Protein partners of the eEF1Bβ subunit of the translation elongation complex eEF1B in the nuclear fraction of human lung carcinoma cells

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Aim. To identify novel protein partners of translation factor eEF1Bβ in nucleus of human lung carcinoma cells. **Methods.** Protein partners of eEF1Bβ in the nuclear fraction of A549 cells were identified by co-immunoprecipitation (co-IP) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Specific protein partners of eEF1Bβ were further selected by using the results of previously published global, quantitative and dynamic mapping of protein subcellular localization with help of ''Mapofthecell'' program. **Results.** 104 high-scored proteins interacting with eEF1Bβ in the nuclear fraction of A549 cells have been identified by mass-spectrometry. Among these proteins, 9 partners of eEF1Bβ were confirmed by the co-fractionation approach. Functional analysis of the partners has divided them on the pro-oncogenic (lung-cancer related) and neutral/anti-oncogenic moieties. These two groups are estimated to be spatially separated in human cancer cells. **Conclusions.** The position of eEF1Bβ as a link between the oncogenic and neutral/tumor-suppressor moieties of its protein partners in nucleus of lung cancer cells is suggested. Deciphering of a possible role of the eEF1Bβ distribution between the pro-cancer or anti-cancer communities of its protein partners can be a subject of further research.

K e y w o r d s: eEF1Bβ, protein-protein interactions, A549 cells, ILF2, ILF3, HNRNPU, CELF1, DDX6, CCNT1, PA2G4, SPATS2L, USP39.

Introduction

eEF1Bβ is a nucleotide-exchanging subunit of translation elongation complex eEF1B. This complex, along with eEF1A protein, play a main role in the elongation cycle of human translating ribosome providing GTP-dependent aminoacyl-tRNA recognition in the A-site [1, 2].

Stability of the eEF1B complex in carcinogenesis was recently questioned [3, 4] suggesting that its subunits including eEF1Bβ

may fulfill non-canonical functions separately from the complex. It is known that eEF1Bβ participates in Cd^{2+}-induced oncogenesis [5], eEF1Bβ mRNA is overexpressed in human tumors [6]. Overexpression of eEF1Bβ was reported to be associated with the chemoresistance of malignant melanoma [7]. Nuclear localization of eEF1Bβ was shown [3, 4] which apparently contradicts to a common belief that no translation occurs in nucleus. However, nuclear eEF1Bβ may play some non-canonical and possibly cancer-related roles which are not directly related to the protein synthesis.

In an attempt to identify these roles we present here a data on the experimental analysis of the eEF1Bβ protein partners in the nuclear fraction of the human lung carcinoma cell line A549. A number of the proteins were found, and, what is most important, nine eEF1Bβ partners identified by co-precipitation were confirmed by the co-localization data in the HeLa cells extract by the Mapofthecell program (http://www.mapofthecell.org). Importantly, these 9 proteins can be functionally divided on the pro-oncogenic and neutral/tumor-suppressing groups separated in cellular space. These two moieties can be connected via eEF1Bβ protein.

Materials and Methods

Obtaining of nuclear fraction

A549 cells were cultured in DMEM (Sigma, USA) growth medium with 10 % FBS (Sigma, USA) and 1 % penicillin/streptomycin (Sigma). Cells were grown up to 80 % confluency and harvested with Trypsin-EDTA and phosphate buffered saline (PBS). Nuclear fraction was obtained as described in [8] with modifications. Cells were resuspended in 1 volume of lysis buffer (10 mM HEPES pH7,9; 1,5 mM $MgCl_2$; 10 mM KCl; 0,5 % NP-40; 0,2 mM PMSF; 0,5 mM DTT) and incubated on ice for 20 min. Suspended cells were centrifuged at 400 g for 10 min. Precipitate was resuspended in 4.5 volumes of the buffer comprising 10 mM HEPES, 0.25 mM sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 % NP-40, 0.5 mM DTT, 0.2 mM PMSF and kept 10 min on ice to provide protein extraction. The suspension was loaded on sucrose cushion (2M) and centrifuged at 400 g for 10 min. Nuclei were resuspended in the lysis buffer and centrifuged at 1500 g for 10 min. The procedure was repeated twice. The nuclear pellet was resuspended in a half of initial cell volume of the nucleus lysis buffer (20 mM HEPES pH 7.9, 25 % glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF), incubated on ice for 30 min and centrifuged at 16000 g for 30 min. The supernatant was used as a protein nuclear fraction.

Co-immunoprecipitation

Nuclear fraction of A549 cells was incubated with Protein G Sepharose (Sigma, USA) for 1 hour at 4 °C for pre-clearing. Mouse anti-eEF1Bβ antibodies (Abnova, Taiwan) (1.5 µg of antibodies per 1 mg of total protein) were added to pre-cleared lysates and the incubation persisted overnight at 4 °C. To precipitate the antibody-protein complex, Protein G Sepharose was added according to the manufacturer's protocol and incubated for 2 hours at 4 °C. The samples were analyzed by 12 % PAGE. Gel was stained with the colloidal CBB-G250 [9]. To control non-specific binding the eluate of

the nuclear fraction loaded on empty Protein G-Sepharose was used. Protein bands of interest were cut and processed for mass-spectrometry analysis (LC-MS/MS).

LC-MS/MS

Mass spectrometry analysis was conducted at the Mass Spectrometry Laboratory of the Institute of Biochemistry and Biophysics (Warsaw, Poland). Proteins from each band were digested with trypsin. MS analysis was performed used a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled with a nanoAcquity (Waters Corporation) LC system. Spectrometer parameters were as follows: polarity mode, positive; capillary voltage, 1.5 kV. A sample was first applied to the nano-ACQUITY UPLC Trapping Column (Waters) using water containing 0.1 % formic acid as the mobile phase. Next, the peptide mixture was transferred to the nanoACQUITY UPLC BEH C18 Column (Waters, 75 μm inner diameter; 250 mm long) and an ACN gradient (5–40 % over 100 min) was applied in the presence of 0.1 % formic acid with a flow rate of 250 nl/min and eluted directly to the ion source of the mass spectrometer. Each LC run was preceded by a blank run to avoid sample carry-over between the analyses.

The acquired MS/MS data were pre-processed with Mascot Distiller (version 2.3.2.0, Matrix Science, London, UK). The initial search parameters were set as follows: enzyme, trypsin; variable modifications, carbamidomethyl, oxidation; peptide mass tolerance, ± 100 ppm; fragment mass tolerance, ± 0.1 Da; max missed cleavages, 1; ions score or expect cut-off, 30; max missed cleavages – 1, Swiss-Prot database with the taxonomy restricted to Homo sapiens (20348 sequences); fragmentation mode, HCD; significance threshold, p<0,05.

Bioinformatic analysis

To visualize molecular interaction network for eEF1Bβ we used Cytoscape 3.2.0 Program [10]. The Cytoscape 3.2.0 interaction database BIOGRID was supplemented with newly identified protein partners of eEF1Bβ in nuclear fraction of the cells and analyzed by MCODE plugin which finds clusters (highly interconnected regions) in any network loaded into Cytoscape. MCODE analysis was performed on the hybrid supercomputer "SCIT-4" of the Glushkov Institute of Cybernetics (GIC) of National Academy of Sciences of Ukraine (http://icybcluster.org.ua).

Analysis of the nuclear protein partners which are co-fractionated with eEF1Bβ in the course of a number of subsequent centrifugations was carried out by use of Mapofthecell program (http://www.mapofthecell.org). All protein partners identified by co-precipitation studies were tested for a possibility of their co-fractionation with eEF1Bβ as described in [11].

Results and Discussion

The co-immunoprecipitation of cellular proteins with anti-EF1Bβ antibodies in nuclear extract of A549 cells was used to sort out the protein partners of the elongation factor eEF1Bβ. Mass-spectrometric identification of the protein partners of eEF1Bβ was carried out as described in Materials and Methods section. 104 proteins were identified as interacting partners of eEF1Bβ in the nuclear fraction of human lung carcinoma cells.

As these proteins obviously belong to different functional classes we attempted to classify the nuclear molecular networks which could involve eEF1Bβ, by using MCODE plugin in the Cytoscape 3.2.0 program [10]. This approach has been successfully used by us recently for clustering the cytoplasmic partners of eEF1Bβ [12].

Surprisingly, Cytoscape 3.2.0 was not capable to reveal any functional cluster of the 104 protein partners of eEF1Bβ in nucleus. At present, we are not aware of the reason behind this.

For independent estimation of the possible interactions it is important to use methodically different approach. Precise co-fractionation of the cellular proteins was recently described as an alternative way to estimate a possibility of their interaction [11]. The Mapofthecell program contains a database of the spatial proteome of cancer cells based on the study of precise subcellular localization of human proteins.

Throughout analysis of the spatial proteome of cancer cells by the Mapofthecell program for the presence of nuclear protein partners of eEF1Bβ, detected earlier by the co-immunoprecipitation procedure, has identified nine protein partners confirmed by both co-immunoprecipitation and co-fractionation studies (Table 1). Their characteristics are given below.

Interleukin enhancer-binding factor 2 (ILF2) is a transcription factor which control the interleukin 2 gene transcription. ILF2 forms a complex with ILF3 protein which modulates its RNA-binding properties of the latter [13]. ILF2 regulates RNA splicing and DNA damage response in multiple myeloma [14].

ILF2 functions as an oncogene, regulates epithelial-mesenchymal transition associated genes in pancreatic carcinoma cells [15], and is involved in hepatocellular carcinoma [16, 17]. Overexpression of ILF2 in gastric

Table 1. **Protein partners of eEF1Bβ identified by both mass-spectrometry and precise subcellular co-fractionation**

№	Gene names	Protein names	Localization		Association with lung cancer
			Cyto	Nucl	
1	ILF2	Interleukin enhancer-binding factor 2	++	++++	Yes
2	ILF3	Interleukin enhancer-binding factor 3	+	++++	Yes
3	HNRNPU	Heterogenous nuclear ribonucleoprotein U	+	++++	Yes
4	CELF1	GUGBP Elav-like family member 1	+	++++	Yes
5	USP39	Ubiquitin specific peptidase 39	+	++++	Yes
6	DDX6	DEAD-Box Helicase 6(	++	++	No
7	PA2G4	p42 isoform of Proliferation-associated protein 2G4	++++	++	No
8	SPATS2L	Spermatogenesis Associated Serine Rich 2 Like protein	++	++	No
9	CCNT1	Cyclin T1	++	++++	No

Intracellular localization is given according to human cell database GeneCards (http://www.genecards.org)

cancer is associated with poor prognosis [18]. Importantly, there is a link of ILF2 with lung cancer [19].

Interleukin enhancer-binding factor 3 (ILF3) is a multifunctional protein, which is involved in RNA metabolism, including transcription, degradation, and translation. In addition, it was shown that ILF3 regulates microRNA expression [see for review 20] It interacts with nuclear export machinery [21] and is involved in viral replication [22].

ILF3 is overexpressed in lung carcinoma cells [23]. Stabilization of ILF3 protein by means of lncRNA-LET downregulation contributes to the hypoxia-induced lung cancer cell invasion [24].

Heterogenous nuclear ribonucleoprotein U (HNRNPU) is involved in pre-mRNA splicing [25], takes part in DNA double-strand break (DSB) signaling and repair [26], is involved into the complex providing the c-myc mRNA stability [27].

Loss of HNRNPU causes development of a neurodevelopmental syndrome [28], HNRNPU is involved in adipose tissue biology [29].

Overexpression of miR-193a-3p leads to down regulation of HNRNPU which indicates a possibility of its involvement to non-small-cell lung carcinoma [30].

GUGBP Elav-like family member 1 (CELF1) is a RNA-binding protein which participates in mRNA targeting [31, 32] and degradation [33]. It is involved in energy homeostasis transition in adipose tissue [34]. Controls alternative splicing in human cells [35]. Participates in microRNA destabilization [36]. CELF1 protected cardiomyocytes from ischemia-induced injury through the promotion of angiogenesis and inhibition of apoptosis [37].

CELF1 protein functions as a central node controlling translational activation of genes driving epithelial-to-mesenchymal transition and ultimately tumor progression [38]. It promotes glioma [39] and oral squamous cell [40] carcinomas. However, non-phosphorylated at S302 CELF1 is, in fact, potent tumor-suppressor [41]. Importantly, there are multiple links of CELF1 to lung cancer [42–47].

Ubiquitin specific peptidase 39 (USP39) is a deubiquitinating enzyme without ubiquitin protease activity, which is a component of spliceosome [48]. USP39 has been implicated in the progression of several cancers [49–51]

Importantly, USP39 is overexpressed in lung cancer cells and promotes their proliferation [52]. USP39 is up-regulated in lung and colon carcinomas and its expression correlates with poor clinical outcome, so USP39-targeting strategies were suggested as the basis of new anticancer therapies [53].

DEAD-Box Helicase 6 (DDX6) functions in cytoplasmic RNA regulation. It is also found in nucleus, with unknown role [54].

DDX6 fulfils a progenitor function through two distinct pathways that include the degradation of differentiation-inducing transcripts and by promoting the translation of self-renewal and proliferation mRNAs [55]. DDX6 represses aberrant activation of interferon-stimulated genes [56]. DDX6 modulates interaction of miR-122 with the 5' untranslated region of hepatitis C virus RNA [57]. DDX6 was involved in radio- and chemo resistance in glioblastoma [58], however, the exact role of DDX6 in this case was not identified.

The short isoform, p42, of Proliferation-associated protein 2G4 (PA2G4) promotes cell differentiation [59]. The isoform p42

Ebp1 functions as a potent tumor suppressor of NSCLC through interruption of Akt signaling [60]. PA2G4 P42 stabilized the interaction of SCF-type ubiquitin ligase FBXW7 with its substrates and promoted FBXW7-mediated degradation of oncogenic targets, enhancing its overall tumor-suppressing function [61].

Limited information is available on *SPATS2-like protein (SPATS2L)*. It is found that SPATS2L can be targeted by microRNA-1269a, downregulation of the latter promotes the occurrence and process of hepatocellular carcinoma [62]. However, the direct link of SPATS2L to cancer was not demonstrated. Interestingly, SPATS2L mRNA is found among highly overexpressed mRNAs in systemic lupus erythematosus [63].

Cyclin T1 (CCNT1) is a regulatory subunit of the CDK9/cyclin-T1 cyclin-dependent kinase complex, which promotes transcription via phosphorylation of RNA polymera-se II [64]. This complex is a target of many anti-cancer drugs [65–67] and is important for anti-viral therapy as well [68–70].

Importantly, five out from nine identified protein partners of eEF1Bβ demonstrated obvious connections to lung cancer. Peculiarly, these five proteins showed quite similar localization in a cellular topology map (Fig. 1). Cyclin T1, which is a transcription factor of general significance, was also adjacent to the lung cancer-related cluster (Fig. 1). Furthermore, the eEF1Bβ partners with no marked pro-oncogenic action were closely co-localized in another segment of the map (Fig. 2). Tumor suppressor PA2G4 was involved in this group as well.

Close co-localization of the proteins in cellular topology map is considered as a sign of their potential ability to be involved in the same complex in cell [11]. Subsequently, one cannot exclude that the ILF2, ILF3, HNRNPU, CELF1, USP39 proteins, or at least some of
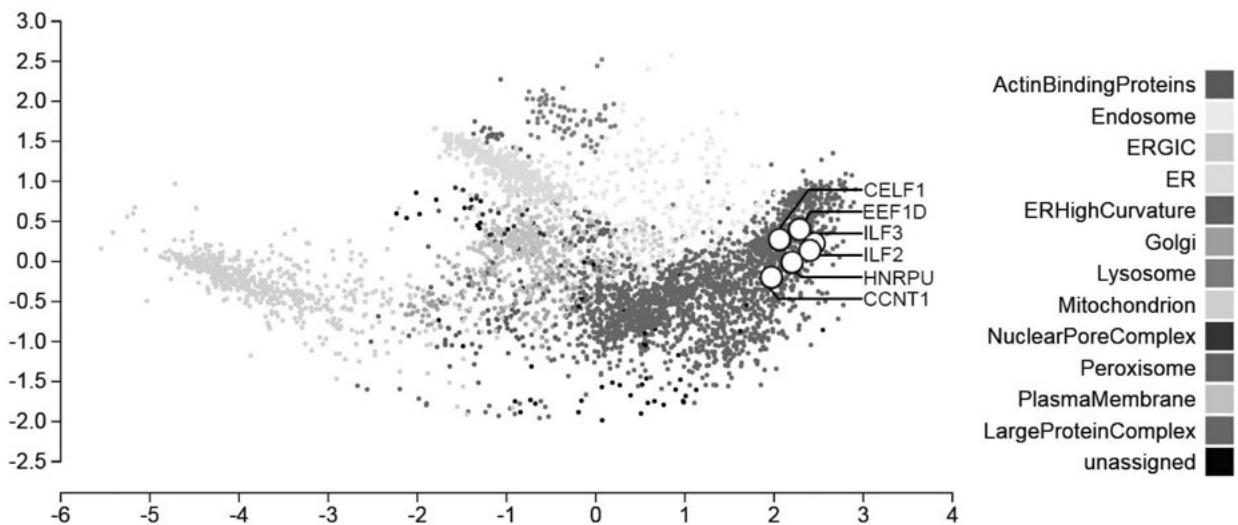

Fig. 1. Consortium of the eEF1Bβ protein partners involved in human lung cancer. "Mapofthecell" database (http://www.mapofthecell.org) (Map 5) was used. EEF1D is a gene name for eEF1Bβ protein.
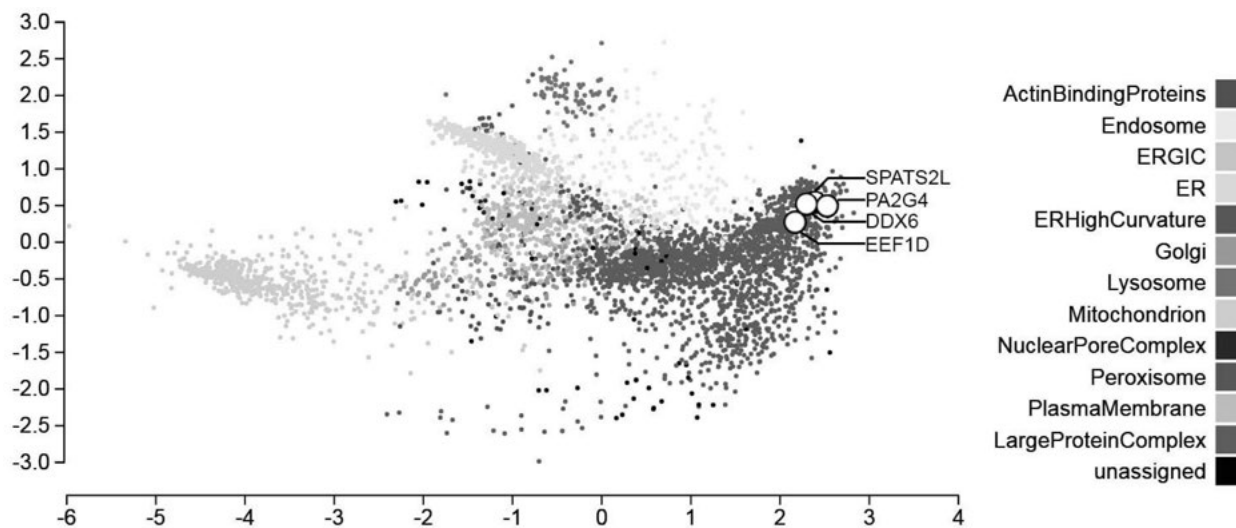
Fig. 2. Consortium of the eEF1Bβ protein partners which are not reported to be involved in human lung cancer. "Mapofthecell" database (http://www.mapofthecell.org) (Map 6) was used. EEF1D is a gene name for eEF1Bβ protein.

them, form a complex in the nucleus of lung cancer cells which interacts with a nucleus-localized portion of the translation factor eEF1Bβ population. Importantly, the complex of ILF2 and ILF 3 was already described [13].

Moreover, recently a complex of ILF2 and HNRNPU with long intergenic noncoding RNA was detected and shown to be important for mouse embryogenesis [71]. To our knowledge, an ability of other eEF1Bβ partners to
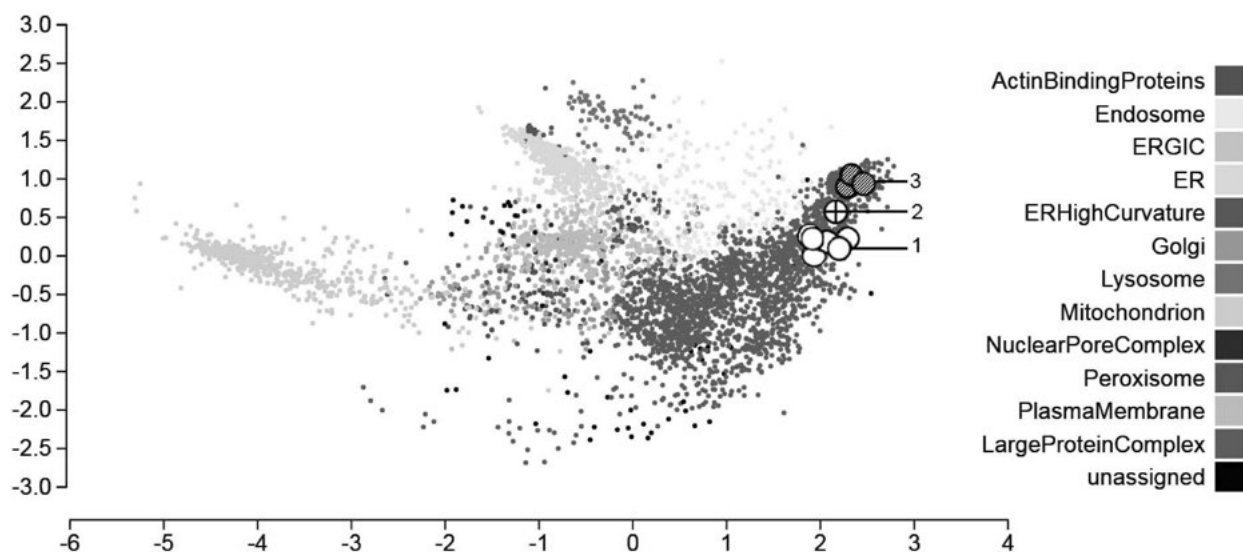
Fig. 3. Groups of pro-oncogenic (1) and neutral/tumor-suppressing (3) proteins can be spatially separated and linked by eEF1Bβ (2). Map 1 of "Mapofthecell" database was used.

interact with each other is not yet deciphered. Importantly, all these proteins show mainly nuclear localization (Table 1). On the contrary, the group of the eEF1Bβ partners involving tumor-suppressor protein PA2G4 demonstrates both nuclear and cytoplasmic localization, which is valid also for eEF1Bβ. One may suggest that cyto-nucleo shuttling of eEF1Bβ might provide as well a re-distribution of its partners between these cellular compartments. Leucine-zipper motif present in the eEF1Bβ molecule [72] can be responsible for these multiple and reversible interactions.

One may suggest that a co-compartmentalization of lung cancer-related eEF1Bβ partners in nucleus may facilitate cumulative oncogenic action of these proteins. This predicts novel, unforeseen before, level of complexity of regulation of cancer-related processes in human cell. The position of eEF1Bβ as an intermediate link between oncogenic and tumor-suppressor moieties of its protein partners (Fig. 3) can make it an important regulatory element of a switch between normal and cancer realities of a cell. This raises a question of identification of the mechanism of the eEF1Bβ release from the eEF1B complex and deciphering the role of eEF1Bβ shuttling between pro-cancer and anti-cancer communities of its protein partners.

Conclusions

104 proteins were identified as interacting partners of eEF1Bβ in the nuclear fraction of human lung carcinoma cells. From this amount, 9 protein partners were found to be co-fractionated with eEF1Bβ during precise cell fractionation procedures. Functional analysis of the partners has divided them on the pro-on-

cogenic and neutral/anti-oncogenic moieties. ILF2, ILF3, HNRNPU, CELF1, USP39 are pro-oncogenic and, moreover, strongly coupled to lung cancer. On the contrary, the cancer relation of DDX6 and SPATS2L is not obvious while PA2G4p42 shows potent tumor-suppressing properties. We believe that the position of eEF1Bβ as an intermediate link between the oncogenic and tumor-suppressor moieties of its protein partners can make it an important regulatory element and possible target of anti-cancer therapy.

Acknowledgments

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Білки-партнери субодиниці eEF1Bβ комплексу елонгації трансляції eEF1B в ядерній фракції клітин карциноми легені людини

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Мета. Виявити нових білків-партнерів фактора трансляції eEF1Bβ в ядрі клітин карциноми легені. **Методи.** Білки-партнери eEF1Bβ, отримані з ядерного екстракту клітин A549 методом ко-імунопреципітації (co-IP), були ідентифіковані за допомогою високоефективної рідинної хроматографії з тандемною мас-спектрометрією (LC-MS/MS). Подальше підтвердження білків-партнерів проводили із використанням опублікованих даних глобального, кількісного і динамічного картування субклітинної локалізації білків за допомогою програми Mapofthecell. **Результати.** 104 білки, які взаємодіють із eEF1Bβ в ядерній фракції клітин карциноми легені A549 були ідентифіковані мас-спектрометрією. Проміж цих білків, 9 партнерів eEF1Bβ були підтверджені даними із прецизійного субклітинного фракціонування. За допомогою функціонального аналізу ці білки-партнери можуть бути поділені на про-онкогенну і нейтральну/анти-онкогенну групи. Передбачено, що ці групи можуть бути просторово розділені в ракових клітинах людини. **Висновки.** Запропоновано, що eEF1Bβ може бути проміжною ланкою між онкогенною і нейтральною/пухлиносупресорною групами партнерів цього білка в ядрі ракових клітин. Розшифрування можливої ролі залучення eEF1Bβ до про-ракової або анти-ракової спільнот білкових партнерів цього білку може бути предметом подальших досліджень.

К л ю ч о в і с л о в а: eEF1Bβ, білок-білкові взаємодії, клітини A549, ILF2, ILF3, HNRNPU, CELF1, DDX6, CCNT1, PA2G4, SPATS2L, USP39.

Белки-партнеры субъединицы eEF1Bβ комплекса елонгации трансляции eEF1B в ядерной фракции клеток карциномы легкого

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Цель. Выявление новых белков-партнеров фактора трансляции eEF1Bβ в ядре клеток карциномы легкого. **Методы.** Белки-партнёры eEF1Bβ, полученные из ядерного экстракта клеток A549 методом ко-иммунопреципитации (co-IP), были идентифицированы с помощью высокоэффективной жидкостной хроматографии с тандемной масс-спектрометрией (LC-MS/MS). Дальнейшее подтверждение белков-партеров проводили с использованием опубликованных ранее данных глобального, количественного ы динамичного картирования субклеточной локализации белков с помощью программы «Mapofthecell». **Результаты.** Методом масс-спектрометрии было идентифицировано 104 белка, взаимодействующих с eEF1Bβ в ядре клеток A549. Из этих белков, 9 партнеров eEF1Bβ были под-твержден данными, полученными при подробном субклеточном фракционировании. С помощью функ-ционального анализа эти белки-партнеры можно раз-делить на про-онкогенную i нейтральную/анти-онко-генную группы. Предсказано, что эти группы могут быть разделены в пространстве в раковых клетках человека. **Выводы.** Выдвинуто предположение, что eEF1Bβ может быть промежуточным звеном между онкогенной и нейтральной/опухоле-супрессорной группами его партнеров в ядре раковых клеток. Расшифровка возможной роли вовлечения eEF1Bβ в про-раковую или анти-раковую группы его белковых партнеров может быть предметом дальнейших исследований.

Ключевые слова: eEF1Bβ, белок-белковые вза-имодействия, клетки A549, ILF2, ILF3, HNRNPU, CELF1, DDX6, CCNT1, PA2G4, SPATS2L, USP39.