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ITSN1 BINDS THE E2-CONJUGATING ENZYME UBC9

Aim. Scaffolding protein of the intersectin 1 (ITSN1) associated with malignant cell transformation. A short isoform of ITSN1 (ITSN1-S) can localize to the nucleus and inhibit breast cancer cell proliferation but the exact mechanisms of ITSN1 nuclear export have not been fully elucidated. SUMOylation of ITSN1, or its interaction with components of SUMO modification, may be one of the regulatory mechanisms contributing to the nuclear-cytoplasmic shuffle of ITSN1 in the cell. ***Methods***. Full-length human *UBC9* sequence was subcloned in *pGEX4T2* vector for *in vitro* GST-binding assays with overexpressed Omni-ITSN1-S in 293 cell line. Lysates of 293 cells with overexpressed FLAG-UBC9 were used for co-immunoprecipitation with endogenous proteins of ITSN1 and ITSN2. ***Results***. Endogenous ITSN1-S form complexes with full-length overexpressed UBC9 in 293 *in vivo*. Further analysis revealed that GST-UBC9 binds human full-length short isoform ITSN1-S *in vitro*. ***Conclusions*.** E2-conjugating enzyme of the SUMOylation, UBC9, is confirmed as a novel protein partner for ITSN1 both *in vitro* and *in vivo*. Considering the tumor suppressor role of a nuclear ITSN1-S in breast cancer and the unique role UBC9 plays in SUMO-modification of proteins, we suggest a possibility of UBC9 and ITSN1 interaction association with malignant transformation, which can be the ground for the further studies.

Keywords: ITSN1 (intersectin 1), UBC9, SUMOylation, protein-protein interactions.

Intersectin 1 (ITSN1) is a multi-domain scaffold protein involved in the regulation of endocytosis, vesicle transport, and activation of various signal transduction pathways. Altered *ITSN1* expression is associated with the proliferation and invasion of glioblastoma, neuroblastoma, and lung cancer cells, as well as with the development of Down syndrome, Alzheimer`s, and Huntington`s diseases. ITSN1 is located on human chromosome 21 and consist of short (ITSN1-S) and long (ITSN1-L) isoforms produced by alternate splicing events that incorporate a guanine nucleotide exchange factor (GEF) domain into the long form [1].

ITSN1-S shuttles between the cytoplasm and nucleus due to nuclear localization sequences (NLS) in the EH domain [2] and the SH3D domain [3]. Additionally, ITSN1-S translocates to the nucleus and interacts with lamin A/C. Through its EH regions, ITSN1-S interacts with another PI3K and PIK3C2A, in the cytoplasm to reduce PIK3C2A-AKT pathway activation and inhibit breast cancer cell migration and invasion [2]. Furthermore, nuclear ITSN1-S inhibits nascent DNA synthesis and replication in breast cancer cells [3]. These results provide additional evidence for the involvement of ITSN1-S as a tumor suppressor with nucleocytoplasmic functions in breast cancer.

The detailed mechanism of ITSN1-S translocations has not been adequately established up to date. One of the regulatory pathways controlling the nuclear-cytoplasmic transport of proteins in the cell that could potentially contribute to the ITSN1-S translocation is SUMOylation: the posttranslational modification, in which small ubiquitin-like modifiers get covalently linked to target proteins [4]. Preliminary bioinformatics data obtained in our lab indicates the presence of potential SUMOylation sites and SIM motifs in a full-length sequence of ITSN1, which could imply interactions with SUMO-proteins and SUMO-modification components alike. The transfer of activated small ubiquitin-like modifier proteins to their targets is mediated by UBC9, the sole E2-conjugating enzyme of the SUMOylation cycle [5]. UBC9-mediated SUMOylation has been implicated in many processes, such as DNA replication/repair, cell division, movement, nuclear transport, and transcription [6]. UBC9 also functions as a cellular chaperone and transcriptional coregulator, thus having a SUMOylation-independent function in protein stability and syn-

thesis regulation [7]. In addition, UBC9 has several properties related to cancer development and progression: a strong association between resistance to chemotherapy and a heightened level of UBC9 expression in breast cancer tumors have been reported [8], along with heightened expression in advanced melanomas, head and neck tumor and lung tumor [9].

Our findings reveal an interaction between UBC9 and ITSN1-S, which suggests ITSN1 is a potential target of SUMOylation. Additionally, considering the scaffolding role that ITSN1 plays in cellular signaling, this finding may contribute to further understanding of the SUMOylation process that involves ITSN1 protein partners and the stability of these complexes in the modification process.

Materials and methods

Expression constructs. The cDNA of FLAG-tagged UBC9 was kindly provided by Dr. Christopher D. Lima (New York, USA) [10]. The full coding sequence of human UBC9 was subcloned in *the pGEX4T2* vector (CE Healthcare). Expression construct encoding isoform Omni-tagged ITSN1-S (+VKGEW) was described previously [11].

Antibodies. Rabbit polyclonal antibodies against the EH2 domain of human ITSN1 (anti-ITSN1) and mouse polyclonal antibodies of ITSN2 (anti-ITSN2) were described previously [12, 13]. Monoclonal anti-FLAG clone M2 was purchased from Covance, Sigma. Secondary horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies were purchased from Promega.

Cell culture and transfection. The 293 cell line was obtained from the Bank of Cell Lines of the R. E. Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology, NASU (Ukraine). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 100 mg/ml streptomycin. According to the manufacturer's instructions, the cells were transiently transfected with the JetPEI (polyethyleneimine, Polyplus Transfection) and processed 24 hours after transfection.

Protein Expression, pull-down assay, and Western blot analysis. The recombinant GST-fused proteins were expressed in *Escherichia coli* BL21 (DE3) pLysE cell and affinity purified using glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer's instruction. GST-tagged proteins (5–20 mg) or GST were bound to beads and incubated for two hours at 4°C with lysates of human cell lines. Cell lysates were prepared in extraction buffer containing 20 mM Tris-HCl pH 7.5, 1 % Triton X-100, 150 mM NaCl, and a protease inhibitor cocktail (Roche). The beads were extensively washed with extraction buffer three times and boiled in Laemmli sample buffer (150 mM Tris-HCl pH 6.8, 2.5 % glycerol, 10 % SDS, 3 % beta-mercaptoethanol and 0.5 % bromophenol blue). The eluted proteins were resolved in SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and blocked for one hour in 5 % non-fat milk, 1xPBS (phosphate-buffered saline), and 0.1 % Triton X-100. Afterward, the membranes were incubated with the corresponding primary antibodies for one hour at room temperature and washed. Detection was performed by horseradish peroxidase-labeled secondary antibodies. Chemiluminescence was captured with Molecular Imager ChemiDocTM XRS+ (BioRad).

Immunoprecipitation. For immunoprecipitation (IP), the cells were lysed in IP buffer (20 mM Tris-HCl pH 7.5, 1 % Nonidet P40, 150 mM NaCl, 10 % glycerol, 2 mM EDTA and protease inhibitor cocktail (Roche). The lysates were mixed with antibodies and protein A/G PLUS-Agarose (Santa Cruz Biotechnology). After incubation for three hours at 4°C, the beads were washed three times with IP buffer. Bound proteins were eluted by boiling in Laemmli sample buffer and analyzed using SDS-PAGE and Western blotting.

Results and discussion

ITSN1 is a multidomain scaffold protein found in various cellular compartments, with notable presence in the cytoplasm, plasma membrane, and endocytic vesicles. Its distribution is intricately linked to its diverse functions in cellular processes such as endocytosis, vesicle trafficking, and signal transduction. In addition to its notable functions in stabilization of macromolecular complexes and cell signaling, ITSN1 has emerged as a significant player in cancer biology, particularly in the context of cancer invasion and metastasis. It has been shown to promote tumor development in malignant glioma, as well as in breast cancer cells, while being localized in nuclei in this particular case [2]. ITSN1 also plays a significant role in metastatic invasion, interacting with WIP and N-WASP proteins and localized in invadopodia [14].

To investigate whether ITSN and UBC9 could be involved in the same protein complexes, coimmunoprecipitation was performed. Two members of the intersectin family, ITSN1 and ITSN2,

were used in this study. To perform this analysis, cells of the 293 cell line with a short-term transfection of FLAG-UBC9 were lysed and used for co-immunoprecipitation using monoclonal antibodies against FLAG. The two members of the ITSN protein family, ITSN1 and ITSN2, share a significant structural similarity, however there is a particular difference in the expression of their longer isoforms. ITSN1-L expression is specific to the brain, whereas ITSN2-L expression is ubiquitous [1].

ITSN1-S was detected in immunoprecipitates obtained with anti-FLAG antibodies from lysate of 293 cells (Fig. 1A).

The interaction of the short and long ITSN2 isoforms was not detected in the precipitates with UBC9 (Fig. 1B), indicating the absence of association between these proteins. Thus, the obtained results revealed the possibility of *in vivo* complex formation between UBC9 and ITSN1 in 293 cells.

The obtained data were confirmed by precipitation *in vitro*. For this, a cDNA fragment encoding the UBC9 sequence was cloned into a vector for bacterial expression containing a GST tag. Recombinant GST-UBC9 protein was expressed in *E. coli* cells, then affinity purified using glutathione-sepharose, and used in an in vitro interaction study. Because ITSN1 has different isoforms that are formed by alternative splicing and characterized by tissue specificity, for the experiment, the ITSN1-S isoform with an additional five amino acid residues VKGEW in the SH3A domain was used, the sequence of which was cloned into a vector for eukaryotic expression from Omni-tag. 293 cells transiently expressing Omni-ITSN1-S(+VKGEW) were lysed and used for GST precipitation. The results showed that GST-fused UBC9 binds recombinant Omni-ITSN1-S(+VKGEW) (Fig. 2).

This finding demostrates the presence of a protein-protein complex between ITSN1 and UBC9 for the first time. Taking into account the unique role UBC9 plays in SUMOylation, this interaction can indicate the fact that ITSN1 undergoes SUMOylation by itself, considering that ability to bind UBC9 by the substrate is crucial to the process of covalent attachment of SUMO-proteins. The identification of ITSN1 as a SUMOylation target implies potential impacts on the stability and dynamics of the complexes it forms during this modification process. This finding also indicates interplay between ITSN1, its protein interactors, and the SUMOylation machinery. Unraveling the specifics of how ITSN1 and its partners undergo SUMOylation may provide valuable insights into the broader landscape of post-translational modifications, with implications for understanding cellular homeostasis and potential therapeutic interventions.

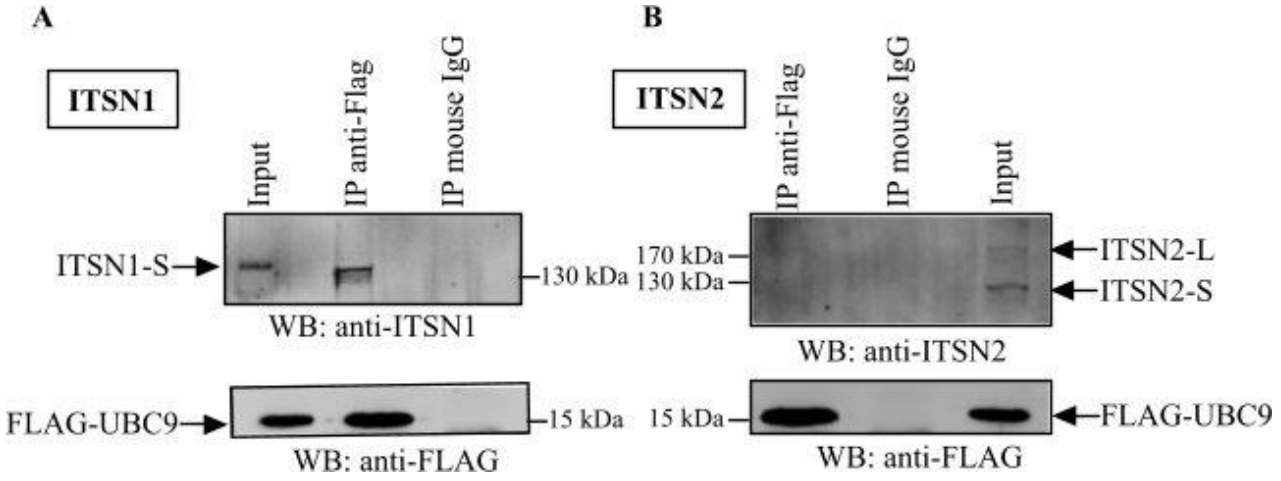

Fig. 1. Endogenous ITSN1-S forms a complex with UBC9. 293 cells were transfected with FLAG-UBC9. Cell extracts were subjected to immunoprecipitation with anti-FLAG antibodies. The precipitated proteins were analyzed by Western blotting with anti-ITSN1 (A) and anti-ITSN2 (B) antibodies. Mouse immunoglobulin G (IgG) was used as a control. IP, immunoprecipitation; WB, Western blotting.

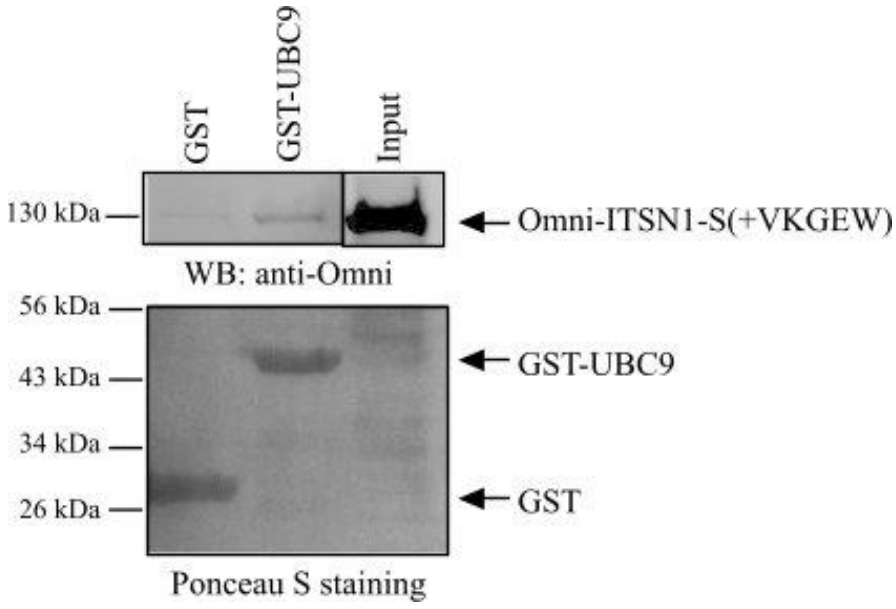

Fig. 2. UBC9 interacts with scaffold protein ITSN1 *in vitro*. The GST-fused UBC9 were expressed bacterially and purified via affinity purification. The GST-UBC9 or GST alone (control) immobilized on glutathione beads were incubated with lysate of 293 expressing Omni-ITSN1-S(+VKGEW). Bound proteins were separated by SDS-PAGE and detected by immunoblotting with anti-Omni antibodies. GST-UBC9 was visualized by Ponceau S staining. WB, Western blotting.

Conclusions

Our data identify Ubc9 as a new ITSN1 interaction partner and introduce the Ubc9-ITSN1 module as a new regulatory layer on the controls of cellular processes. This discovery prompts further exploration into the specific molecular mechanisms dependent on the Ubc9-ITSN1 interaction and its further impact on signaling pathways.

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ITSN1 ФОРМУЄ КОМПЛЕКС ІЗ E2-КОН'ЮГУЮЧИМ ФЕРМЕНТОМ UBC9

Мета. Цитоплазматичний скаффолдний протеїн інтерсектин 1 (ITSN1) асоційований зі злоякісною трансформацією клітин. Коротка ізоформа ITSN1 (ITSN1-S) здатна локалізуватися в ядрі та інгібувати проліферацію клітин раку молочної залози, проте механізми ядерного експорту ITSN1 остаточно не з'ясовані. Сумоїлування ITSN1 або його взаємодія з компонентами системи SUMO-модифікації може бути одним із регуляторних механізмів, що сприяє ядерно-цитоплазматичному транспорту ITSN1 у клітині. ***Методи***. Повнорозмірну послідовність UBC9 людини субклонували у вектор pGEX4T2 для GST-преципітації *in vitro* з надекспресованим Omni-ITSN1-S у клітинах лінії 293. Лізати 293 клітин із надекспресією FLAG-UBC9 використовували для коімунопреципітації з ендогенними протеїнами ITSN1 та ITSN2. ***Результати.*** Ендогенний ITSN1-S утворює комплекс *in vivo* з повнорозмірним надекспресованим UBC9 у клітинах лінії 293. Подальший аналіз показав, що рекомбінантний GST-UBC9 преципітує *in vitro* коротку ізоформу ITSN1-S людини. ***Висновки.*** E2-кон'югуючий фермент сумоїлування UBC9 є новим протеїновим партнером ITSN1 *in vitro* та *in vivo*. Враховуючи роль ядерного ITSN1-S в інгібуванні пухлинного росту раку молочної залози та критичну роль UBC9 у SUMO-модифікації білків з можливістю регуляції їх ядерного транспорту, ми прогнозуємо, що дана взаємодія може стати основою для подальших досліджень впливу сумоїлювання на функції ITSN1.

Ключові слова: ITSN1 (інтерсектин 1), UBC9, сумоїлування, білок-білкові взаємодії.