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Mass-spectrometric and bioinformatic analysis of eEF1Bγ interactome in the cytoplasmic fraction of A549 cells

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Aim. To study protein networks containing the translation elongation factor eEF1B gamma (eEF1Bγ) in lung carcinoma cells. **Methods.** The protein partners of eEF1Bγ in the cytoplasmic fraction of human lung adenocarcinoma A549 cells were identified by co-immunoprecipitation (co-IP) followed by subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protein interaction network for eEF1Bγ was determined by a Cytoscape 3.2.0 program using a MCODE plugin. **Results.** 222 high-scored proteins interacting with eEF1Bγ in the cytoplasm of A549 cells have been identified. Possible functional networks involving these protein-protein interactions were predicted using bioinformatic approaches. **Conclusions.** Five protein networks were identified as possible targets of eEF1Bγ in lung cancer cells. Apart from translation, eEF1Bγ was shown to be potentially involved in cell cycle regulation, nucleosome remodeling, transcription, mRNA splicing and processing, and oxidative stress response.

Keywords: eEF1Bγ, protein-protein interactions, A549 cells

Introduction

eEF1Bg is a non-catalytic subunit of the eEF1B complex responsible for GDP/GTP exchange in translation elongation factor eEF1A. Apart from eEF1Bg, eEF1B contains two catalytic subunits, eEF1Bα and eEF1Bβ. Altogether eEF1A and the eEF1B complex provide efficient and accurate translation of mRNA on ribosomes in cytoplasm of eukaryotic cells. However, the data on the existence of a free pool of the eEF1B subunits in human cancer tissues [1.2] suggested their non-translational functioning as well. The nature of these non-canonical functions remains mostly unidentified.

Recently, the information on cellular processes that can engage the oncogenic eEF1Bβ subunit in cancer cells has been obtained [3, 4]. While a direct information about an oncogenic role of the eEF1Bg subunit is absent, there are several reports on its overexpression in cancer cells [5–8]. This suggests a possibility of cancer-related functioning of this pro-

tein. Here, we used the combination of co-immunoprecipitation, mass-spectrometry and bioinformatics to investigate a cytoplasmic interactome of the eEF1Bg protein in human cancer cells. In particular, we aimed to shed light on non-translational cancer-related processes which can involve eEF1Bg in lung cancer.

The obtained results show that eEF1Bγ may be involved in cell cycle regulation, nucleosome remodeling, mRNA splicing and processing, viral mRNA transcription and oxidative stress response in lung cancer cells.

Materials and Methods

Preparation of cytoplasmic fraction of human lung cells

Human lung cancer cells A-549 were harvested with Trypsin-EDTA. Cytoplasmic fraction was isolated as in [3]. Briefly, the cells were lysed with 1.5 volume of buffer containing 10 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 0.5 % NP-40, 0.2 mM PMSF, 0.5 mM DTT and kept on ice for 20 min. Then, the cells were centrifuged 10 min at 400 g. The supernatant was subjected to centrifugation at 16000 g for 30 min. The obtained supernatant was used as cytoplasmic extract. The absence of nuclear fraction admixture was verified by Western blotting with anti-Poly(ADP-ribose) polymerase and anti-Histone 3.3 antibodies.

Co-immunoprecipitation

The cytoplasmic extract was pre-cleared with Protein G Sepharose (Sigma, USA) for 1 h at 4 °C. Anti-eEF1Bγ antibodies (Abnova, Taiwan) (1.5 μg of antibodies per 1 mg of total protein) were added to pre-cleared lysates and incubated overnight at 4 °C. After addition of Protein G Sepharose slurry the incubation persisted for 2 h at 4 °C with continuous shaking. Then the resin was washed with ten resin volumes of the lysis buffer and treated further in accordance with the manufacturer's protocol. After elution of eEF1Bγ-associated proteins, the 12 % PAGE was performed [9]. The protein bands of interest were cut and processed for mass-spectrometry analysis (LC-MS/MS).

Mass-spectrometry LC-MS/MS

The cytoplasmic extract incubated with plain G-Sepharose was used as a control of nonspecific binding. The electrophoretic bands that were not present in the control or were much more extensive than in the control were cut and processed for mass spectrometry analysis at the Mass Spectrometry Laboratory of the Institute of Biochemistry and Biophysics (Warsaw, Poland) using LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) equipped with nanoAcquity (Waters Corporation) LC system, with ions score or expect cut-off, 30 and significance threshold, $p < 0.05$, as described before [3, 4].

Bioinformatics analysis

Cytoscape 3.2.0 Program [10] interaction database BIOGRID was supplemented with newly identified protein partners of eEF1Bγ and analyzed by MCODE plugin which finds highly interconnected regions (clusters) in any network loaded into Cytoscape. These clusters have been shown to represent protein complexes and/or parts of pathways [11]. For the sake of clarity, such known protein partners of

eEF1Bγ as eEF1A1, eEF1A2 and UBC (polyubiquitin-C) were excluded from the database as they interact with a huge number of cell proteins and create a very complicated network of protein-protein interactions that is not associated with eEF1Bγ directly [3]. Also, we simplified the task by taking for analysis only the first (direct) partners of eEF1Bγ partners determined by the Program algorithm. MCODE analysis was performed on the hybrid supercomputer "SCIT-4" at the Glushkov Institute of Cybernetics (GIC) of National Academy of Sciences of Ukraine (http://icybcluster.org.ua).

Results and Discussion

Literature data indicate that eEF1Bg may have connections with different types of cytoskeleton [12, 13] and interact with viral components [14, 15], it may be involved in transcription process [16–18] and oxidative stress response [19].

Our study was aimed to examine whether these and other cellular processes implicate eEF1Bγ in lung cancer cells. We identified 222 proteins as the interacting partners of eEF1Bγ in the cytoplasm of human lung adenocarcinoma A549 cells by using co-immunoprecipitation and subsequent LC-MS/MS identification of the interacting proteins. These protein partners were used for further analysis by the Cytoscape program to predict functional clusters, which may involve these proteins.

First, human BioGRID database was employed to generate by the MCODE the protein interaction network of eEF1Bγ based on existing (published) data. The resulting network contained 11 proteins (including eEF1Bγ). Three of them showed direct interactions with eEF1Bγ (Fig. 1). Those are eEF1Bβ (Gene ID 1936), FLNC (filamin C, gamma, Gene ID 2318) and NUDT21 (Cleavage and polyadenylation specificity factor subunit 5, Gene

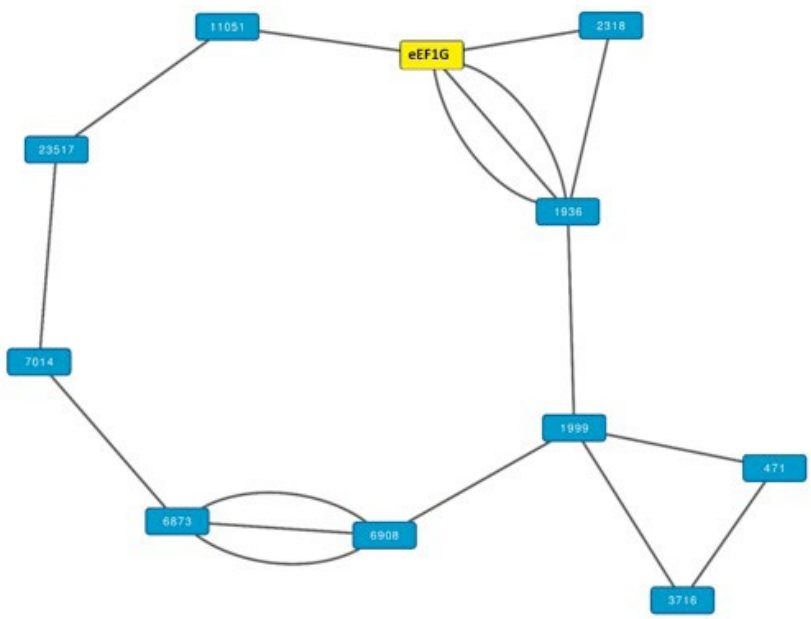

Fig. 1. Protein cluster containing eEF1Bγ, generated by MCODE in the Cytoscape 3.2.0 Program from Human BioGRID database solely on the basis of literature data.

ID 11051). The other members of this protein network are: SKIV2L2 (Ski2 Like RNA Helicase 2, Gene ID 23517), TERF1 (Telomeric repeat-binding factor 1, Gene ID 7014), TAF2 (TATA-box binding protein associated factor 2, Gene ID 6873), TBP (TATA-box binding protein, Gene ID 6908), ELF3 (ETS-related transcription factor Elf-3, Gene ID 1999), JAK1 (Janus kinase 1, Gene ID 3716) and ATIC (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, Gene ID 471). The majority of these proteins participate in synthesis and degradation of mRNA and its regulation (20–24).

Second, a protein network was generated by MCODE after complementing the BioGrid database with newly identified 222 partners of eEF1Bγ. Resulting protein cluster contained 55 proteins (including eEF1Bγ) (Fig. 2). Several functional protein sub-clusters were identified among the main cluster. *Sub-cluster A* included the proteins associated with mRNA splicing and processing. *Sub-cluster B* contained the proteins participating in nucleosome remodeling via changes of post-translational modifications of histones and DNA binding. The proteins from *sub-cluster C* were involved in cell cycle events. *Sub-cluster D* mostly comprised the members of translation apparatus, and *sub-cluster E* contained the transcription factors associated with oxidative stress response. The detailed analysis of the

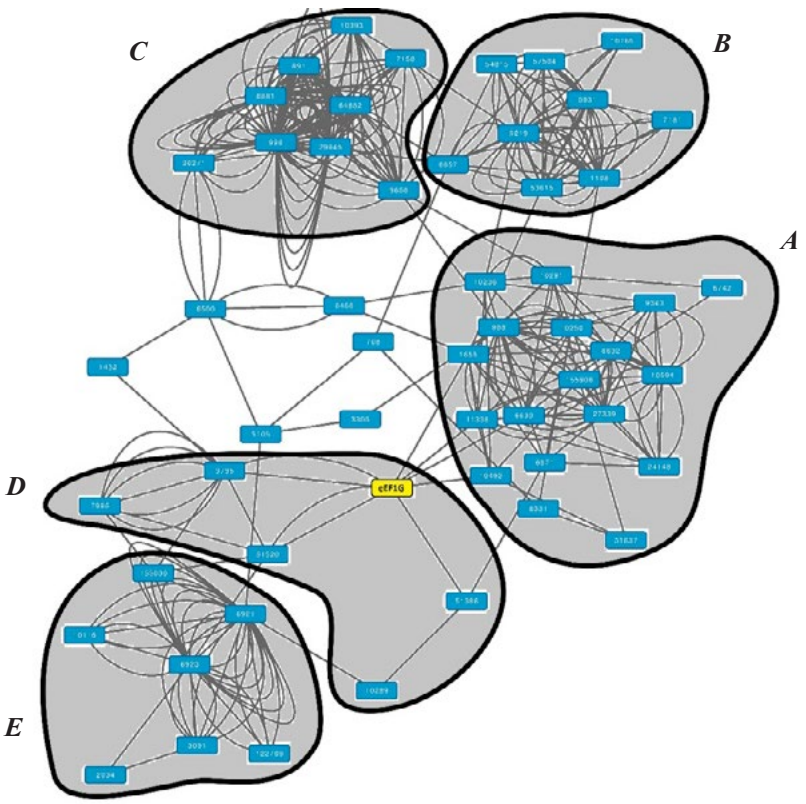

Fig. 2. Protein cluster containing eEF1Bγ, generated by MCODE in the Cytoscape 3.2.0 Program from Human BioGRID database supplemented with co-IP/MS-MS experimental data. Sub-clusters: *A* — the proteins associated with mRNA splicing and processing, *B* — nucleosome remodeling which proceeds via changes in the post-translational modifications of histones, *C* — the proteins involved in cell cycle events, *D* — translation and viral RNA replication, *E* – oxidative stress response.

protein components of the sub-clusters is shown below.

Sub-cluster A contained 3 experimentally identified in this paper protein partners of eEF1Bγ — DDX5 (Probable ATP-dependent RNA helicase DDX5, Gene ID 1655), SYNCRIP (Heterogeneous nuclear ribonucleoprotein Q, Gene ID 10492) and U2AF2 (Splicing factor U2AF 65 kDa subunit, Gene ID 11338) . The proteins CDC5L (Cell division cycle 5-like, Gene ID 988), SRRM1 (Serine/arginine repetitive matrix 1, Gene ID 10250), SNRPD1 (Small nuclear ribonucleoproteinD1polypeptide 16 RDa, Gene ID 6632), PRPF19 (Pre-mRNA processing factor 19, Gene ID 27339), rev P19 (Protein Rev p19, Gene ID 155908) and SNPD2 (Small nuclear ribonucleoprotein D2 polypeptide 16.5 kDa, Gene ID 6633) formed the central core of *sub-cluster A*. The outer layer of this group contained the proteins HNRNPR (Heterogeneous nuclear ribonucleoprotein R, Gene ID 10236), SF3A (Splicing factor 3A, subunit 1, Gene ID 10291), EFTUD2 (Elongation factor Tu GTP binding domain containing 2, Gene ID 9343), SSBP1 (Single stranded DNA binding protein 1, Gene ID 6742), PRPF8 (Pre-mRNA processing factor 8, Gene ID 10594), PRPF6 (Pre-mRNA processing factor 6, Gene ID 24148), YBX3 (Y box binding protein 3, Gene ID 8531), TADA2A (Transcriptional adaptor 2A, Gene ID 6871), SYNCRIP (Heterogeneous nuclear ribonucleoprotein Q, Gene ID 10492), U2AF2 (U2 small nuclear RNA auxiliary factor 2, Gene ID 11338), DDX5 (Probable ATP-dependent RNA helicase DDX5, Gene ID 1655), and C14orf166 (Chromosome 14 Open Reading Frame 166, Gene ID 51637).

Association of eEF1Bγ with *sub-cluster A* suggests its participation in the mRNA splicing and processing events. Peculiarly, the splicing complexes are normally localized in the nucleus, whereas the interaction of eEF1Bγ with the members of *sub-cluster A* is observed in the cytoplasmic fraction of the cells. One of the possible explanation is that eEF1Bγ participates in cyto-nuclear transport of the components of the sub-cluster A. As indicated in the Materials and Methods section, the absence of admixture of the nuclear proteins in the cytoplasmic fraction was routinely controlled by Western blotting with anti-Poly(ADP-ribose) polymerase and anti-Histone 3.3 antibodies. This significantly diminishes the possibility of artefact presence of the components of the splicing machinery in cytoplasm.

According to bioinformatics analysis, *sub-cluster B* is linked to *sub-cluster A* via several proteins: CHD4 (Chromodomain helicase DNA binding protein 4, Gene ID 1108), MTA2 (Metastasis associated 1 family, member 2, Gene ID 9219) and MBD3 (Methyl-ChG binding domain protein 3, Gene ID 53615). There are six more proteins in this *sub-cluster*: NR2C1 (Nuclear receptor subfamily 2, group C, member 1, Gene ID 7181), RBBP7 (Retinoblastoma Binding Protein 7, Gene ID 5931), KDM5B (Lysine-specific demethylase 5B, Gene ID 10765), MTA3 (metastasis associated 1 family, member 3, Gene ID 57504), GATAD2A (GATA zinc finger domain containing 2A, Gene ID 54815), and SOX2 (SRY (Sex determining region Y)-box 2, Gene ID 6657). The main function of *sub-cluster B* is suggested to be a nucleosome remodeling which proceeds via changes in the post-translational modifications of histones.

Thus, eEF1Bγ can be possibly involved in controlling the pre-translational gene expression events.

The proteins SOX2 and MTA3 functionally link *sub-clusters B and C*. The core of *sub-cluster C* contains five proteins: CCNB1 (cyclin B1, Gene ID 891), CDC16 (Cell Division Cycle 16, Gene ID 8881), ANAPC1 (Anaphase Promoting Complex Subunit 1, Gene ID 64682), ANAPC4 (Anaphase Promoting Complex Subunit 4, Gene ID 29945), and CDC27 (Cell Division Cycle 27, Gene ID 996). All these proteins, except cyclin B1, are the components of the Anaphase Promoting Complex (APC), which maintains the metaphase–anaphase transition due to the degradation of cyclin B1. Cyclin B1 is associated with G2/M transition of mitotic cell cycle. Other two proteins of *sub-cluster C* are TP53BP1 (Tumor Protein P53 Binding Protein 1, Gene ID 7158) and MDC1 (Mediator of DNA-Damage Checkpoint 1, Gene ID 9656). The last one is connected with *sub-cluster A* by two links. Importantly, possible association of eEF1Bγ with the cell cycle regulation components has been reported [25].

The protein FBXO5 (F- box protein 5, Gene ID 26271), which can inhibit APC complex due to its ubiquitin ligase activity, connects *sub-cluster C* with *sub-clusters A* and *D* through SKP1 (S-phase kinase-associated protein 1, Gene ID 6500). The SKP1 protein takes part in ubiquitination of FBXW4 (F-Box and WD Repeat Domain Containing 4, Gene ID 6468), which in turn interacts with two proteins of *sub-cluster A*, one of them (DDX5) was experimentally identified as a partner of eEF1Bγ in this paper.

The protein MAPK14 (mitogen-activated protein kinase 14, Gene ID1432) links *sub-clusters C* and *D*. This kinase belongs to the family of MAP-kinases, which integrate ubiquitous cellular signals and participate in regulation of transcription, differentiation, proliferation and cell development. MAPK14 is implicated in the control of the genotoxic stress response and in the stress-induced transcription and cell cycle regulation [26]. According to the scheme, in *sub-cluster D* this protein interacts with SKP1 (S-Phase Kinase-Associated Protein1, Gene ID 6500), which participates in regulation of ubiquitination, and KARS (lysyl-tRNA synthetase, Gene ID 3735). KARS is associated with protein biosynthesis and also interacts with gag Pr55 (Gag polyprotein (Human immunodeficiency virus 1), Gene ID 155030). KARS interaction with gag Pr55 assists effective packaging of $tRNA_3^{Lys}$, which works as a primer for initiation of the reverse transcription, to viral particles [27]. The protein AIMP2 (Aminoacyl tRNA Synthetase Complex-Interacting Multifunctional Protein 2, Gene ID 7965) from *sub-cluster D* interacts with KARS and LARS (Leucyl-TRNA Synthetase, Gene ID 51520) in the macromolecular aminoacyl-tRNA synthetase complex. Interestingly, another component of HIV-1 virus, the protein rev p19, is also found in the interactome of eEF1Bγ (*sub-cluster A*). Thus, on the non-translational side, the data on *sub-clusters D* and *A* provide independent support to the notion that eEF1Bγ can take part in functioning of HIV-1 virus [14]. In particular, it may play a role of structural platform for spatial immobilization of some viral and host-cell proteins leading to more effective viral replication.

The protein PCK1 (Phosphoenolpyruvate Carboxykinase 1, Gene ID 5105) connects *sub-clusters A, B, C,* and *E*. This protein is at the main checkpoint in control of gluconeogenesis. PCK1 is linked to *sub-cluster A* via the protein HSPA1L (Heat Shock 70kDa Protein 1-Like, Gene ID 5105). PCK1 is connected to *sub-claster B* through the protein C1QBP (Complement Component 1, Q Subcomponent Binding Protein, Gene ID 708), a multifunctional protein, associated with inflammation and infection processes, apoptosis, transcription and pre-mRNA splicing regulation, and ribosome biogenesis [28]. C1QBP interacts with the protein SOX2 from *sub-cluster B* that links *sub-clusters C* and *B*. It also interacts with the protein YBX3 (Y Box Binding Protein 3, Gene ID 8531) from *sub-cluster A,* which can act as a transcription factor specifically binding certain DNA sequences, in particular ds-DNA. PCK1 is linked to *sub-cluster E* through interaction with the proteins ELOC (Elongin-C, Gene ID 6921) and ELOB (Elongin-B, Gene ID 6923). ELOC and ELOB are the regulatory subunits of the transcription factor B (SIII) complex, which activates RNA-polymerase II–mediated transcription elongation. Interestingly, eEF1Bγ was reported to interact with RNA-polymerase II [16].

Sub-cluster E also includes LRR1 (Leucine-rich repeat protein 1, Gene ID 122769), a negative regulator of 4-1BB-mediated signaling, which leads to the NK-kappaB and JNK1 activation [29]. An independent study showed that eEF1Bγ is a positive regulator of NF-κappaB signaling pathway, with unknown mechanism of action [17]. Another component of this *sub-cluster E* is HIF1A (Hypoxia Inducible Factor 1, Alpha Subunit (Basic Helix-Loop-Helix Transcription Factor, Gene ID 3091), a subunit of transcription factors complex HIF-1, which is a main regulator of anti-hypoxic homeostatic cell response. This factor is responsible for the transcription activation of many genes involved in energetic metabolism, angiogenesis, apoptosis as well as of the genes that product an enhanced oxygen transition or raise anti-hypoxic metabolic adaptation level [30]. The protein EPAS1 (Endothelial PAS Domain Protein 1, Gene ID 2034) is a link between HIF1A and ELOB. This protein is a transcription factor associated with induction of the genes regulated by oxygen. The protein FEM1B (Fem-1 Homolog B, Gene ID 10116) belongs to a family of death receptor-associated proteins essential for apoptosis. Thus, *sub-cluster E* mainly contains the transcription factors participating in the induction of the oxidative stress response, which can play adaptive or apoptotic role.

Several papers suggested possible participation of eEF1Bγ in oxidative stress response [19, 31, 32], however the details of such connection were not presented. Our data predict existence of the direct links between eEF1Bγ and transcription factors involved in induction of this response. This connection can be used as a new target for drugs with a capacity of jugulating oxidative stress response in human cells.

Translation initiation factor eIF1B (*sub-cluster D)* interacts with transcription factor ELOC (*sub-cluster E)* and with translation initiation factor eIF3L (Eukaryotic Translation Initiation Factor 3, Subunit L, Gene ID 51386). Factor eIF3L interacts with eEF1Bγ closing

the loop of interactions in *sub-cluster D*. On the other hand, eIF3L interacts with TADA2A (*sub-cluster A)* which is a member of the histone-acetylase complex PCAF. It is one more link that connects eEF1Bγ with *sub-cluster A*, the function of which is associated with the nucleosome structure modification.

Several identified protein partners of eEF1Bγ are associated with human diseases. The proteins CCNB1 and FBXO5 from *sub-cluster C* are associated with tetraploidy [33]. The proteins CCNB1, MDC1 and MTA3 (*sub-cluster C*) are linked to breast cancer [34–36]. The protein PRPF8 (*sub-cluster A)* is related to myeloid neoplasms [37]. The proteins CDC5L and PRPF19 from *sub-cluster A* participate in the development of poikiloderma with neutropenia [38]. The proteins CDC27 (*sub-cluster C*) and SSBP1 (*sub-cluster A*) are associated with herpes infection [39,40]. The proteins MAPK14 (*sub-cluster D*) and HIF1A (*sub-cluster E*) are involved in vascular disease [41, 42]. DDX5 (*sub-cluster A*) participates in the necrosis development [43].

Surprisingly, seven proteins from *sub-clusters A, B, C* and protein PCK1, which connects these sub-clusters, are involved in corneal diseases, particularly retinoblastoma. It opens an interesting possibility of the eEF1Bγ involvement in the development of human corneal diseases.

As eEF1Bγ has no evident catalytic activity, we suggest that the potential molecular mechanisms of its participation in non-translational events could involve its ability to form multimeric structures. In such way, eEF1Bγ can serve as a scaffold for various protein partners participating in a variety of cellular processes.

Conclusions

222 proteins were identified by co-immunoprecipitation, with subsequent LC-MS-MS, as interacting with eEF1Bγ in the cytoplasm of human lung adenocarcinoma A549 cells. The eEF1Bγ protein partners were used to construct possible functional networks by the Cytoscape 3.2.0 program. We identified five protein networks (sub-clusters), which can involve eEF1Bγ in human cancer cells. They are linked to mRNA splicing and processing; nucleosome remodeling; cell cycle regulation; viral RNA transcription; oxidative stress response. Thus, our data support and detail the previously reported cases of eEF1Bγ linkage to the processes of cell cycle, transcription of viral RNAs and oxidative stress response. Moreover, they indicate, for the first time, a possible participation of eEF1Bγ in the mRNA maturation and nucleosome remodeling in human cancer cells.

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Мас-спектрометричний та біоінформаційний аналіз інтерактома eEF1Bγ в цитоплазматичній фракції клітин A549

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Мета. Виявити білкові мережі, до яких може входити фактор елонгації трансляції eEF1Bγ в клітинах карциноми легені. **Методи.** Білки-партнери eEF1Bγ у цитоплазматичній фракції клітин аденокарциноми легені людини A549 були ідентифіковані за допомогою коімунопреципітації із наступною рідинною хроматографією та тандемною мас-спектрометрією (LC-MS/MS). Білкові мережі, до яких входить eEF1Bγ, визначали за допомогою програми Cytoscape 3.2.0 із плагіном MCODE. **Результати.** Ідентифіковано 222 білки-партнери eEF1Bγ в цитоплазматичній фракції клітин A549. Функціональні мережі, які можуть формуватися цими білками, були визначені біоінформатично. **Висновки.** На основі експериментальних даних виявлено п'ять білкових мереж, у яких може брати участь eEF1Bγ в клітинах аденокарциноми легені людини. Показано, що крім трансляційних компонентів, ці мережі формуються білками, задіяними у регуляції клітинного циклу, ремоделюванні нуклеосом, транскрипції, сплайсингу і процесінгу мРНК та клітинної відповіді на оксидативний стрес.

Ключові слова: eEF1Bγ, білок-білкові взаємодії, клітини A549.

Мас-спектрометрический и биоинформационный анализ интерактома eEF1Bγ в цитоплазматической фракции клеток A549

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Цель. Выявить белковые сети, членом которых может быть фактор элонгации трансляции eEF1Bγ в клетках карциномы легкого. **Методы.** С помощью ко-иммунопреципитации с последующей жидкостной хроматографией и тандемной масс-спектрометрией были идентифицированы белки-партнеры eEF1Bγ в цитоплазматической фракции клеток аденокарциномы легкого людини A549. Белковые сети, в состав которых входит eEF1Bγ, определяли с помощью программы Cytoscape 3.2.0 с плагином MCODE. **Результаты.** Идентифицированы 222 белка-партнера eEF1Bγ в цитоплазматической фракции клеток A549. Функциональные сети, которые могут формироваться этими белками, были определены биоинформатически. **Выводы.** На основании экспериментальных данных найдено пять белковых сетей, в которых может участвовать eEF1Bγ в клетках аденокарциномы легкого человека. Показано, что кроме трансляционных компонентов, эти сети формируются белками, задействованными в регуляции клеточного цикла, ремоделировании нуклеосом, транскрипции, сплайсинга и процессинга мРНК и клеточного ответа на оксидативный стресс.

Ключевые слова: eEF1Bγ, белок-белковые взаємодействия, клетки A549.

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