRNA and protein complexes (35-38). Particularly, Nup214-Nup88 complex is required for the CRM1-mediated nuclear export of the 60S preribosomal subunit (36), hinting to the possible impact on the overall translation of mRNA. On the other hand, the interactions of FLII with the proteins playing roles in the translational regulation such as TIAL1 might also be functionally relevant. TIAL1, also named TIAR, was found to bind several mRNAs encoding translation factors such as eukaryotic initiation factor 4A (eIF4A) and eIF4E (translation initiation factors), eEF2 (a translation elongation factor), and c-Myc (which transcriptionally controls the expression of numerous translation regulatory proteins), suppressing their translation and participating to the global inhibition of translation machinery in response to low levels of short-wavelength UV irradiation (39). FLII possibly exerts its regulatory functions by interacting with these RNA-binding proteins through its N-terminal LRR domain, and with actin cytoskeleton via its C-terminal gelsolin related repeats, thereby mediating the traffic of the mRNP complexes along the actin filaments, and modulating the translational processes. In support of this hypothesis, we have been able to detect the interaction of the FLII LRR domain with Nup88 by *in vitro* GST pull-down assay (data not shown).

Guided by the results of FLII interactome obtained in this study, further studies allowing the confirmation and functional demonstration of these relevant interactions will provide new elements for the comprehensive understanding of the precise mechanisms of action of FLII.

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