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RNA-binding protein SAM68 interacts with endocytic proteins and actin cytoskeleton modulators

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SAM68 is a nuclear RNA-binding protein involved in the regulation of mRNA processing. SAM68 overexpression is observed in multiple types of cancer. Recently, the possible link between RNA-binding protein SAM68 and scaffold protein ITSN1 has been identified. The aim of the study was to confirm the probability of direct binding between SAM68 and ITSN, analyze the effect of ITSN1 on SAM68-mediated alternative splicing, and identify novel SAM68 partners among endocytic proteins and actin cytoskeleton modulators. The interactions were revealed in pull-down assays using purified recombinant proteins or cell lysates. ITSN1 knockdown in HeLa cells was performed using the shRNA approach. The expression of isoforms produced by alternative splicing was measured using RT-PCR. It was demonstrated that SAM68 directly interacted with ITSN1 in vitro. Next, it was found that ITSN1 knockdown in HeLa cells induced SRSF1 intron 3 retention increasing the expression of the proto-oncogenic isoform of SRSF1 by three times. It was also shown that SH3 domains of AMPH1, BIN1, CTTN1, TKS4, and TKS5 precipitated SAM68 from lysate of 293 cells. As a result, SAM68 directly binds to ITSN1 and interacts with endocytic proteins and actin cytoskeleton modulators, whereas SAM68-mediated splicing in HeLa cells may be regulated by ITSN1.

Keywords: SAM68, ITSN1, SRSF1, alternative splicing.

Introduction. SAM68 (Src associated in mitosis of 68 kDa, *KHDRBS1*) is an RNA-binding protein involved in the regulation of transcription, alternative splicing, RNA maturation, and signalling processes in a cell [1]. SAM68 central KH domain mediates RNA binding, whereas proline-rich regions, tyrosine motifs, and RG repeats located in the non-structured N- and C-terminal regions provide the binding to protein partners. Multiple studies reveal the proto-oncogenic properties of SAM68, while its overexpression is observed in glioblastoma, breast, prostate, colorectal, cervical and other types of cancer, whereas SAM68 depletion inhibits cells proliferation [2].

SAM68 interacts with various splicing factors and spliceosome-associated proteins and binds A/U rich sequences in RNA. This protein is involved in the alternative splicing of various mR-

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NAs, promoting exon inclusion (*CD44*, *BCL-X*, and *AR-V*), exon exclusion (*SMN1/2*, *mTOR*), and intron retention (*SRSF1*, *CCND1*, and *BIRC5*) [1]. In most cases, SAM68 induces the formation of proto-oncogenic isoforms associated with proliferation and migration of cancer cells [2].

Although SAM68 is preferentially a nuclear protein, it also localizes in the cytoplasm and at the plasma membrane, where it modulates the activity of several receptor-mediated signalling pathways (insulin, leptin, Toll-like, TNF α , and EGF activating cascades) [3]. As a result, in addition to nuclear proteins, SAM68 interacts also with various SH3 domain-containing proteins comprising adapter proteins (GRB2, CRK, NCK1), SRC kinases (SRC, FYN, BRK), and other regulatory enzymes (methyltransferase PRMT1/2, PLC γ) [1, 4]. The interactions contribute to SAM68 posttranslational modification altering its RNA- and protein-binding properties, as well as its cellular localization [1].

Recently, using pull-down and immunoprecipitation assays, it was found that SAM68 could form a complex with protein ITSN1 [5]. ITSN1 is a scaffold protein involved in clathrin-mediated endocytosis and actin cytoskeleton rearrangements, as well as regulation of some cell signalling pathways [6]. In addition, ITSN1 was found to localize in a cell nucleus, although its nuclear function is still unknown [7]. ITSN1 overexpression stimulates oncogenic transformation of rodent fibroblasts, induces invadopodia formation, whereas ITSN1 overexpression inhibits proliferation, growth, and migration of lung cancer cells [6].

As SAM68-ITSN1 interplay could be involved in the regulation of the overlapping cellular signalling pathways, the current work aimed to confirm the direct interaction between the proteins *in vitro* and to analyze the possible effect of ITSN1 on SAM68-mediated alternative splicing events in HeLa cells. Finally, the putative interactions between SAM68 and other cytoplasmic SH3 domain-containing proteins involved in the regulation of clathrin-mediated endocytosis and actin cytoskeleton remodelling were analyzed.

Materials and Methods. *Expression constructs.* For the production of the constructs encoding SAM68 fused to His-tag, the coding sequence of SAM68 was inserted into the pET22b vector. The plasmid encoding GST-ITSN1_{SH3} was described previously [5]. Constructions encoding ITSN1-specific shRNAs were prepared according to the RNAi Consortium (TRC) protocol [8] using the pLKO vector. The following antisense sequences were used: shRNA1: 5'-TTT AAT TGG TCA TTG AGT ATC-3'; shRNA2: 5'-ATT ATT CAT GTC AGC TAG TGC-3'.

Antibodies. Mouse monoclonal anti-GST, anti-SAM68, and anti-GAPDH antibodies were purchased from Invitrogen, Sigma, and Santa-Cruz Biotechnology, respectively. Secondary HRP-labeled anti-mouse antibodies were obtained from Promega.

Cell culture. 293 and HeLa cells were obtained from the ATCC and maintained in DMEM with 10 % FBS, 100 mg/ml streptomycin and 50 mg/ml penicillin.

Pull-down assays. The recombinant GST- and His-fused proteins were produced in *Escherichia coli* BL21 cells and purified using glutathione-sepharose 4B (GE Healthcare) and Ni²⁺-NTA-agarose (Qiagen), respectively, according to the manufacturer protocols. The pull-down experiments and sample preparation were performed, as described previously [5].

Western blotting. Protein samples were resolved by PAGE-SDS and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5 % non-fat milk in TBS-T (0.1 % Tween 20) for 1 h and incubated with corresponding primary and secondary antibodies. Chemiluminescence was detected using ECL reagents with ChemiDoc XRS+ (Bio-Rad).

shRNA knockdown. Knock-down experiments in HeLa cells were performed as described previously in [8]. In brief, lentiviral particles containing shRNA-coding constructs were produced in 293 cells. HeLa cells were transduced with the viral supernatants and were incubated with 1 µg/ml puromycin for 72 h. Control HeLa cells were transduced with viral particles encoding the empty pLKO vector.

Real-time PCR. Total RNA was extracted from HeLa cells using TRI Reagent® (Molecular Research Center, Inc) according to the manufacturer's recommendations. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using ImProm-II™ Reverse Transcription System (Promega). Quantitative real-time PCR was performed via GoTaq® qPCR Master Mix (Promega) on CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). For the detection of two possible transcripts produced during the alternative splicing, isoform-specific primers were used. Expression levels of all studied isoforms were estimated according to $\Delta\Delta C_T$ approach described previously using GAPDH expression level for normalization [9].

Statistical analysis. Unpaired two-tailed *t*-test was used for the statistical comparison. GraphPad Prism 6 software was used for the calculation. All the experiments were performed in triplicates.

Results and discussion. To confirm the probability of the direct interaction between SAM68 and ITSN1, purified recombinant proteins SAM68-His and GST-ITSN1_{SH3} were used in pull-down analysis. His-tagged SAM68 was immobilized on Ni²⁺-NTA agarose and incubated with purified GST or GST-fused tandem of five SH3 domains of ITSN1. Pull-down results demonstrated that SAM68 precipitated ITSN1 SH3 domains and did not precipitate GST (Fig. 1). The observed data confirmed that ITSN1 directly binds SAM68.

To analyze the functional interaction between ITSN1 and SAM68 in cells, the effect of ITSN1 knockdown on SAM68-mediated splicing events was analyzed. ITSN1 knockdown in HeLa cells was performed using two different ITSN1-specific shRNAs. The efficiency of ITSN1 knockdown was checked by qPCR (Fig. 2, *a*) and Western blot analysis (Fig. 2, *b*) that showed a ~50 % decrease in ITSN1 expression at mRNA and protein levels.

Four alternative splicing events that are known to be regulated by SAM68 were analyzed in HeLa cells with ITSN1 knockdown. They included the selection of alternative splice site in *BCL-X* exon 2 (isoform *BCLX-S*), *CD44* exon v5 inclusion (*CD44-v5*), *CCND1* intron 4 retention (*CCND1-b*), and *SRSF1* intron 3 retention (*SRSF1-L*). The ratio between the relative expression of two possible transcripts produced during the alternative splicing was estimated (Fig. 3).

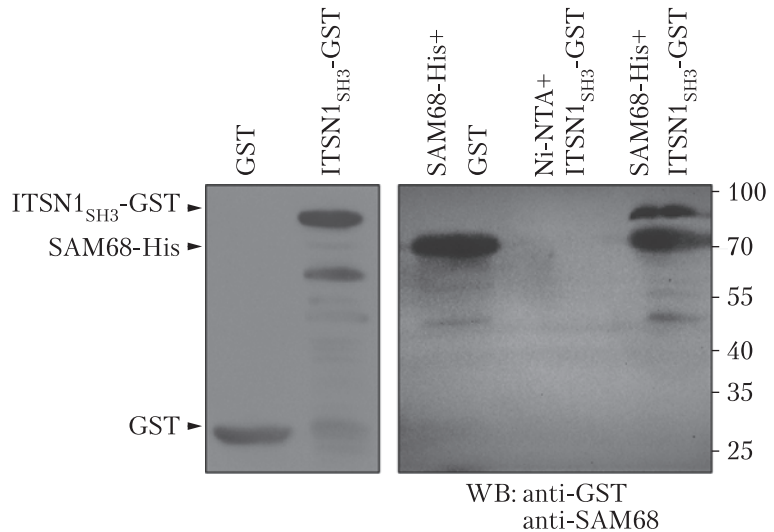


Fig. 1. ITSN1 SH3 domains directly interact with RNA-binding protein SAM68

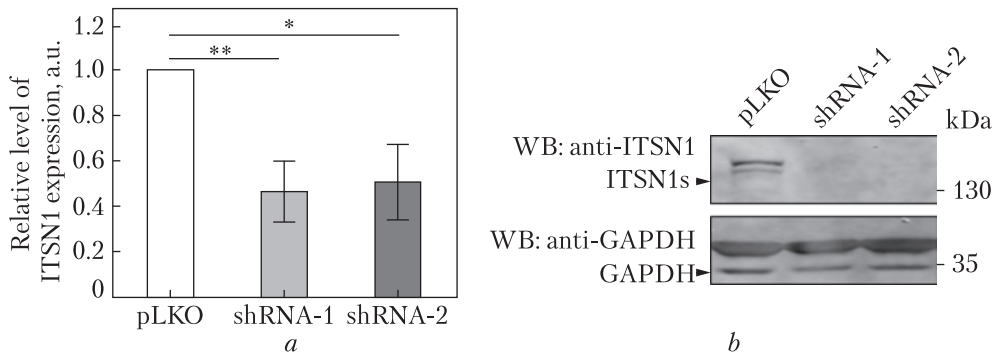


Fig. 2. The efficiency of ITSN1 knockdown in HeLa cells. *a* – normalized ITSN1 expression level. *b* – ITSN1 protein synthesis level. * – $p < 0.05$, a.u. – arbitrary units, WB – Western blotting

It was found that ITSN1 knockdown in HeLa cells caused no change in the expression of *BCLX*, *CD44* and *CCND1*, as the ratio between transcripts in shRNA-expressing cells was similar to control cells (Fig. 3). However, ITSN1 knockdown significantly increased *SRSF1* intron 3 retention, associated with the increased expression of the proto-oncogenic isoform of splicing factor *SRSF1* (*SRSF1-L*). The ratio between *SRSF1-L* and *SRSF1-S* expression levels increased by three times, as compared to control cells. The obtained results suggest that ITSN1 might regulate SAM68 activity in HeLa cells, in particular, modulating SAM68-mediated alternative splicing of *SRSF1* pre-mRNA, although a further analysis is required to confirm the finding.

As other proteins that regulate endocytosis and actin filaments rearrangement possess SH3 domains, it was suggested that they could also interact with SAM68. The putative interaction between SAM68 and several SH3 domain-containing proteins was studied *in vitro* using GST pull-down assay. GST-fused SH3 domains of AMPH1, BIN1, CTTN, TKS4, and TKS5 were immobilized on glutathione-sepharose and incubated with lysates of 293 cells. The SH3 domain of SRC kinase was used as a positive control, while recombinant GST alone was used as a negative control. PAGE-SDS analysis of the precipitated proteins demonstrated that SAM68 interacted with AMPH1, BIN1, CTTN, TKS4 (SH3-3 domain), and TKS5 (SH3-5 domain) (Fig. 4). The data indicate that the selected endocytic proteins and actin cytoskeleton modulators interact with RNA-binding protein SAM68.

The obtained results clearly demonstrated the direct interaction between RNA-binding protein SAM68 and scaffold protein ITSN1, confirming previous results showing that ITSN1 and SAM68 could interact *in vitro* and in 293 cells [5]. Moreover, it could be suggested that the interaction is mediated by ITSN1 SH3 domains and SAM68 proline motifs.

The present work showed a novel function of ITSN1 protein associated with the regulation

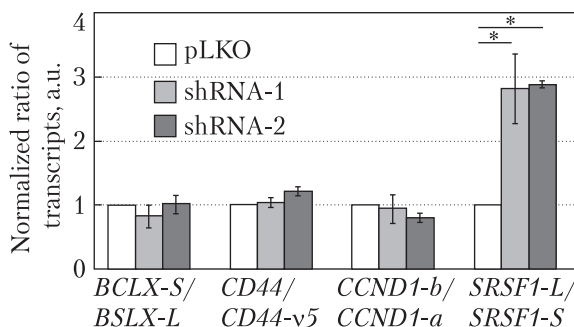


Fig. 3. The effect of ITSN1 knockdown on SAM68-mediated alternative splicing events in HeLa cells. * – $p < 0.05$, a.u. – arbitrary units

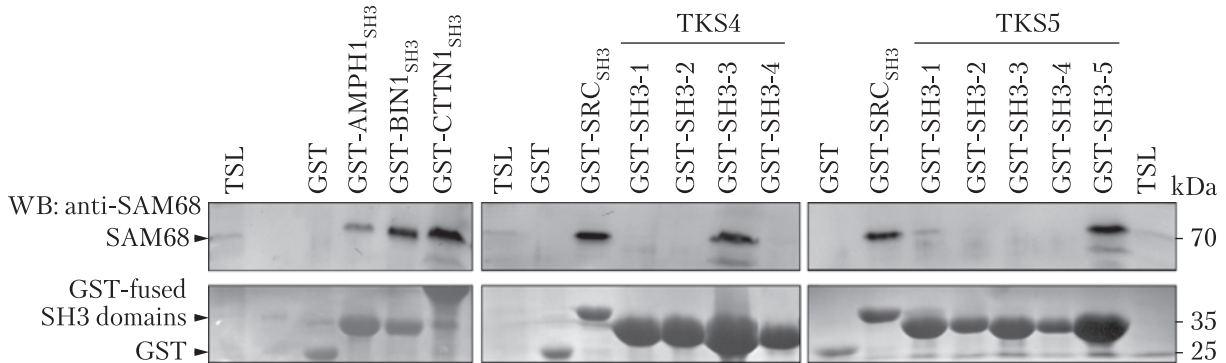


Fig. 4. SAM68 interacts with SH3 domains of AMPH1, BIN1, CTTN1, TKS4, and TKS5 *in vitro*

of alternative splicing. Significant change (a 50 % increase) in the expression of full-length isoform of SRSF1 was observed. Proto-oncogene SRSF1 (ASF/SF2) is a splicing factor that is overexpressed in various human cancers [10]. The 3'UTR region of ASF/SF2 pre-mRNA contains intron 3 that is normally retained in the mature full-length transcript. Exclusion of the intron is associated with the recognition of a premature termination codon and degradation of the SRSF1 mRNA by the nonsense-mediated mRNA decay [11]. SAM68 binds SRSF1 pre-mRNA, induces intron 3 retention, and increases SRSF1 expression activating epithelial-to-mesenchymal transition in colon cancer cells SW40 [12]. The obtained data demonstrated that ITSN1 knockdown increased the production of proto-oncogenic full-length isoform of SRSF1 that correlates with the fact that the decreased ITSN1 expression is observed in lung cancer cells A549 [13]. As SAM68 is a modulator of SRSF1 mRNA splicing, it could be suggested that ITSN1 is involved in the regulation of SAM68 activity. The regulatory process might be associated with the direct interaction between ITSN1 and SAM68. On the other hand, as SRSF1 expression is activated by MAPK-Erk1/2-mediated phosphorylation of SAM68, it could be also expected that ITSN1 knockdown could stimulate MAPK-Erk1/2 signalling pathway that was previously observed in mouse lung cells [14].

Finally, the obtained results extended SAM68 protein interactome demonstrating that SAM68 could interact with endocytic proteins AMPH1, BIN1, as well as actin cytoskeleton modulators CTTN1, TKS4 and TKS5. As SAM68 was found to modulate the activation of several membrane-associated receptors, it could be suggested that the interaction between SAM68 and BIN1 or AMPH1, which are known to induce plasma membrane bending, could regulate the endocytosis-mediated internalization of membrane receptors. It was shown that SAM68 induces migration of cancer cells and mice fibroblasts [2], suggesting that SAM68 could interact with proteins that modulate actin cytoskeleton rearrangement, in particular, CTTN1, TKS4, and TKS5, as well as AMPH1 and BIN1 which control the initiation of actin polymerization [15]. The interactions may be required for the recruitment of certain mRNA to the sites of podosomes formation. However, a further analysis is needed to find out the functional role of the identified interactions.

RNA-binding protein SAM68 directly interacted with ITSN1 SH3 domains *in vitro*. ITSN1 knockdown in HeLa cells stimulated *SRSF1* intron 3 retention increasing expression of the proto-oncogenic isoform of splicing factor SRSF1. SH3 domains of AMPH1, BIN1, CTTN1, TKS4,

and TKS5 precipitated RNA-binding protein SAM68 from lysate of 293 cells. The obtained data expanded SAM68 protein interactome and suggested that SAM68-mediated alternative splicing could be regulated by ITSN1.

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РНК-ЗВ'ЯЗУВАЛЬНИЙ БЛОК SAM68
ВЗАЄМОДІЄ ІЗ БІЛКАМИ ЕНДОЦИТОЗУ
ТА МОДУЛЯТОРАМИ АКТИНОВОГО ЦИТОСКЕЛЕТА

Метою дослідження було підтвердити можливість прямого зв'язування між SAM68 та ITSN1, проаналізувати вплив ITSN1 на SAM68-опосередкований альтернативний сплайсинг і виявити нових партнерів SAM68 з-поміж ендоцитозних білків та модуляторів реорганізації актинового цитоскелета. Взаємодії проаналізовано за допомогою pull-down методик з використанням очищених рекомбінантних білків або лізатів клітин 293. Нокдаун ITSN1 у клітинах лінії HeLa проводили, використовуючи дві шпилькові РНК. Експресію ізоформ, що утворюються в ході альтернативного сплайсингу, проаналізовано за допомогою ПЛР в реальному часі. Показано, що SAM68 прямо взаємодіє з ITSN1 *in vitro*. Далі виявлено, що нокдаун ITSN1 у клітинах лінії HeLa підвищує рівень збереження інтрону 3 *SRSF1* на 50 %, сприяючи експресії протоонкогенної ізоформи SRSF1. Встановлено, що SH3 домени білків AMPH1, BIN1, CTTN1, TKS4 та TKS5 преципітують SAM68 із лізатів клітин 293. SAM68 безпосередньо зв'язується з ITSN1 і взаємодіє з ендоцитозними білками та модуляторами перебудов актинового цитоскелета, а SAM68-опосередкований сплайсинг у клітинах лінії HeLa може регулюватися ITSN1.

Ключові слова: SAM68, ITSN1, SRSF1, альтернативний сплайсинг.

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РНК-СВЯЗЫВАЮЩИЙ БЕЛОК SAM68
ВЗАИМОДЕЙСТВУЕТ С БЕЛКАМИ ЭНДОЦИТОЗА
И МОДУЛЯТОРАМИ АКТИНОВОГО ЦИТОСКЕЛЕТА

Целью исследования было подтвердить возможность прямого связывания между SAM68 и ITSN1, проанализировать влияние ITSN1 на SAM68-опосредованный альтернативный сплайсинг и найти новых партнеров SAM68 среди ендоцитозных белков и модуляторов реорганизации актинового цитоскелета. Взаимодействия проанализированы с помощью pull-down методик с использованием очищенных рекомбинантных белков или лизатов клеток 293. Короткие РНК, образующие шпильки, использовали для нокдауна ITSN1 в клетках линии HeLa. Экспрессия изоформ, образующихся в ходе альтернативного сплайсинга, проанализирована с помощью ПЦР в реальном времени. Показано, что SAM68 прямо взаимодействует с ITSN1 *in vitro*. Далее обнаружено, что нокдаун ITSN1 в клетках линии HeLa повышает уровень сохранения интрона 3 *SRSF1* на 50 %, способствуя экспрессии протоонкогенной изоформы SRSF1. Установлено, что SH3 домены белков AMPH1, BIN1, CTTN1, TKS4 и TKS5 преципитируют SAM68 из лизатов клеток 293. SAM68 непосредственно связывается с ITSN1 и взаимодействует с ендоцитозными белками и модуляторами перестроек актинового цитоскелета, а SAM68-опосредованный сплайсинг в клетках линии HeLa может регулироваться ITSN1.

Ключевые слова: SAM68, ITSN1, SRSF1, альтернативный сплайсинг.