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THÈSE

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et l'université nationale Tarass Chevtchenko de Kyiv

présentée par :

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Rôle de l'intersectin-1 au cours du trafic membranaire: identification de nouveaux partenaires moléculaires.

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by Olga GUBAR

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Role of Intersectin 1 in membrane trafficking: Identification of new molecular partners.

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Résumé en Français

Thèse de doctorat

Présentée par Olga GUBAR

Rôle de l'intersectin-1 au cours du trafic membranaire: Identification de nouveaux partenaires moléculaires.

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-Département de génétique fonctionnelle, Institut de biologie moléculaire et génétique, Université de Kiev, Ukraine, co-directeur : Pr. A. Rynditch.

Prologue

J'ai réalisé une thèse en co-tutelle entre l'Université de Strasbourg en France et l'Université de Kiev en Ukraine. Le laboratoire du Pr. A. Rynditch à Kiev s'intéresse aux mécanismes d'endocytose dépendants de la clathrine ainsi qu'à la signalisation qui l'accompagne. Les travaux se focalisent essentiellement sur les Intersectins (ITSN), une famille de protéines d'échafaudage qui existent sous de multiples formes de variants d'épissage. Le laboratoire du Dr. M.F. Bader à Strasbourg s'intéresse à la sécrétion neuroendocrine et s'attache à comprendre plus particulièrement les mécanismes moléculaires qui régissent et contrôlent l'exocytose régulée par le calcium ainsi que l'endocytose compensatrice qui s'en suit. Ces deux mécanismes doivent être étroitement régulés puisque l'apport de membrane consécutif à l'exocytose est rapidement compensé par une recapture de vésicules pour maintenir une surface cellulaire constante et réapprovisionner le stock de vésicules nécessaires à la pérennité de la libération hormonale. Bien que ce processus de couplage soit clairement identifié, les mécanismes moléculaires le régulant restent inconnus. L'idée d'une collaboration entre ces deux laboratoires est née suite aux données de l'équipe Strasbourgeoise montrant que l'ITSN1-L régule l'exocytose d'hormones dans les cellules de phéochromocytome de rat (PC12) *via* l'activation de Cdc42. L'ITSN1 étant impliquée dans les mécanismes d'endocytose dépendants de la clathrine, cette famille de protéines nous est alors apparue comme un candidat idéal pour assurer le couplage fonctionnel entre l'exocytose régulée et l'endocytose compensatrice des granules de sécrétion dans les cellules neuroendocrines. Ce postulat a ainsi constitué notre idée de départ et a motivé notre collaboration. Au cours de ma thèse, les objectifs ont quelques peu été changés.

I. Introduction

L'homéostasie cellulaire est intimement liée au trafic membranaire, processus dynamique qui permet les échanges de lipides et de protéines entre les compartiments cellulaires mais aussi entre la cellule et le milieu extracellulaire. Ces échanges membranaires sont primordiaux pour diverses fonctions cellulaires, notamment l'assimilation de substances nutritives, la mobilité, et la sécrétion.

L'intersectin-1 (ITSN1), la première représentante de la famille d'Intersectins, est une protéine d'échafaudage multifonctionnelle, impliquée dans les processus d'endocytose dépendants de la clathrine et de la caveoline, dans l'exocytose régulée par le calcium et dans le recyclage des vésicules synaptiques. Elle est aussi impliquée dans diverses voies de signalisation ainsi que dans la survie cellulaire. Des mutations entrainant une perturbation de fonction de l'ITSN1 ont été associées à diverses pathologies dont le syndrome de Down et certaines affections neurodégénératives telles que les maladies d'Alzheimer et de Huntington. ITSN1 est également impliquée dans la tumorigenèse, notamment du gliome et du neuroblastome.

L'ITSN1 est soumise à de multiples évènements d'épissage alternatif. Les deux isoformes principales et majoritairement exprimées dans la cellule sont les formes courte (S) et longue (L). La forme courte de l'intersectin (ITSN-S) comprend deux domaines d'homologie à l'Epsine (EH), une région 'coiled-coil' et cinq domaines d'holomologie à Src (SH3). La forme longue (ITSN-L) appartient à la famille des facteurs d'échange Dbl connus pour leur activité oncogénique. Elle possède en plus trois domaines C-terminaux additionnels : un domaine d'homologie à Dbl (DH), un domaine d'homologie à la Pleckstrin (PH) et un domaine C2. Cette organisation moléculaire confère à la forme longue, en plus de ses propriétés de protéine d'échafaudage, la propriété de facteur d'échange nucléotidique pour la GTPase monomérique Cdc42 grâce aux domaines DH-PH.

En plus de ces deux isoformes principales, de nombreux autres évènements d'épissages alternatifs ont été identifiés pouvant donner naissance jusqu'à 34 autres isoformes de l'ITSN1 exprimés de façon plus minoritaires.

Bien que les Intersectins soient impliquées dans les processus d'endocytose et d'exocytose, leur mode précis d'action, tout comme la fonction des variantes d'épissage ne sont pas encore connus.

Mon projet de thèse a finalement consisté à identifier de nouveaux partenaires de l'ITSN1 et de son variant d'épissage sans exon 35 (ITSN1-LΔ35) et de déterminer la fonction de ces nouveaux complexes dans l'exocytose et l'endocytose. Ce travail a abouti à la caractérisation biochimique et fonctionnelle de l'interaction entre RhoU, une petite protéine G atypique, et les ITSN1 pleine longueur et Δ35. De plus, j'ai montré la capacité de l'ITSN1 à interagir avec l'Oligophrénine1 (une protéine GAP favorisant l'activité GTPase de certaines protéines Rho) impliquée dans un syndrome de retard mental. Enfin j'ai pu démontrer la possibilité pour l'ITSN1 de former des homo- et hétéro-dimères.

II. Résultats

II.1. Caractérisation de l'interaction entre l'ITSN1 et la GTPase RhoU.

Les GTPases monomériques sont des interrupteurs moléculaires qui cyclent régulièrement entre un état inactif lié au GDP et un état actif lié au GTP. Elles participent à la réorganisation du cytosquelette d'actine et sont indispensables à une multitude de processus cellulaires tels que que la division, la migration, le développement neuronal et le trafic membranaire intracellulaire. Elles sont également largement impliquées dans différentes phases du développement tumoral. Leur passage à l'état actif est assuré par des facteurs d'échange nucléotidique (protéine GEF pour Guanine nucleotide Exchange Factor) assurant l'échange du GDP par le GTP. Leur inactivation s'opère *via* des protéines favorisantes l'hydrolyse du GTP (protéines GAP pour GTPases Activating Proteins).

Des résultats préliminaires du Dr S. Ory avaient permis d'identifier par précipitation d'affinité suivie d'une analyse du complexe par spectrométrie de masse, l'ITSN2 comme partenaire de RhoU. RhoU est une GTPase monomérique de la famille Rho qui possède, comme tous les membres de cette famille, un domaine de liaison aux nucléotides de guanine (GDP ou GTP). Cependant, elle possède des propriétés atypiques : elle a la capacité de se charger spontanément en nucléotide *in vitro*, son mode d'adressage membranaire dépend d'une palmitoylation (plutôt que des farnésylations ou des géranylations habituelles à cette famille de protéines) et elle présente des domaines supplémentaires en région N- et C-terminale. Notamment, RhoU possède une extension N-terminale riche en prolines capable d'interagir avec des adaptateurs moléculaires à domaines SH3 comme Grb2 ou Nck1. Ce domaine permet à RhoU de participer à la transmission du signal du récepteur à l'EGF et

pourrait réguler son activation, nécessaire au contrôle de la migration, de l'adhérence, de la polarité et de la transformation cellulaire.

L'ITSN2 étant très homologue à l'ITSN1, j'ai tout d'abord vérifié la capacité éventuelle d'ITSN1 de lier RhoU *in silico*, via l'application d'évaluation des motifs d'interaction protéique *Scansite*, Les résultats obtenus affichent le domaine SH3A d'ITSN1 comme une cible d'interaction possible avec les motifs riches en proline de RhoU. Je me suis alors attachée à confirmer cette interaction et à en définir les domaines d'interaction. Ainsi, j'ai pu montrer par immunoprécipitation que les formes courte et longue de l'ITSN1 sont capables d'interagir avec RhoU et que cette interaction requiert les domaines SH3A, C et E. L'interaction ne dépend pas de l'activité de RhoU mais de son extension N-terminale riche en prolines, en particulier de son deuxième motif riche en prolines. J'ai également montré que l'ITSN1 présente une localisation subcellulaire différente en présence de RhoU. En effet, l'ITSN1 n'est que partiellement membranaire (particulièrement au niveau des puits de clathrine), alors qu'elle est largement retrouvée dans le cytoplasme. En revanche, en présence de RhoU, elle est recrutée au niveau de la membrane plasmique et des endosomes de recyclage. D'un point de vue fonctionnel, cette interaction se traduit par une levée de l'inhibition de l'endocytose de la transferrine observée lors de l'expression de l'ITSN1. Ces données montrent pour la première fois qu'un facteur d'échange peut interagir avec une GTPase Rho indépendamment de ses domaines DH-PH et ouvrent de nouvelles perspectives quant à la relation entre les facteurs d'échange et des GTPases Rho atypiques. De plus, l'ITSN1L étant un facteur d'échange de Cdc42, son interaction avec RhoU amène un degré de complexité supplémentaire quant à l'interconnexion entre les cascades moléculaires faisant intervenir ces GTPases monomériques. A l'heure actuelle, le rôle précis de l'interaction RhoU-ITSN1 reste inconnu, mais au vu des données de la littérature, il serait intéressant d'explorer plusieurs processus tels que la signalisation et/ou le trafic du récepteur à l'EGF, la migration des cellules cancéreuses, et éventuellement le développement des épines dendritiques.

II.2. Cas d'un variant d'épissage de l'ITSN1 : l'ITSN1-LΔ35

Parmi les multiples événements d'épissage alternatif de l'ITSN1, je me suis particulièrement intéressée au variant dont l'exon 35 est éliminé (ITSN1-LΔ35). Il en résulte une délétion de 32 acides aminés situés à cheval entre le domaine DH et le linker reliant le domaine DH au domaine PH. Afin d'évaluer si cette délétion a des conséquences sur la structure de ce tandem DH-PH, nous avons construit un modèle 3D en se basant sur la structure résolue des domaines pleine longueur DH-PH d'ITSN1-L. Ce modèle a permis de montrer que l'absence de transcription du codon 35 entraîne la délétion de l'hélice α6 dans le domaine DH. Cette hélice α6 sert à maintenir une certaine distance entre le domaine DH et le domaine PH. Pour certains facteurs d'échange le domaine PH établit un contact avec la GTPase et participe ainsi à la détermination de la spécificité d'interaction. Nous avons donc supposé que l'ITSN1-LΔ35 pourrait avoir une spécificité distincte de l'isoforme principale vis-à-vis des GTPases. Cependant, nous ne pouvons pas exclure que ce rapprochement du domaine PH vers le domaine DH puisse créer un obstacle stérique pour l'interaction avec la GTPase.

Les données obtenues au cours de ma thèse concernant l'interaction et la capacité d'activation de Cdc42 ne montrent aucune différence entre le tandem DH-PH pleine longueur et le tandem DH-PHΔ35 . En revanche, la spécificité d'interaction est modifiée puisque le tandem DH-PH de l'ITSN1 n'interagit pas avec la GTPase RhoU alors que celui du variant Δ35 en est capable. Une des fonctions communes aux deux protéines concernent leur capacité à induire la transformation cellulaire *in vitro*. Par extension, j'ai voulu déterminer si les profils d'expression de RhoU, de l'ITSN1-L et de l'ITSN1-LΔ35 étaient comparables dans certains tissus cancéreux humains. Par RT-PCR et « PCR nichée » à partir d'ARN total extrait d'échantillons de tumeurs humaines, j'ai pu déterminer que les transcrits des deux isoformes d'ITSN1 (L et LΔ35) sont présents dans les cancers du sein, de la prostate et de la glande médullosurrénale (phéochromocytomes) et que RhoU est présent dans les phéochromocytomes (seul tissu testé dans le cas du messager de RhoU).

II.3. Caractérisation de l'interaction entre l'ITSN1 et une Rho GAP, l'Oligophrénine

L'Oligophrénine 1 (OPHN1) est une protéine GAP (GTPase Activating Protein) qui catalyse l'inactivation spécifique de certains membres de la famille Rho comme RhoA, Rac1 et Cdc42. Elle est impliquée dans le développement des dendrites et dans la recapture des vésicules synaptiques. Les travaux préliminaires de l'équipe Strasbourgeoise ont aussi montré que l'OPHN1 est nécessaire à l'endocytose compensatrice dans les cellules chromaffines. L'OPHN1 contient des domaines riches en prolines qui permettent l'interaction avec des domaines SH3. Avec l'ITSN1, l'OPHN1 me paraissait un bon candidat pour coupler l'exocytose à l'endocytose. Il me semblait donc important de déterminer si l'ITSN1-L pouvait interagir avec l'OPHN1. J'ai pu en effet montrer que le domaine SH3A de l'ITSN1 lie l'OPHN1. De plus ces deux protéines co-immunoprécipitent et colocalisent dans les cellules PC12 (une lignée issue d'un phéochromocytome de rat). En réponse à la stimulation de la sécrétion, l'OPHN1 est recrutée à la membrane plasmique indiquant que la stimulation pourrait permettre l'assemblage d'un complexe nécessaire à l'endocytose compensatrice.

L'ensemble de ces résultats permet d'établir pour la première fois l'existence d'un complexe ITSN1-L-OPHN1 dans les cellules neuroendocrines et indique que l'assemblage de complexes multimoléculaires pouvant réguler de façon opposée certaines GTPases Rho peuvent être au cœur du couplage de l'exocytose et de l'endocytose.

II.4. Les isoformes d'ITSN1 interagissent entre elles.

La caractéristique principale des protéines d'échafaudage est d'assembler de volumineux complexes multiprotéiques afin de coordonner certains processus cellulaires ou voies de signalisation. Il est connu que certaines protéines d'échafaudage forment des dimères voire s'oligomérisent pour assurer leur fonction. De façon intéressante, les données de notre laboratoire en Ukraine montrent que l'ITSN1-S interagit avec une de ses isoformes mineures, l'ITSN-22a.

Au vu de ces données, nous avons supposé que les isoformes principales d'ITSN1 pourraient également former des complexes dans les cellules. Effectivement, l'ITSN1-S et l'ITSN1-L co-précipitent dans toutes les lignées cellulaires testées (HEK293, HEK293T et PC12). En accord avec ce résultat, ces deux isoformes d'ITSN1 colocalisent parfaitement dans les cellules HEK293T. C'est aussi très probable qu'il existe des

complexes entre les deux formes courtes (ITSN1-S) ou entre les deux formes longues (ITSN1-L), et peut-être dans toutes les combinaisons imaginables entre les deux isoformes principales et une multitude des isoformes mineures (sauf si le domaine d'interaction est perdu lors de l'épissage). De plus, notons qu'il a été montré que les isoformes d'ITSN1 et ITSN2 sont également capables d'interagir entre elles, permettant sans doute la formation de supercomplexes moléculaires, qui serviraient de plateformes pour certains processus cellulaires tels que le trafic membranaire ou la signalisation.

III. Conclusion

L'ensemble de mes travaux de doctorat a permis d'identifier deux nouveaux partenaires de l'ITSN1 : RhoU et l'OPHN1. Ils indiquent aussi que, bien que minoritaires, les variants d'épissage de l'ITSN1 pourraient avoir des conséquences insoupçonnées en modifiant leur spécificité d'interaction. Les Intersectins, l'OPHN1 et RhoU ont des fonctions diverses mais toutes semblent être impliquées dans certaines phases du trafic vésiculaire. Ainsi, mes travaux qui s'inscrivent dans le contexte global du laboratoire visant à élucider les mécanismes de la libération hormonale et de l'endocytose compensatrice, ouvrent de nouvelles perspectives sur la fonction des régulateurs des GTPases Rho et des GTPases Rho atypiques. De plus, nous montrons que l'ITSN1 est capable de former des complexes entre ses différentes isoformes. De cette façon, les ITSN pourraient former de supercomplexes protéiques pouvant servir au trafic membranaire.

IV. Liste des publications et communications :

IV.1. Publications :

1) **Gubar O.**, Houy S., Billuart P., Tsyba L., Kropyvko S., Gasman S., Rynditch A. "GTPase-activating proteing oligophrenin 1 is a new partner of multifunctional adaptor protein intersectin 1" *Biopolymers and cell*. -2012. – T. 28, № 5. (in Ukrenian).

2) **Gubar O.**, Kropyvko S., Tsyba L., Gasman S., Rynditch A. "Alternatively spliced short and long isoforms of adaptor protein intersectin 1 form complexes in mammalian cells." *Biopolymers and cell*. -2012. – T. 28, № 6.

3) **Gubar O**, Tryoen-Toth, P., Croisé P., Calco, V., Rynditch A., Gasman, S. and Ory, S. "RhoU binds to Intersectins and regulates their function" (en préparation)

4) **Gubar O.**, Morderer D., Croisé P., Ory S., Tsyba L., Gasman S., & Rynditch A. "Intersectin : the crossroads beetween secretory vesicle exocytosis and endocytosis". *Frontiers in Endocrinol.* (en préparation)

5) Houy S., **Gubar O**., Calco V., Tryoen-Tóth P., Bader MF., Ory S. & Gasman S. : Exocytosis and endocytosis in neurosecretory cells: two unseparable friends! *Frontiers in Endocrinol.* (en préparation)

IV. 2. Présentation de poster:

1) **Septembre 2011:** « The role of new intersectin-1L splice variants in Rho-GTPases-regulated pathways ». The 4th international IMBG conference for young scientists "Molecular biology: advances and perspectives", Kyiv, Ukraine. 14-17 septembre 2011.

2) **Mai 2012:** « Role of the intersectin 1 splice variant Δ35 in exo-endocytosis. ». 15éme colloque du Club exo-endocytose, Isle-sur-la-Sorgue, France. 31 mai-2 juin 2012.

3) **Septembre 2012:** «The new intersectin 1L splice variant ITSN1-LΔ35 is expressed in different cancer types ». The 1st Multidisciplinary Symposium "Molecular Oncology : from Laboratory Bench to Medicine", Kyiv, Ukraine. 17-22 septembre 2012.

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Ms Olga Gubar was a member of the **European Doctoral College** of the University of Strasbourg during the preparation of her PhD, from 2008 to 2013, class name Rosa Parks. She has benefited from specific financial supports offered by the College and, along with her mainstream research, has followed a special course on topics of general European interests presented by international experts. This PhD research project has been led with the collaboration of two universities: the Taras Shevchenko National University of Kyiv, Ukraine, and the University of Strasbourg, France.

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Table of contents

List of abbreviations.

AD – Alzheimer's disease

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP2 – adaptor protein 2

BAR – Bin–Amphiphysin–Rvs domain

CA – constitutively active

CCP – clathrin-coated pit

CCR – coiled coil region

CME – clathrin-mediated endocytosis

DH – Dbl homology domain

DN – dominant negative

DS – Down syndrome

EGFR – epidermal growth factor receptor

EH – EPS15 homology domain

EMT – epithelio-mesenchymal transition

EPS15 – EGFR pathway substrate 15

ERK – extracellular signal-regulated kinase

FAK – focal adhesion kinase

FCHO – Fer/Cip4 homology domain only proteins

GAP – GTPase activating protein

GDI – guanine nucleotide dissociation inhibitors

GEF – guanine nucleotide exchange factor

HD – Huntigton disease

Htt – huntingtin

IB – immunoblotting

IP – immunoprecipitation

ITSN – intersectin

ITSN1-S and ITSN1-L short and long isoforms of ITSN1, respectively

JNK – c-Jun N-terminal kinase

LMP2A - Epstein–Barr virus latent membrane protein 2A

MAPK – mitogen-activated protein kinase

MEK – mitogen-induced extracellular kinase

MMP – matrix metalloproteinase

NMDA – N-Methyl-D-aspartic acid or N-Methyl-D-aspartate

NPF-motifs - asparagine-proline-phenylalanine motifs

N-WASP – neural Wiskott-Aldrich syndrome protein

OPHN1 – oligophrenin 1

PAK – p21-activated kinase

PH – pleckstrin homology domain

PI3K-C2β – class II phosphoinositide-3-kinase C2β

RTK – receptor tyrosine kinase

RT-PCR – reverse transcription polymerase chain reaction

SH3 – Src homology domain 3

SNAP – soluble NSF attachment protein

SNARE – soluble NSF attachment protein (SNAP) receptor

SV – synaptic vesicle

T-ALL – T-cell acute lymphoblastic leukaemia

I. Prologue

Endocytosis and exocytosis are processes absolutely necessary for cell survival. Endocytosis provides uptake of nutrients, internalization of receptors and signal transduction, recycling of synaptic vesicles, immune defense... Exocytosis provides release of hormones, neuromediators and other biologically active substances.

Both endocytosis and exocytosis need assembling of complicated protein complexes which require assistance of scaffold proteins. Intersectin 1 is a multidomain scaffold highly implicated in endocytosis and, according to recent research data, in exocytosis. Intersectin 1 has two main isoforms: short and long. The short one (ITSN1-S) is ubiquitously expressed and consists of two EH domains, coiled-coil region and five SH3 domains. The long one (ITSN1-L) is expressed predominantly in neurons and has three additional C-terminal domains: DH, PH and C2. DH (Dbl homology) domain is a GEF (guanine nucleotide exchange factor) for Cdc42, a small GTPase from the Rho family. Recent findings of our group in Ukraine predicted 34 minor splice variants for ITSN1. I was particularly interested in one of the multiple splicing events, which affected DH-PH domains. Indeed deletion of exon 35 leads to deletion of 32 amino acid residues in DH domain and decreases spacing between DH and PH domains. We supposed that this ITSN1 isoform may have different functional activity that in turn could have impact at downstream cellular processes. At the same time, the group of Stéphane Gasman was showing the importance of ITSN1-L in calcium regulated exocytosis and started to get interested in the mechanism regulating the compensatory endocytosis of secretory granules that follow exocytosis. The collaboration between Alla Rynditch and Stéphane Gasman started here, based on the idea that ITSN1-L or some of its alternative splicing forms (ITSN-Δ35 in particular) could couple secretory granule exocytosis to endocytosis in neuroendocrine cells. It was the start of my Ph.D project.

So my main goal was originally to characterize this ITSN1-LΔ35 isoform, beginning from the evaluation of the mRNA expression level in different cell lines to the biochemical properties and a possible role in regulated exocytosis and compensatory endocytosis. But as it often happens with thesis projects, it was radically changed during the performance. ITSN1-L, especially its DH-PH domains, which we needed for the multiple biochemical and *in vivo* assays, appeared a very difficult protein to work with. Moreover, our target cell lines, such as primary culture of bovine chromaffin cells, hardly survived upon the overexpression of ITSN1-LΔ35, so we had to abandon the idea to perform any functional *ex vivo* assay on exo- or endocytosis in neuroendocrine cells. Even for the biochemical tests *in vitro*, the DH-PH domains and its Δ35 isoform were extremely hard to purify, which significantly slowed down the advancement of my thesis.

A second goal of the project, included “just-in-case-of”, was to discover probable functions and/or partners of other domains of ITSN1 and investigate their role in endo- and exocytosis. The achievement of purposes was possible using the combination of molecular and cellular biology approach. Molecular biology methods such as RT-PCR, GST pull down, immunoprecipitation, *in vitro* and *ex vivo* GEF assays, and cellular biology methods such as immunocytochemistry, transferrin uptake assay, GH release and DBH internalization assays were to be used.

That is how the second, “just-in-case-of” goal suddenly became the principal one. Thus only a small subchapter of this manuscript is devoted to ITSN1-LΔ35, whereas the principal results refer to the new partners of ITSN1, small GTPase RhoU and GAP OPHN1. We have also attempted to reveal their common function with ITSN1. We tried to keep in line with the membrane trafficking concept, but now it seems that we touched but a small part of their common role and the real significance of our findings lie beyond exo- and endocytosis and only awaits to be investigated.

In addition there is also a small chapter about ITSN1 isoform complexes, which imply large scaffold supercomplexes formation of different isoform composition.

However, even if the way was not straight and easy, we have obtained good prominent and novel results, which open a large field for further investigations.

I wish you a nice reading!

II. Introduction.

1. Role of endo- and exocytosis in the cell.

Membrane trafficking comprises a multitude of vesicular transport processes indispensable for the cell life. Endo- and exocytosis are important components of membrane trafficking, directed to or from the cell, respectively. Endocytosis assures nutrient uptake, membrane receptors cycling and signaling. It is also indispensable for the immune system, as it provides foreign particles phagocytosis. Endo- and exocytosis together ensure antigen presentation and neurotransmission. Exocytosis also provides the release of hormones and other biologically active molecules. But this list comprises only a small part of cellular functions which require these two processes.

1.1. The mechanism of clathrin-dependent endocytosis.

The most studied and well-defined type of endocytosis is clathrin-mediated endocytosis (CME), which is the uptake of material into the cell from the surface using clathrin-coated vesicles (1 and 2). The pathway is versatile, as many different cargoes can be packaged using a range of accessory adaptor proteins. Clathrin-mediated endocytosis is used by all known eukaryotic cells. Clathrin-coated vesicle formation proceeds through five main stages: initiation, cargo selection, coat assembly, scission and uncoating (Fig. 1). Following cargo selection and initiation of pit formation, soluble clathrin triskelia polymerize into hexagons and pentagons. Clathrin does not bind directly to the membrane or to cargo receptors and thus relies on adaptor proteins and complexes (such as adaptor protein 2 (AP2)) and accessory proteins (such as AP180 and epsin) to be recruited to the plasma membrane. The endocytosed material is sorted into endosomes and either recycled (sent back to the surface) or targeted to more mature endosomes and later compartments (such as lysosomes and multivesicular bodies). The initiation stage includes the formation of a putative nucleation module that defines the sites on the plasma membrane where clathrin will be recruited and vesicles will bud. This nucleation module is thought to assemble only at the plasma membrane because of a preference for PIP_2 (Phosphatidylinositol 4,5-bisphosphate). It includes Fer/Cip4 homology domain only (FCHO) proteins, EGFR pathway substrate 15 (EPS15) and intersectins. These proteins are thought to recruit AP2, which mediates cargo selection. Clathrin is then recruited to the plasma membrane by AP2 and also by other accessory adaptor proteins. After the assembling of clathrin coat, the vesicle budding and scission depends on the GTPase dynamin. Dynamin is recruited by BAR (Bin–Amphiphysin–Rvs) domain-containing proteins, such as amphiphysin, endophilin or sorting nexin 9, which have a preference for the curvature of the vesicle neck and are

likely to help the neck formation. Finally, once detached from the parent membrane, the clathrin coat is disassembled from its lattice arrangement back to triskelia by the ATPase heat shock cognate 70 (HSC70) and its cofactor, auxilin (or cyclin G-associated kinase (GAK) in non-neuronal tissues). At the same time the phosphatase synaptojanin provides changes in the phosphoinositide composition of the vesicle. Then the vesicle can fuse with its target compartments (1 and 2).

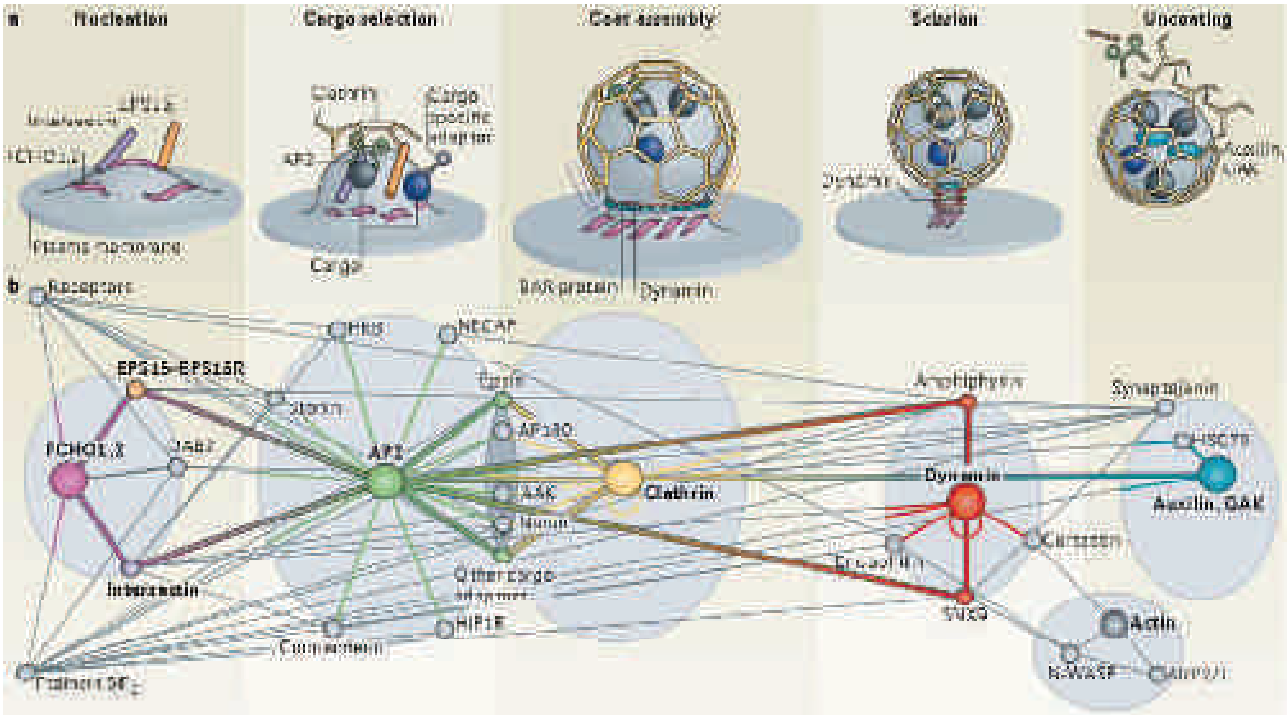

Fig. 1. The clathrin-coated vesicle cycle (from McMahon et al., 2011). a) The proposed five steps of clathrin-coated vesicle formation. b) The clathrin network. The protein–protein interactions underlying the different stages of vesicle progression are shown. Major hubs are obvious because of their central location in the network and the large number of interacting molecules. They are essential for pathway progression and are denoted by the central colored circles. Possible pathways of progression between hubs are shown with thicker lines.

This is only a common scheme of CME, which does not include a multitude of variations and modulation possibilities. For example, the specificity or efficiency of vesicle formation can be changed by adding accessory modules to the core modules. These accessory modules are cargo-, compartment- or cell-specific.

One of specific functions of CME in metazoans is receptor internalization (thus controlling the quantity of membrane receptors and activity of some signaling pathways (3)), which may be constitutive (not dependent on ligand binding) or stimulated (ligand-induced). Moreover, some signal transduction requires not only ligand binding but also receptor internalization (4 and 5). CME is also often used by pathogens (bacteria, viruses and toxins) to enter the cell where they mimick

cellular receptor ligands and often modify the CME because of their large size. For example, viruses and bacteria require additional actin polymerization for their uptake (1).

The loss-of-function mutations of the key components of membrane trafficking are embryonic lethal in metazoans. Moreover several perturbations of clathrin-mediated endocytosis proteins have been reported in numerous human disorders, such as cancer, myopathies, neuropathies, metabolic and genetic syndromes, and psychiatric and neurodegenerative diseases (1).

There are some other types of endocytosis, which are often united under the name of clathrin-independent endocytosis or non-CME. They comprise several processes which are characterized with one or more key features (for example, caveolin-dependent or lipid rafts-associated). It is not at all clear whether non-CME is a single pathway or, as appears more likely, a mixture of pathways, possibly with partially overlapping characteristics (6).

1.2. Exocytosis in neurons and neuroendocrine cells.

In multicellular organisms two types of exocytosis have been described: 1) Ca^{2+}-regulated exocytosis and 2) constitutive exocytosis. Constitutive exocytosis is performed by all cells and serves for the release of components of the extracellular matrix, or just delivery of newly-synthesized membrane proteins. Ca^{2+}-regulated exocytosis is typical for the cells that are specialized in secretion, such as endocrine cells or neurons.

The general scheme of Ca^{2+}-regulated exocytosis comprises the translocation of the vesicles to the exocytic site (the active zone in neurons), their docking, priming and fusion with the plasma membrane in response to Ca^{2+}-influx (Fig. 2 – by the example of synapse) (7). In neurons the active zone is highly structured to provide the maximal efficiency of secretion. The protein matrix, which consists of cytoskeleton and scaffold proteins, assures effective synaptic vesicles (SV) docking, whereas electron-dense projections from this matrix serve for tethering and maintaining the ready-to-use SV pool (8).

The core of exocytic machinery is formed by SNARE (soluble NSF attachment protein (SNAP) receptor) complexes. Vesicle SNARE (v-SNARE: VAMP2/synaptobrevin) on vesicle membranes and target SNAREs (t-SNAREs: syntaxin and SNAP-25) on the target plasma membranes interact to form a highly stable trans-SNARE complex to fuse opposing membranes and to release vesicle cargo. Firstly, secretory vesicles are docked at the active zone of a presynaptic terminal with unassembled SNARE complexes and are then primed for release by partial SNARE-complex assembly that is catalyzed by Munc18, Munc13, and RIM. After Ca^{2+}-influx, Ca-binding

protein synaptotagmin induces the fusion step promoting full assembly of SNARE complexes, which leads to fusion-pore opening and release of the vesicle contents (9-12).

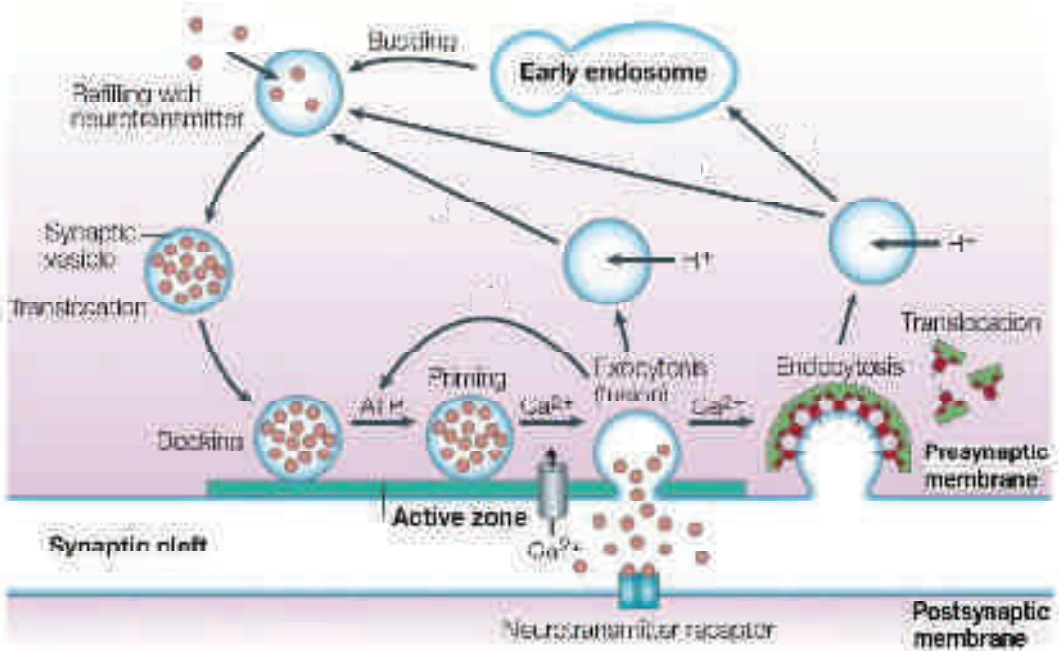

Fig. 2. Exocytic and endocytic membrane trafficking events at the presynaptic membrane – the synaptic vesicle cycle (from Gundelfinger et al., 2003).

It should be emphasized that recent data show high involvement of monomeric GTPases in different stages of exocytosis, which will be discussed in the chapter devoted to Rho GTPases (Chapter II.2.4.2.1).

In neurons and neuroendocrine cells, secretion can occur through two main modes (2 and 13):

1) the 'kiss-and-run' mode: the vesicle content is released (in some cases only partially) through a narrow fusion pore;
2) full collapse: the vesicles completely empty their contents and flatten out, becoming the part of the membrane.

In neuroendocrine cells a third intermediate type of secretion was described: cavicapture (for granule cavity capture) in which the fusion pore dilation allows the full release of small molecules (e.g. catecholamines) and the partial release of neuropeptides while the fusing granule omega shape is preserved (14 and 15).

Endo- and exocytosis are always tightly connected as cellular homeostasis requires membrane surface area stability. Thus exocytosis is always followed by compensatory endocytosis, while the membrane parts internalized by endocytosis are restored with recycling vesicles.

1.3. Compensatory endocytosis.

As for the compensatory endocytosis, it is also Ca^{2+}-dependent and is stimulated alone with the exocytosis. In the case of kiss-and-run and cavicapture the vesicle entity restoration is dynamin- but not clathrin-dependent (15). In the case of full fusion it was shown that in neuroendocrine cells the former vesicle membrane is preserved (does not diffuse in plasma membrane) and is entirely recaptured by compensatory CME (14). Synaptic vesicle components (for example, SNAREs, synaptotagmin and neurotransmitter transporters) are sorted into clathrin-coated pits by binding AP2 and cargo-specific adaptors. Recycled synaptic vesicles are translocated to endosomes, where they are reloaded with neurotransmitters for another round of release, or they may be refilled just directly after the uncoating (Fig. 2) (1 and 7). Two main types of compensatory endocytosis were described in neurons: slow clathrin-dependent and fast clathrin-independent. In addition, after prolonged stimulation the third type, so-called "bulk endocytosis", may occur, forming large vesicles – analogues of multiple synaptic vesicles (13). The mode of exo- and endocytosis, which will prevail, depends on the mode, time and strength of the stimulation, temperature and cell type/synapse maturation (13).

1.4. Scaffold proteins in membrane trafficking.

Even if the core process of both endo- and exocytosis is extensively studied, much less is known about their accessory modules as well as the mechanisms of their regulation, selectivity and kinetics. However it has been shown that the scaffold proteins play important roles in the regulation and organization of the membrane trafficking events. First of all I would like to precise the definition of the term "scaffold" which is often mixed up with the closely related term "adaptor". Scaffold proteins are molecules that bind multiple signaling components and promote their communication or interaction with each other. Classical scaffolds (e.g. Ste5) usually do not possess any type of enzymatic activity and they can be considered as specific elements that selectively facilitate signaling between their bound components. Conversely, adaptor proteins usually connect two other proteins and frequently direct them into specific cellular locations (16). In endocytosis adaptors link receptors to the clathrin triskelia (1), whereas the scaffold proteins facilitate receptor endocytosis and at the same time function as platforms for the assembly of protein signaling complexes (17). Thus endocytic scaffolds combine the role of adaptors, linking receptors to the clathrin-coated pits (CCP), and promote signaling complexes assembling as classic scaffolds. It should be mentioned that the scaffolds are not a specific family of proteins, as they seem to have evolved independently and are extremely diverse in their organization. It is rather a functional concept of a protein linking other proteins due to its multiple modular interaction domains (18).

To accomplish their function scaffolds use four main mechanisms (Fig. 3). The most frequently scaffolds tether interacting partners close in space and enhance effective local concentrations. In this case they can also promote correct mutual orientation of the interacting partners. The scaffolds can also mediate signaling complex assembly in a combinatorial manner, meaning that a certain active component can participate in signaling through different pathways using distinct scaffold proteins. The function of the signaling modules can be regulated by certain scaffold dynamic regulation, without the need to regulate individually each of the signaling pathway components. Finally some scaffolds can also modify the conformation of their partners, or in turn the conformation of the scaffold can also be modified by its binding partners (16).

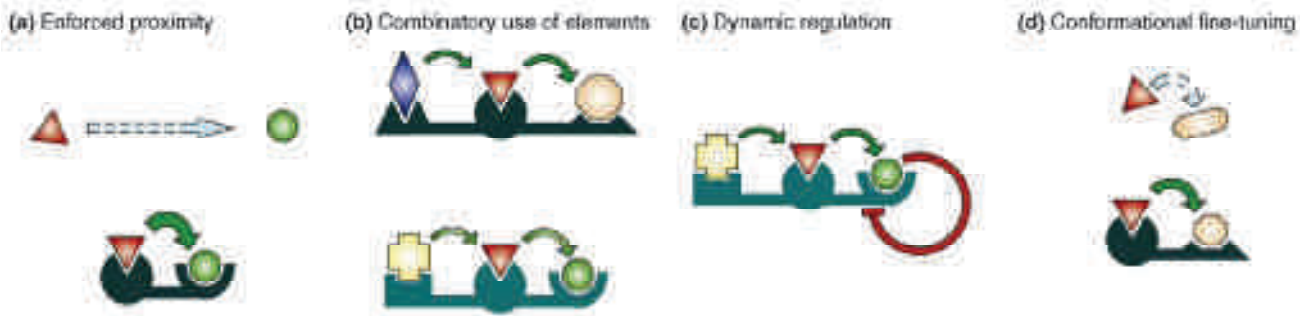

Fig. 3. Schematic representation of the four main scaffold mechanisms (from Zeke et al., 2009).

Except signaling complexes assembling and abovementioned receptor linking to CCP, the other functions of scaffolds include small GTPases signaling and coordination of the communication at cell-cell signaling junctions, such as neuronal synapses. The scaffolds may also direct protein ubiquitylation (18).

2. INTERSECTIN1: one of the key proteins in membrane trafficking and cellular signaling

Intersectin1 (ITSN1) is a multidomain and multifunctional scaffold protein, which is involved in clathrin- and caveolin-mediated endocytosis, exocytosis, Ras-MAPK signaling, JNK activation, small GTPases activity regulation, epidermal growth factor receptor (EGFR) ubiquitylation, dendritic spine development and neuron survival, - and this list is not exhaustive. The variety of ITSN1 function will be discussed in this chapter.

2.1. Gene

ITSN1 is highly conserved in metazoans. It is expressed in mammals (mouse Ese1, rat EHSH1) (19 and 20) and its orthologues exist in Xenopus laevis (Itsn1) (21), Drosophila melanogaster (Dap160) (22) and Caenorhabditis elegans (ITSN) (23).

Human *ITSN1* gene comprises 250 kb and is located at chromosome 21 q22.1-q22.2 region between D21S320 and D21S325 markers (24 and 25). The gene consists of 41 exons, and its transcription is directed from centromere to telomere (26). The orthologous ITSN genes of nematodes and arthropods contain 8 and 11 exons, respectively.

Two main isoforms of ITSN1 are generated due to alternative splicing at exon 30. In mammals the short transcript of about 5,3 kb is ubiquitously expressed whereas the long transcript of 15 kb is enriched in neurons. The short and the long transcripts encode a protein of 1220 aa (136 kDa) and 1721 aa protein (194 kDa), respectively (24). It should also be emphasized that only vertebrates have neuron-specific ITSN1-L isoform, whereas invertebrate orthologues of ITSN1 correspond to its short isoform.

2.2. Domain composition

The short isoform of ITSN1 (ITSN1-S) consists of two N-terminal Eps15 homology domains (EH1 and EH2), a coiled coil region (CCR) and five Src homology domains (SH3A-E) (Fig. 5). The long isoform (ITSN-L) contains three additional C-terminal domains, a Dbl homology domain (DH), a pleckstrin homology domain (PH) and a C2 domain (24).

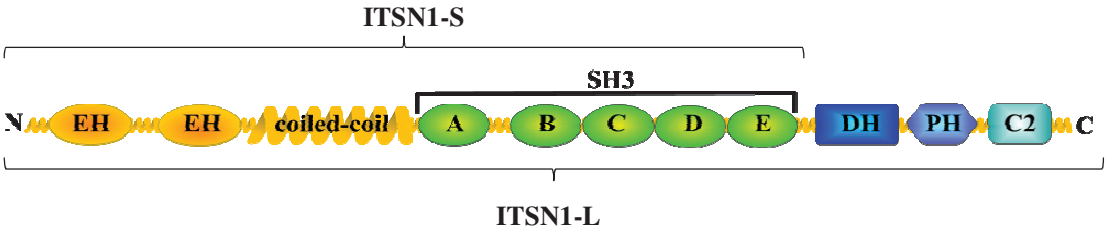

Fig. 4. ITSN1 domain composition.

Two EH domains extend from Glu21 to Pro100 and from Ser221 to Arg310 respectively (24). The EH domains usually comprise about 100 aa and are responsible for protein-protein interactions. The EH domains bind NPF-motifs (asparagine-proline-phenylalanine). This binding is realized by conserved Leu and Trp. Structurally EH domains consist of two helix-loop-helix motifs (EF-hands) that are bound by antiparallel β-fold. The EH domains containing proteins are mostly implicated in endocytosis, synaptic vesicle recycling, actin rearrangement and intracellular signaling (27).

CCR (also called KLERQ – by the abbreviations of most abundant amino acids) is a positively charged α-helix, which consists of about 370 aa (11% K, 12% L, 20% E, 13% R, 15% Q) (20).

Five SH3 domains of ITSN1 (Val740-Glu806, Val913-Gly971, Val1002-Ser1060, Lys1074-Pro1138, Ala1155-Phe1214) consist of about 50-70 aa and bind Pro-rich motifs (type II – PXXPX(R/K)) (24; 28 and 29). They promote protein complexes assembling and are commonly found in scaffolds and other proteins implicated in cell signaling pathways, cytoskeletal organization and membrane traffic (18 and 29).

The DH domain of ITSN1 is 180 aa long and extends from Leu1244 to Glu1428 (24). These domains possess guanine-nucleotide exchange activity catalyzing the exchange of GDP for GTP within Rho GTPases by promoting GTPase intermediates that are devoid of nucleotide and Mg^{2+}. After the nucleotide depletion GTP is preferentially loaded into Rho GTPases during nucleotide exchange as GTP is present in the cell at considerably higher concentrations than GDP. The DH domain fold comprises 10–15 α-helixes and 3_{10}-helixes that are roughly arranged along six main axes to form a helical bundle that has been compared in appearance to a chaise longue. The ‘seat back’ created by a U-shaped arrangement of α-helices is responsible for the specificity of the DH domain. The DH domain interacts with the switch regions of the GTPase by its three critical regions (CR1-3). Switch region 1 of the GTPase interacts with CR1 and CR3, switch 2 contacts with CR3 and a part of C-terminal α6 helix (30). The DH domain of ITSN1 is a specific guanine nucleotide

exchange factor (GEF) for Cdc42 (31). The amino acids responsible for the specificity are Leu1376 and Met1369, whose side chains establish Van der Waals bonds with Phe56 of Cdc42 (32). The activity of the DH domain in the full size ITSN1 is sterically inhibited by the linker after the adjacent SH3E domain (33 and 34). This is consistent with previous reports showing that the interaction of ITSN1 SH3 domains with N-WASP (neural Wiskott-Aldrich syndrome protein) or Numb enhances ITSN1-L GEF activity *in vivo* (31 and 35).

The PH domain of ITSN1 extends from Met1515 to Glu1571 (24). In most cases the length of PH domain is about 100 aa. It is a phosphoinositide binding domain whose C-terminal position towards the DH domain is evolutionally conserved (though there are several exceptions). Thus one of the possible PH domain roles is the membrane localization of the adjacent DH domain and also an alosteric regulation of the latter. In most cases the PH domain reinforces the nucleotide-exchange properties of the DH domain and sometimes the PH domain directly interacts with the GTPase thus taking part in the GEF specificity determination. Moreover in some cases the PH domain was shown to bind downstream members of the GTPase cascade (30). The PH domain of ITSN1 does not influence the GEF activity of the DH domain *in vitro* but enhances Cdc42 activation *in vivo* (36). A crystallographic structure of the DH-PH domains of ITSN1 shows that the PH domain does not interact with Cdc42 (32). The PH domain of ITSN1 does bind the phosphoinositides but without any striking specificity, except for some preference for the PIP_2 (Phosphatidylinositol 4,5-bisphosphate) (37). But the ITSN1 PH domain interaction with phosphoinositides is not enough to localize the protein, and it was shown that the EH domains and the CCR are responsible for the membrane localization of the full-size protein (36; 38 and 39).

The C2 domain extends from Arg1597 to Glu1679 (24). The mean length of C2 domains is about 130 aa. The C2 domains bind Ca^{2+} and subsequently bind phospholipids. The C2 domains can also interact with other proteins in a calcium-dependent manner. The majority of C2-containing proteins take part in cellular signaling (secondary lipid messenger creation, protein phosphorylation, GTPases activation, ubiquitin ligation etc.) and membrane traffic (40). The function of ITSN1 C2 domain is currently unknown.

ITSN family of proteins includes a second member, ITSN2, which exists only in vertebrates and is quite similar to ITSN1. It also undergoes alternative spicing which gives rise to the short and the long isoform, whose domain compositions are similar to those of ITSN1. But unlike the latter, both ITSN2 transcripts are ubiquitously expressed. ITSN2 isoforms colocalize with the endocytic complex components, namely Eps15. The homology of the EH1 and 2 domains between ITSN1 and ITSN2 is 87,1% and 72,6% respectively. The homology between the SH3 domains of the two

proteins is 80% (and for the SH3A domains – only 62%), and 85,4% between the PH domains – (41).

2.3. Alternative splicing

Besides the main splicing event at exon 30 which produces the short and the long isoform of the protein (24), a multitude of minor splicing events as well as an alternative promoter for ITSN1 were described.

An alternative promoter was identified in intron 5 of human ITSN1 gene. It generates the transcripts encoding ITSN1-S isoform without the EH1 domain. However, it is not known, whether these transcripts are translated into proteins (42).

Among multiple minor splice events described for ITSN1, the majority is with high probability subjected to the nonsense-mediated decay, as the introduced by alternative splicing stop-codon lies more than 50 nucleotides upstream of an exon-exon junction (42-44). This presumes that alternative splicing serves not only for the creation of ITSN1 protein variations but also for the regulation of ITSN1 expression level.

Thus I will describe only those alternative transcripts which have good chances to give rise to a protein (Fig. 5). There are four minor splicing events for ITSN1-S and one for ITSN1-L, which occur with relatively high frequency and abundance. Most of them seem to be conserved in mammals (at least, between human, rat and mouse) (20 and 43). The first splicing event from the N-terminus affects the spacing between EH1 and EH2 domains, excising 37 aa from the linker (43). The second one is brain-specific and leads to the insertion of five amino acids into the SH3A domain (20 and 43). The resulting neuron-specific isoform of the SH3A has different affinity to the majority of partners (45 and 46). The third splicing event results in the deletion of exons 25 and 26, which leads to the loss of the SH3C domain (20 and 43). Finally, the fourth splicing event generates the shortest of known ITSN1 transcripts, called 22a after the alternative exon number, encoding a protein which consists of the two EH domains, the CCR, the SH3A domain and the exon 22a-specific C-terminal sequence (47).

As for the splicing events specifically affecting the long isoform of ITSN1, only one among the three described is highly reliable and conserved in human and mice. This is an excision of exon 35, which encodes 31 aa in the DH domain and 25 aa in the DH-PH spacer (42 and 43). This transcript constitutes about 10% of total ITSN1-L transcripts and is detected together with the full-size ITSN1-L in fetal lung, liver and muscle, but mostly in fetal and adult brain (42). This

alternative splicing event is of particular interest as it affects the GEF domain of ITSN1-L, which could modify its catalytic activity.

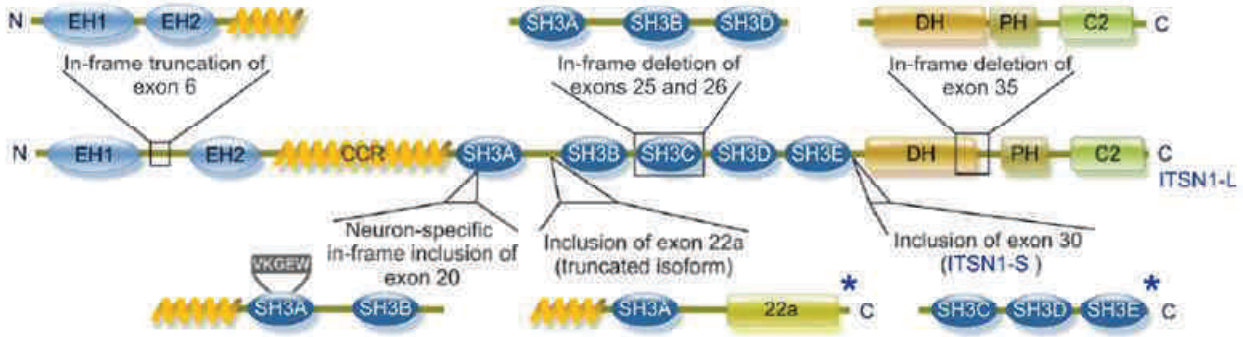

Fig. 5. Domain composition and alternative splicing of mammalian ITSN1 (from Tsyba et al., 2011). Schematic representation of alternative splicing events affecting mouse and human ITSN1. Alternative splicing events are indicated above and below the ITSN1-L domain structure. Exon numbering is according to NM_003024. Asterisks indicate a stop codon.

Three of four previously described slicing events for ITSN1-S also concern ITSN1-L, with a possibility of generation up to 16 different ITSN1-L transcripts. But to date only five ITSN1-L variants have been detected by RT-PCR (42).

Regarding the multitude of alternative splicing events affecting ITSN1, we can presume that this variety may serve several purposes like regulating the level of gene expression or providing tissue and binding specificity. A potential involvement in development has been proposed for some minor transcripts (43 and 46), but this issue should be further investigated.

2.4. Partners and cellular functions

The diverse domain composition of ITSN1 presumes a multitude of versatile partners and functions, which were extensively reviewed by Tsyba et al., 2011, and O'Bryan, 2010 (48). I will briefly survey the main of them.

2.4.1. ITSN1 in involved in membrane trafficking and signaling.

2.4.1.1. ITSN1 implication in endocytosis and exocytosis

ITSN1-S is an important component of the endocytic machinery. Its EH and SH3 domain composition is characteristic for a multitude of endocytic proteins. The ITSN1 implication in endocytosis is also supported by its localization at CCP (31) and interaction with a variety of endocytic proteins (Fig. 6).

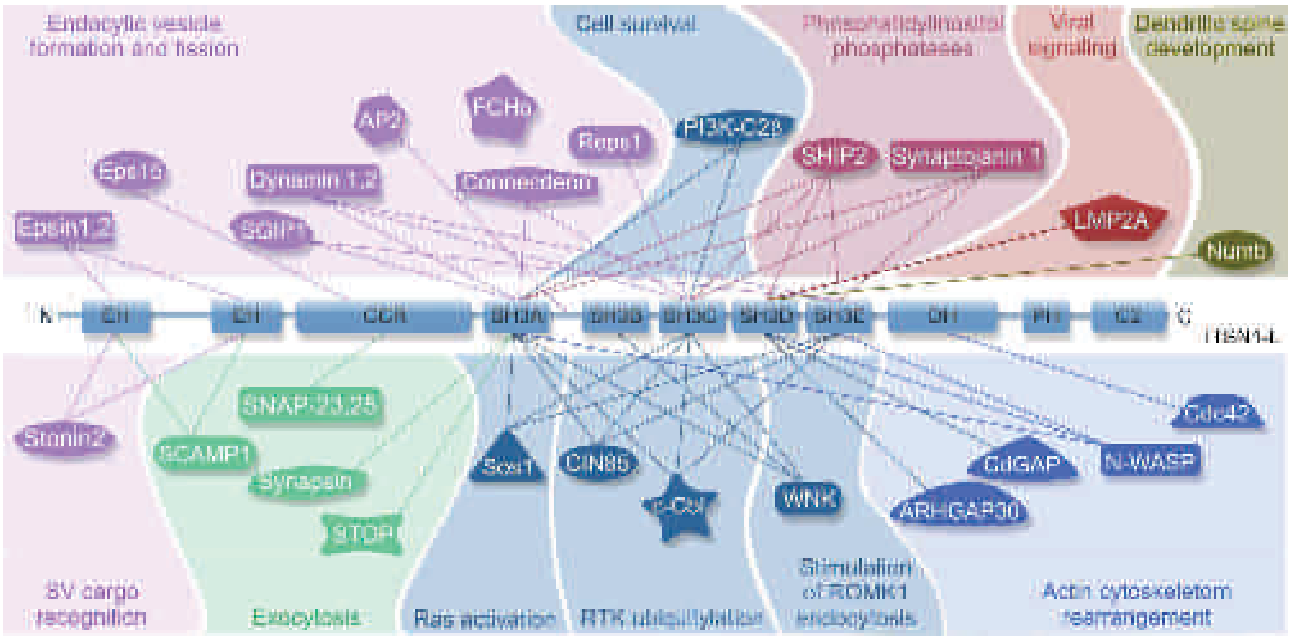

Fig. 6. Schematic representation of the proteins that interact with ITSN1 (from Tsyba et al., 2011; modified with the permission). Lines indicate the domains of ITSNs involved in these interactions. The interaction sites responsible for the binding of FCHO have not been determined.

The EH domains interact with epsin, an endocytic accessory protein (21). Epsin binds AP2, Eps15, clathrin and PIP2, inducing membrane deformation. It also promotes ubiquitinated cargo recognition (49). The other partner of ITSN1 EH domains is stonin 2, a highly conserved cargo-specific synaptic vesicle sorting adaptor, which interacts with Eps15 and synaptotagmins I and II (50-52).

Through the CCR ITSN1 forms heterodimers with Eps15 (EGF receptor pathway kinase substrate clone 15), a protein implicated in endocytosis, endosomal protein sorting and cytoskeletal organization. At CCP it interacts with ubiquitinated cargo and epsin and clusters AP2 (19 and 53).

The SH3A, C and E domains of ITSN1 interact with dynamins I and II. Dynamins are large GTPases involved in CME and other types of endocytosis by promoting membrane scission and vesicles budding (19-22). Moreover, it was shown that a perturbation of ITSN1-dynamin interaction inhibits endocytosis at the fission step in giant synapses of Lamprey. ITSN1 was also shown to regulate dynamin redistribution from SV cluster to the periactive zone (54). Synaptojanin, a phosphatidylinositol-phosphatase, involved in vesicle uncoating in neurons, is also a partner of ITSN1 SH3A, C and E domains (21 and 46). SHIP2 is another inositol-5-phosphatase that binds ITSN1 SH3 domains. It is a negative regulator of insulin signaling and involved in EGFR endocytosis. (55). SHIP2 is recruited by ITSN1early at the CCP and dissociates before fission (56).

The membrane-deforming protein SGIP1 (Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1) and the signaling adaptor Reps1 (RalBP associated Eps15-homology domain protein) interact with ITSN1 SH3 domains and all three proteins localize together at the CCP (57).

Connecdenn is another ITSN1 SH3 domain partner (58). It is a Rab35 GEF, which regulates cargo-specific exit from early endosomes and also interacts with AP2 (59).

ITSN1 interaction with WNK (With-no-lysine kinases) is crucial for WNK-dependent ROMK1 (renal outer medullar potassium 1) endocytosis in distal nephrons (60).

Finally, the linker between SH3A and B domains of ITSN1 is shown to directly interact with AP2 α- and β-appendage domains. This interaction inhibits binding of synaptojanin to ITSN1, whereas the perturbation of ITSN1-AP2 interaction inhibits the onset of SV recycling in large lamprey synapse (61). Clathrin also co-precipitates with ITSN1 from the rat brain lysate, but no direct interaction was shown (38). Finally, recently the ITSN1 interaction with FCHO proteins, the early CCP nucleators, was described (62).

Thus, with such a plethora of endocytic partners, ITSN1 appear an important player at all stages of CME: from the initiation and nucleation complex formation (together with FCHO) to the vesicle scission (with dynamin) and uncoating (synaptojanin). In addition, together with different adaptors (AP2, epsin, Eps15, SGIP1) ITSN1 promotes cargo recognition. Both overexpression and silencing of ITSN1 were shown to inhibit CME (19; 41; 63 and 64). Moreover, ITSN1 SH3A domain overexpression inhibits intermediate stages of CME (CCP formation) whereas other SH3

domains overexpression inhibits later endocytosis stages (membrane fission), which confirms ITSN1 implication throughout the CME (65).

Besides CME, ITSN1 is shown to be implicated in caveolin-mediated endocytosis, which is an important part of transcytosis in endothelial cells. ITSN1 depletion prevents caveolae fission, whereas ITSN1 overexpression leads to the abnormalities in caveolae morphology and impaires their internalization (66).

Several exocytic proteins were also reported to interact with ITSN1. The EH domains of ITSN1 bind secretory carrier membrane protein 1 (SCAMP1), which is a major component of secretory vesicles in exocrine cells (67). It was also shown to regulate the fusion pore dilation and closure in regulated exocytosis in PC12 cells (68 and 69). The CCR of ITSN1 interacts with SNAP-25 and SNAP-23, major t-SNAREs in neurons and non-neuronal cells, respectively (20). The SH3A domain of ITSN1 binds synapsin, a synaptic exocytic protein which is involved in vesicle clustering, maintaining the reserve pool, vesicle delivery to active zones, and synchronizing release events (54 and 70). Thus ITSN1-S is a potent exo-endocytosis coupling scaffold.

2.4.1.2. ITSN1 knockouts

Several knockout organisms and loss-of function mutations have been described for ITSN1. Loss-of-function mutants for *dap160* (dynamin-associated protein of 160 kDa), a drosophila homologue of ITSN1, are lethal at midlarval stage. The rescued to adult viability mutant flies are uncoordinated and display temperature-sensitive paralysis and temperature-sensitive defects in endocytosis together with impaired vesicle morphology, diminished vesicle number and an accumulation of endocytic intermediates. These mutations also lead to the decrease of dynamin, synaptojanin and endophilin levels in drosophila neuromuscular junctions. The synapses are malformed and unable to sustain high-frequency transmitter release (71 and 72). *Dap160* mutants have the same effect as *eps15* mutants (53).

In contrast to flies, C. elegans *itsn-1*is non-essential for viability (23 and 73). *Itsn-1*-null worms display normal locomotion and development under physiological conditions. The levels of EHS-1 (Eps15) and DYN-1 (dynamin) were not altered in mutant worms. But they show hypersensitivity to aldicarb, an acetylcholine esterase inhibitor that causes rapid hypercontraction and eventual death of wild-type worms. *Itsn-1*-null C. elegans also demonstrates defective endogenous synaptic activity. The motor neurons in the mutants show the accumulation of large and irregular vesicles and membrane-bound vesicles at the endocytic sites.

Itsn1-null mice are also viable, though exhibit vesicle trafficking abnormalities. However 12-13% of homozygous pups are unhealthy and often die before the age of two months. The exocytosis in chromaffin cells and the SV endocytosis in neurons are reduced. In addition, the early endosomes are enlarged in *Itsn1*-null brains (74). Recently it was also shown that in *Itsn1*-null mice the intercortical tracts failed to cross the midline. These mice also displayed severe deficits in learning and memory (75).

2.4.1.3. Function in neurons

These reports presume an important function of ITSN1 in synaptic transmission, though this function is not fully evolutionally conserved. However ITSN1 is shown to be implicated in SV retrieval in all studied models. Indeed, for many organisms presynaptic localization of ITSN1 was shown. In drosophila Dap160 is localized to the periactive zone of synaptic nerve terminals (22 and 53). In nematode ITSN1 is also detected in the presynaptic region (23). In lamprey giant synapse it is redistributed to periactive zone upon stimulation (54). However for the mammalian neurons the evidence is somewhat contradictory. Thus Thomas et al. show that ITSN1 is absent from the presynaptic terminals and is involved in dendritic endocytosis rather than in neurotransmission (64). Whereas Pechstein et al. show that ITSN1 is accumulated both at pre- and postsynaptic sites of mouse hippocampal neurons and is required for SV recycling (61). The involvement of ITSN1 in the SV endocytosis is further supported by a biochemical data, as ITSN1 has been identified as one of the core components of synaptotagmin-associated endocytic protein complex in rats. This complex is localized at the presynaptic release face in the chick ciliary ganglion calyx synapses (76). Recently it has been shown that SH3A domain of ITSN1 interacts with microtubule-associated protein STOP, which is implicated in the control of synaptic vesicles pool and synaptic plasticity (77).

ITSN1 is also involved in postsynaptic receptor endocytosis. It is a component of PSD (postsynaptic density) pellets from the rat brain (35). In C. elegans ITSN1 is shown to regulate postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors regulation and trafficking (78). In contrast to nematodes, in rat brain ITSN1 co-precipitates with NMDA(N-Methyl-D-aspartic acid or N-Methyl-D-aspartate)-type glutamate receptors (NR1 and NR2B), but not with the AMPA-type glutamate receptor GluR1 (35).

2.4.1.4. ITSN1 role in signaling.

Together with the membrane trafficking function, ITSN1 plays an important role in cell signaling. Via the SH3A, C and E domains ITSN1 interacts with Sos1 (and mSos1), a GEF for the

small GTPase Ras, and activates Ras/MAP (mitogen-activated protein) kinase pathway (79). ITSN1 is also shown to induce JNK (c-Jun N-terminal kinase)-dependent transcription factor Elk-1 activation in two different ways: Ras-dependently or by a Ras-independent mechanism (80). Indeed, the ITSN1 EH domains overexpression leads to MAPK-independent Elk-1 activation and also to the oncogenic transformation of rodent fibroblasts (81).

The SH3A, C and E domains of ITSN1 also interact with Cbl, and this interaction promotes activated EGFR ubiquitylation and degradation. Moreover, ITSN1 silencing leads to the attenuation of activation of the ERK (extracellular signal-regulated kinase)-MAP kinase pathway (63). ITSN1 also binds Ruk/CIN85, a scaffold promoting c-Cbl activation (82), thus suggesting a triple ITSN1-CIN85-Cbl complex existence, which would provide effective EGFR ubiquitylation.

The knockdown of ITSN1 leads to decrease in MEK (mitogen-induced extracellular kinase) and ERK1/2 phosphorylation and consequently induces the activation of the mitochondrial apoptotic pathway in endothelial cells (83).

ITSN1 also regulates the neuron survival through the interaction with PI3K-C2β (class II phosphoinositide-3-kinase C2β) and consequent activation of PI3K-C2–AKT survival pathway. Silencing of ITSN1 decreased N1E-115 cell survival during differentiation (84).

Thus ITSN1-S is important both for the membrane trafficking and cellular signaling.

2.4.2. ITSN1-L – a unique scaffold with catalytic activity

The long isoform of ITSN1 – ITSN1-L, which is enriched in neurons, possesses a unique for a scaffold catalytic activity – it is a GEF for the small GTPase Cdc42 (20; 31 and 38). This feature implies additional (to that of the short isoform) function at another qualitative level – a role in actin cytoskeleton regulation. For a better understanding of the ITSN1-L importance, the functions of the Rho family of small GTPases should be introduced.

2.4.2.1. Rho family of small GTPases and their regulators.

General information

The family of Rho GTPases belongs to the Ras superfamily of small GTPases. It includes the proteins of relatively small size (around 190-250 aa, exclusions – up to 700 aa). Their mechanism of activation is often compared to a molecular switch which is turned on upon binding to GTP and turned off after hydrolysis of GTP into GDP. Rho GTPases regulate actin rearrangements and thereby are implicated in all cellular functions which require this process such

as cell polarity, migration, adhesion, membrane trafficking and many others. They are also involved in cellular signaling (85 and 86).

The Rho family comprises 20 members that possess the same Rho-type GTPase-like domain. Most Rho proteins consist only of this GTPase-like domain with short N- and C-terminal extensions. The majority of them undergo C-terminal post-translational modifications by prenylation and/or palmitoylation which define their subcellular location and association with specific membranes. However some family members have additional well-defined domains and no post-translational modification (85).

Recently the phylogenetic structure of Rho family was revised. At the place of 6 subfamilies (RhoA, Rac, Cdc42, Rnd, RhoBTB and Miro) and 3 standing aside GTPases (RhoD, Rif and RhoH) (85 and 86), novel classification comprises 8 subfamilies in four clusters (Fig. 7): cluster I contains the Rho (A-C), Rnd (1-3) and RhoD/RhoF subgroups; cluster II comprises Rac/RhoG, Cdc42/RhoJ/RhoQ and RhoU/RhoV subgroups; cluster III (RhoH) and cluster IV (RhoBTB1-2). The Miro subfamily and RhoBTB3 have been excluded from the Rho family (87).

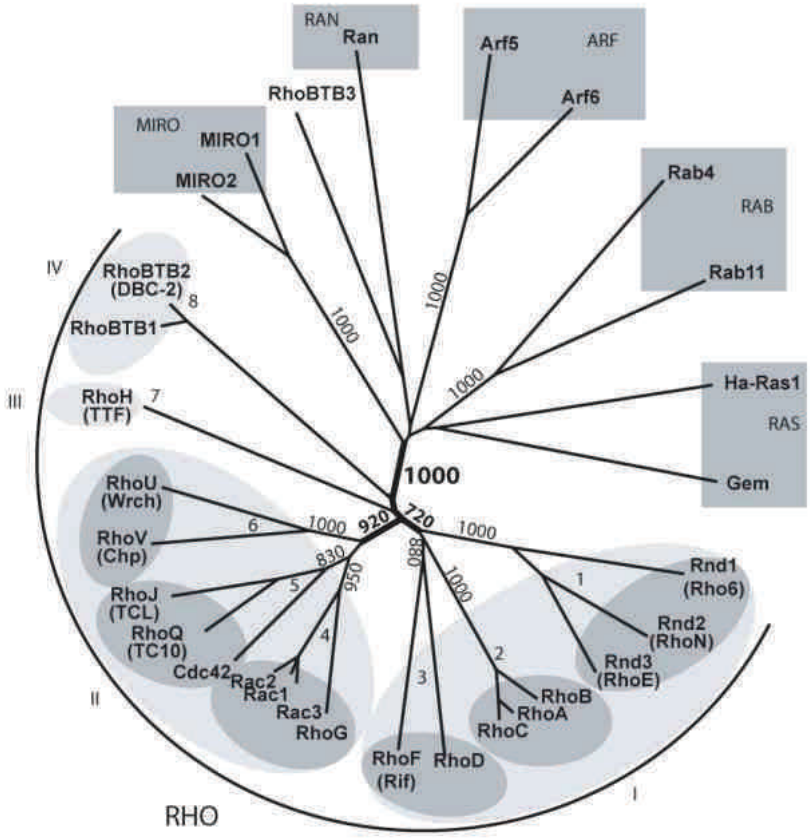

Fig. 7. Phylogenetic tree of Rho-family GTPases and representatives of other Ras superfamily GTPases (from Boureux et al., 2007).

Function

Today the most studied Rho family members are RhoA, Rac1 and Cdc42. Most Rho GTPases are implicated in actin cytoskeleton reorganization but each in different way. For example, Rac proteins induce the formation of lamellipodia, whereas Cdc42 subfamily and Rif induce the formation of filopodia. Rho-like proteins contribute to contractility and formation of stress fibers and focal adhesions, whereas Rnd1–Rnd3 activity leads to the loss of stress fibers. Rnd subfamily is also characterized by the lack of GTPase activity thereby lasting constitutively active (86 and 88).

Together with actin cytoskeleton rearrangement, the other main function of Rho GTPases is signaling (e.g. from growth factor or adhesion receptors) that regulate gene transcription, cell cycle entry, differentiation and cell survival (86). The combination of both activities (actin cytoskeleton regulation and signal transduction) allows Rho GTPases to play a key role in processes such as cell polarity and migration (89).

Rho GTPases have also important role in central nervous system development from the earliest stages. They play an important role in various aspects of neuronal development, including neurite outgrowth and differentiation, axon pathfinding, and dendritic spine formation and maintenance. They also take active part in neuronal migration. For example Rac1 functions in axonal guidance and migration, Cdc42 and Rnd subfamily are required for radial migration, whereas RhoA is downregulated in this process (90-92). Thereby abnormalities in Rho signaling are often the cause of mental retardation (93).

As for the functions of some less known Rho family members, RhoH is haematopoetic-specific and involved and T-cell signaling; RhoU and Chp1 are involved in cell adhesion and cytoskeletal dynamics, and RhoBTBs are potential tumor supressors and form part of cullin3-ubiquitinilation complex (94). As for RhoU, its structure, regulation and functions will be specifically introduced in the results section.

Role in endocytosis

Regarding the subject of my study I was especially interested in Rho GTPases function in membrane trafficking, namely endocytosis and exocytosis. At present it seems that Rho GTPases are not indispensable for clathrin-mediated endocytosis (CME), but instead it appears that they contribute to the organization and efficiency of the process. For instance, Cdc42 is implicated in early stages of CME in cells with dense cortical actin network (e.g. T-lymphocytes or apical surface of endothelial cells). RhoA and Rac1 are also involved in early stages of the CME, whereas RhoD, RhoB, TCL and probably again Cdc42 function at different stages of endosomal traffic (Fig. 8) (95 and 96). But the main function of Rho GTPases is endocytic signaling (e.g. they are involved in

epidermal EGFR and other receptor tyrosine kinases (RTK) signaling during their endocytosis) (95). There are also lots of indirect indications of Rho family involvement in CME (e.g. activation of their effectors or regulators during this process), as for example for Cdc42, the activation of its effectors Toca-1 and N-WASP (97).

Rho GTPases are also highly involved in other types of endocytosis. For example Cdc42, Rac1 and RhoA are required for type I phagocytosis, moreover Rac1 activation is sufficient for the particle internalization. Type II phagocytosis and interleukin-2 receptor clathrin-independent endocytosis require RhoA. The other type of non-CME needs Cdc42. Cdc42 and Rac1 together are important for phagocytic clearance of apoptotic cells and macropinocytosis (95).

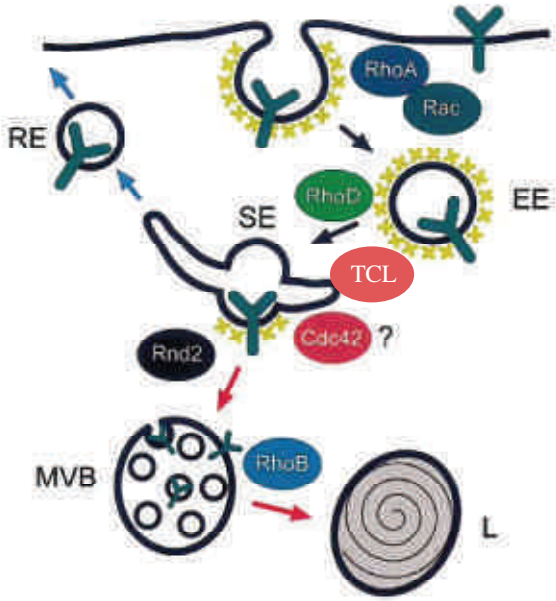

Fig. 8. Diagram summarizing the known and proposed sites of action of Rho GTPases in intracellular endocytic traffic (from Qualmann et al., 2003, modified). Abbreviations: EE, early endosome ; SE, sorting endosome ; L, lysosome ; RE, recycling endosome. Blue Y – receptor, yellow crosses – clathrin coat.

Role in exocytosis

The exact mechanism of function of Rho GTPases in exocytosis remains unclear although RhoA, Rac1 and Cdc42 are highly implicated in and even indispensable for this process. They are involved in insulin granule exocytosis (98), pancreatic digestive enzyme secretion (99), neurosecretion (100) and other types of polarized and regulated exocytosis (101). In regulated exocytosis RhoA maintains the cortical actin network that keeps the reserve pool of vesicles in near plasmalemmal zone and prevents them from docking and priming, whereas Cdc42, Rac1 and ARF6 promote actin remodeling and lipid biosynthesis at later steps of exocytosis (Fig. 9) (100-105). In polarized exocytosis (e.g. bud growing in yeast or metalloproteinases secretion in invadopodia)

RhoA together with Cdc42 are involved in vesicle tethering via the interaction with exocyst components and IQGAP (101).

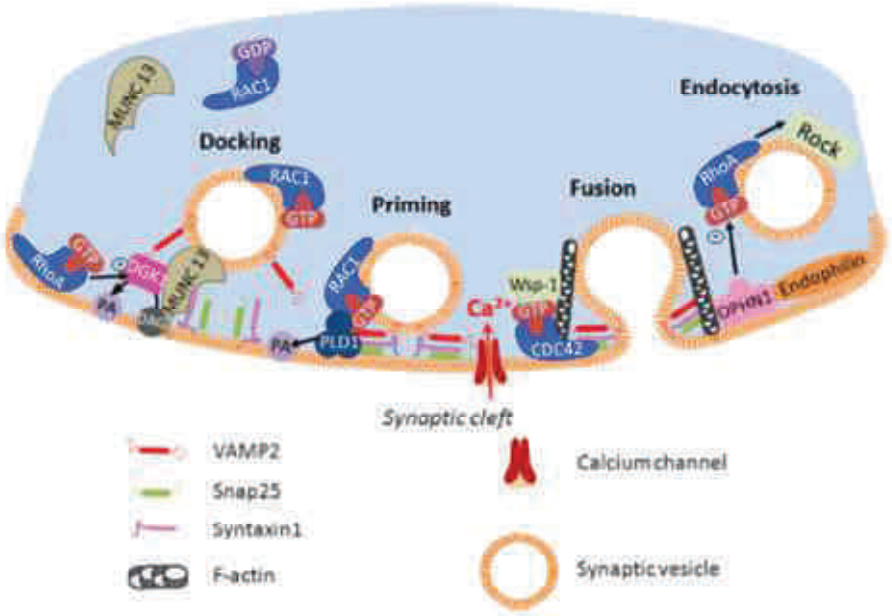

Fig. 9. Hypothetical model for the role of Rho GTPases at different stages of exocytosis in neurons (from Momboisse et al., 2011).

Implication in pathology

Due to the complexity and importance of tasks executed by Rho family GTPases, any disturbance of their function leads to a certain disorder. To date they are known to be implicated in CNS disorders (mental retardation and neurodegeneration) and cancers (their upregulated level is often observed in cancers and their hyperactivation can cause oncogenic transformation; they are also involved in cancer migration and invasion) (106-109). But it is worth mentioning that Rho GTPases are rarely mutated themselves and their dysfunction is mostly the consequence of wrong regulation. Interestingly, the manipulation of Rho GTPases is a step in a number of disease-causing bacterial infections (106).

Regulation

Each GTPase launches its own downstream cascade. But besides this direct action, there are various GTPase crosstalk events inside Rho family as well as between this family and other members of Ras superfamily. Therefore not one but multiple Ras and Rho GTPases interacting in certain order (whether antagonistic or cooperative) are required for accomplishing such complex cellular processes as migration or adhesion, signal transduction, membrane trafficking etc. This crosstalk can occur at three different levels of the pathway: through regulation of activity, through

regulation of protein expression and stability, and through regulation of downstream signaling pathways. First and third levels are assured by the diversity of upstream regulators as well as downstream targets for each Rho GTPase whose combination guarantees the accurate result (110-112).

Traditionally Rho GTPases are thought to be regulated by switching them from inactive to active state and vice versa. But recent findings have shown some additional ways, especially for less studied and atypical family members. These ways include transcriptional regulation, regulation by lipid modification and subcellular localization (86).

According to a classic regulation scheme, Rho GTPases exist in two main states: inactivated GDP-bound and activated GTP-bound which has high affinity for downstream effectors. Until now, three types of Rho regulators are known (Fig.10):

GAPs (GTPase activating proteins), which stimulate the relatively slow intrinsic GTPase activity leading to GTP hydrolysis and so switching off the GTPase

GDIs (guanine nucleotide dissociation inhibitors) which sequester GDP-bound GTPase thereby serving as inhibitors and at the same time conserving the ready-to-be-activated GTPase pool (30);

GEFs (guanine nucleotide exchange factors), which promote GDP exchange for GTP thereby serving as activators;

The GAPs provide rapid and specific inactivation of the target GTPases. More than 70 GAPs were described in eukaryotes. Their activity is often regulated by protein interactions. The GAPs are indispensable for neuronal development, cell growth and differentiation. They are implicated in Rho cross-talk, namely where the fast switching from one Rho pathway to another is required (113 and 114).

The GDIs interact with the isoprenoid moiety of the GTPase, shielding it from exposure to water by inserting it into a hydrophobic pocket in the C-terminal half of the molecule, and relocalize it from the membrane to the cytosol. Thus they act as chaperones, assuring correct GTPase folding in hydrophilic environment and maintaining a stable pool of inactive Rho GTPases. This pool may comprise 90–95% of the Rho proteins in the cell. The GDIs also protect Rho GTPases from degradation. In the answer to the specific signals, inactivated Rho GTPases may be rapidly delivered to the target membrane and reactivated. The Rho GTPases may be released from the GDIs by phosphoinositide lipids, phosphorylation, specific protein interactions or by GEFs (115).

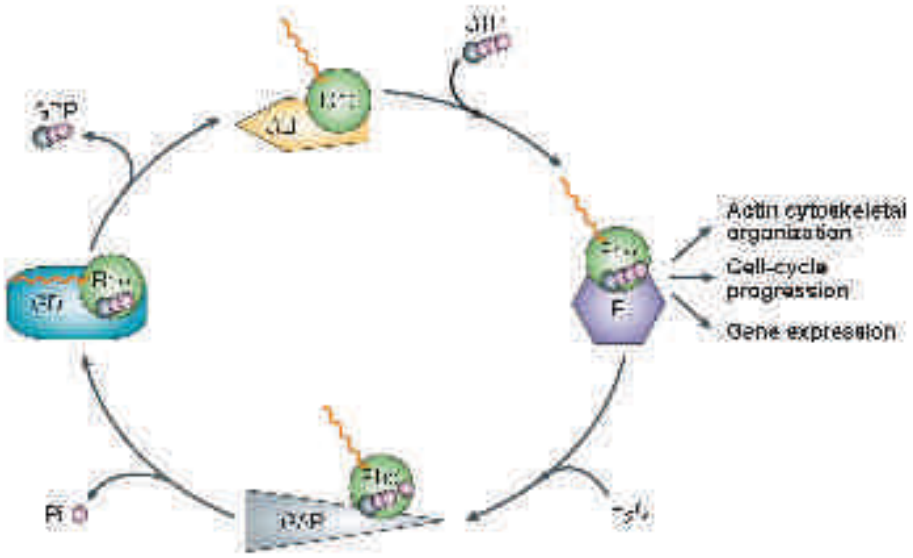

Fig. 10. Rho-GTPase activity regulation (from Rossman et al., 2005). Pi – inorganic phosphate.

Rho GEFs comprise two families, Dbl-GEFs and Dock/CZh-GEFs, which differ in the conserved domains that mediate membrane attachment and catalyze nucleotide exchange on the GTPase. Dbl family GEFs are of particular interest for my study. It comprises about 69 family members with distinct domain composition but united by the obligate presence of DH-PH domains (Fig. 11). The DH domains of the full-size proteins are often inhibited *in vivo*. Truncation, phosphorylation or protein-protein interaction (e.g. target GTPase effector binding) reveals the autoinhibition. The GEFs are also regulated by subcellular localization. Sometimes Rho GEFs possess two distinct DH domains, or an additional Ras GEF domain, or even a GTPase domain, which implies their role in the GTPase crosstalk. Rho GEFs provide fine-tuned spatio-temporal regulation of the target GTPases in key cellular processes, such as epithelial morphogenesis, cytokinesis, cell migration, cell polarity, neuronal development and phagocytosis (30; 116). Dbl GEFs misregulation was reported for some cancers, developmental and neurological disorders (e.g. faciogenital dysplasia, juvenile onset amyotrophic lateral sclerosis) (30). Some pathogenic bacteria mimic host GEFs to control host cell morphology, dynamics and metabolism (e.g. SopE/WxxxE family of bacterial type III effectors). Thus Salmonella SopE directly activates Rac1 and Cdc42 to induce membrane ruffling at the site of bacteria entry. It also provokes the host inflammatory response which helps Salmonella to outcompete the natural microbiota of the intestine (117).

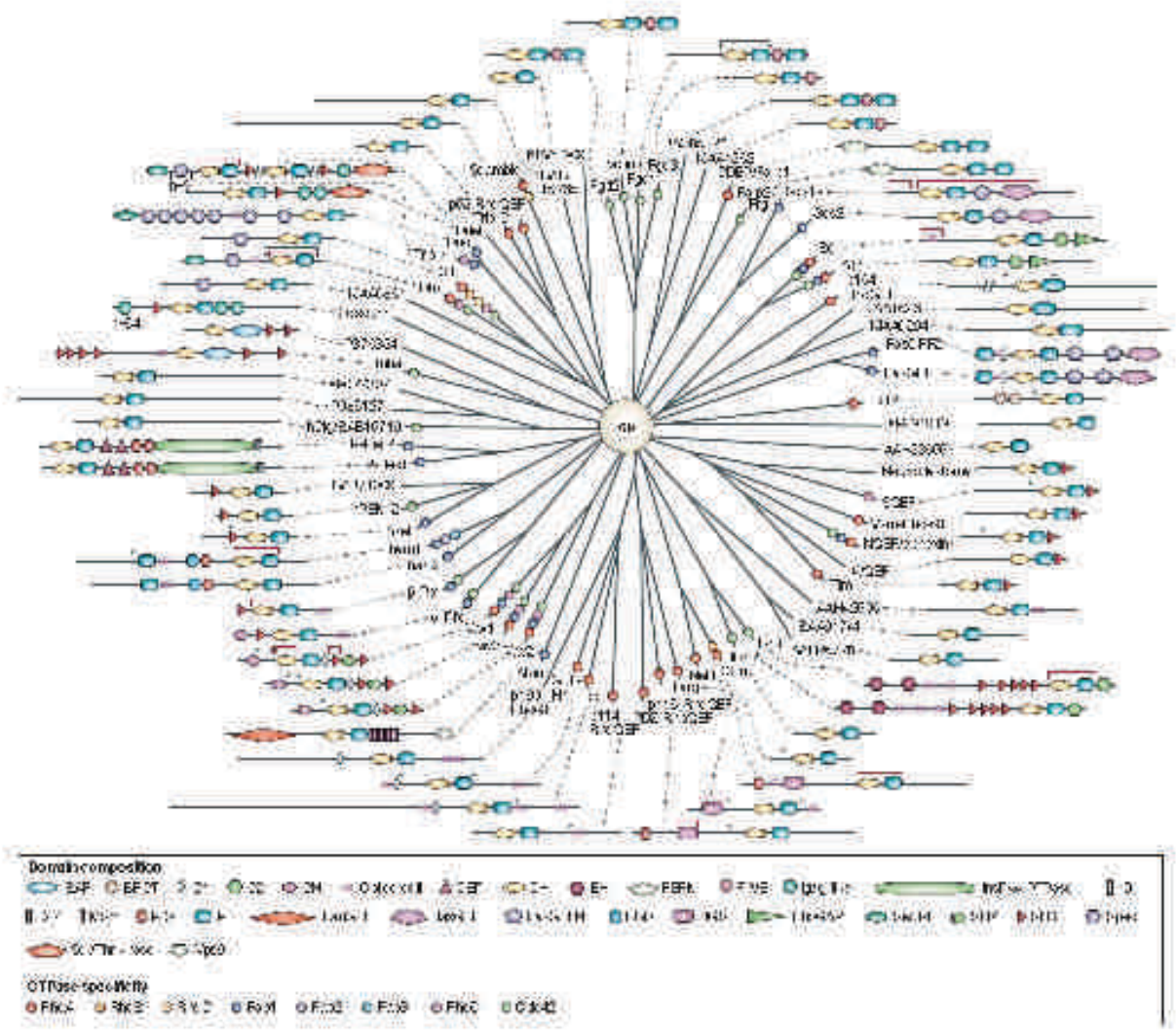

Fig. 11. The Dbl family (from Rossman et al., 2005). The Dbl homology (DH) domains of the 69 unique Dbl proteins in humans have been aligned to produce this phylogenetic tree. Dispersed around the tree branches are illustrations showing the domain composition and domain organization for each member. Black arrowheads above a GEF indicate truncations that are known to activate either the exchange activity or a related cellular function of the corresponding GEF. Black arrowheads with brackets delineate similarly active fragments. Red brackets indicate regions of Dbl proteins with known three-dimensional structures. The colored spheres placed on the tree branches designate the reported Rho GTPase specificity of the corresponding Dbl-family member.

2.4.2.2. ITSN1-L function.

As a specific GEF for Cdc42, ITSN1-L possesses several distinct functions in addition to those common with ITSN1-S.

ITSN1-L interacts with N-WASP, an effector of Cdc42, whose activation leads to actin assembling through Arp2/3 complex (Fig. 6). Moreover, N-WASP binding to the SH3 domains of ITSN1-L upregulates its GEF activity *in vivo*, resulting in actin assembly through N-WASP-ITSN1-L-Cdc42 interaction (31).

ITSN1 also interacts with two GAPs, CdGAP and ARHGAP30 (118 and 119). It was demonstrated that binding to ITSN1 inhibits CdGAP activity (118). Thus ITSN1-L appears an ideal platform for Cdc42 activation, recruiting its effector and inactivating its inhibitor.

The most well-established role of ITSN1-L together with Cdc42 is in dendritic spine development. Dendritic spines are small actin-rich protrusions from dendrites of neurons that form the postsynaptic component of most excitatory synapses in the brain. Spine growth and structural plasticity are associated with synaptic plasticity *in vitro* and learning *in vivo* (48 and 120). ITSN1-L is shown to regulate dendritic spine development together with N-WASP, Cdc42 and EphB2 receptor-type tyrosine kinase. Dominant-negative (DN) ITSN1-L overexpression (SH3 domains only or protein lacking DH-PH domains) inhibited spine formation in the same way as dominant negative Cdc42: leading to the thin and long spine formation (121). Another partner of ITSN1 SH3D domain, Numb, is also implicated in dendritic spine morphogenesis together with ITSN1-L and EphB2. It promotes ITSN1-L GEF activity towards Cdc42 (35). ITSN1-L is localized to somatodendritic regions of cultured rat hippocampal neurons. Its knock-down (similarly to the DN overexpression) inhibited somatodendritic endocytosis and disrupted spine maturation during development, leading to the formation of filopodia-like thin protrusions, whereas the number of mushroom-like spikes was significantly decreased (64).

Our group had recently described a new function of ITSN1-L as a GEF for Cdc42 in exocytosis in neuroendocrine cells. ITSN1-L activates Cdc42 at docking sites for secretory granules. This leads to the local polymerization of actin, which facilitates the late stages of exocytosis. Indeed, silencing of ITSN1 (as well as Cdc42) significantly inhibits regulated exocytosis in PC12 and primary bovine chromaffin cells whereas the overexpression of C-terminal part of ITSN1-L (DH-PH-C2 domains) promotes exocytosis and peripheral actin polymerization in neuroendocrine cells (122 and 123).

Thus ITSN1-L combines the properties of ITSN1-S, which is a prominent player in endocytosis and cellular signaling, with its own specific properties, such as a role in exocytosis and dendritic development.

2.5. ITSN1 implication in pathology.

ITSN1 misregulation is associated with a number of pathologies.

ITSN1 gene is mapped at the chromosome 21 in Down syndrome (DS) critical region and it was supposed that this gene may be implicated in DS-specific phenotype development (24). Indeed, ITSN1-L is overexpressed in the brains of DS fetuses (25). Moreover an association between the endocytic abnormalities and pathological processes in DS and early Alzheimer's disease (AD) (which usually affects DS individuals) was demonstrated. Thus it was shown that abnormal endocytosis precedes β-amyloid deposition in the brain in sporadic AD. Early endosomes are markedly enlarged in DS neurons and they are also the earliest neuropathological alteration identified in sporadic AD (124). This is consistent with ITSN1 role in endocytosis and the consequences of its overexpression. However, the exact function of ITSN1 in DS and AD is still unknown.

ITSN1 is also associated with another neurodegenerative disorder: Huntington disease (HD). HD arises from expansion of a polyglutamine (polyQ) tract in the protein huntingtin (Htt) resulting in aggregation of mutant Htt into nuclear and/or cytosolic inclusions in neurons. It was shown that ITSN1 activation of JNK-MAPK pathway increases Htt aggregates formation, whereas the silencing of ITSN1 attenuates this process. In turn, Htt inhibits ITSN1-mediated signaling in response to EGFR (39). Moreover, ITSN1 overexpression leads to another polyQ protein aggregation: the androgen receptor, which undergoes polyQ-expansion in Kennedy's disease (39). Thus it presumes the role of ITSN1 in the pathogenesis of other polyQ-associated neurodegenerative diseases.

Besides neurological disorders, ITSN1 is also implicated in cancer cells survival and migration. Thus ITSN1-S downregulation induces apoptosis of glioma cells (125). ITSN1-S is also implicated in glioma migration and invasion probably through some signaling pathways involving cofilin, LIM kinase 1, p21-activated kinase (PAK), focal adhesion kinase (FAK), integrin β1, and matrix metalloproteinase 9 (MMP-9) (126). ITSN1-S together with PI3K-C2β is involved in neuroblastoma tumorigenesis. Silencing of ITSN1 significantly inhibits the anchorage independent growth of neuroblastoma tumor cells *in vitro* as well as tumor formation in xenograft assays, an effect rescued by PI3K-C2β overexpression (127).

Recently it has been demonstrated that Epstein–Barr virus latent membrane protein 2A (LMP2A) interacts with ITSN1 together with Syk kinase and Shb adaptor. The interaction with LMP2A regulates ITSN1 phosphorylation. Thus LMP2A may regulate ITSN1-associated signaling pathways (128).

Thus ITSN1 is associated with the pathogenesis of various diseases, though the exact mechanism of its involvement remains to be elucidated. This implies ITSN1 as a prominent target to disrupt the pathological processes.

RESULTS

III. Part I: small GTPase RhoU is a new partner of ITSN1

ITSN2 has been found as a partner of RhoU by mass spectrometry analysis of a RhoU complex isolated by GST pull down (S. Ory, unpublished). ITSN2 is highly homologous to ITSN1. Furthermore the RhoU sequence analysis by Scansite, an online source for the research of protein interaction and phosphorylation motifs, gives ITSN1 SH3 domains as first hit, before Nck1, a known partner of RhoU (129). As ITSN1 was my protein of interest, I aimed at confirming and characterizing its potential interaction with RhoU.

1. Introduction: RhoU – an atypical Rho GTPase.

General properties

RhoU, also known as Wrch1 (Wnt-1 responsive Cdc42 homolog) is a Rho GTPase recently discovered as a gene responsive to Wnt-1 signaling in mouse mammary epithelial cells (130). Initially it has been considered as a member of Cdc42 subfamily as it shares 57% sequence identity with Cdc42 but phylogenic study suggested that RhoU, together with RhoV (Chp1), evolved separately of Cdc42/Rac (87). RhoU is considered as an atypical Rho GTPase because of several specific properties:

i) RhoU has 46 amino acids N-terminal extension which comprises two (mouse) or three (human) PXXP proline-rich motifs and a short C-terminal extension of 21 amino acids (130);

ii) it possesses atypical C-terminal CAAX motif (CCFV), which is palmitoylated and not prenylated. This palmitoyl moiety is necessary to its membrane targeting (131);

iii) RhoU has high intrinsic exchange activity in vitro, questioning the necessity for a GEF for its activation in cells (129).

iv) RhoU is transcriptionally regulated. It is upregulated by Wnt-1 in mouse mammary epithelial cells (130), by RANKL during osteoclast differentiation (132), by gp130 cytokines via STAT3 in mouse embryonic fibroblasts (133), by Notch in T-cell (134) or during initiation of cell migration (135) It can be also downregulated in response to 1,25$(OH)_2$ vitamin D in mesenchymal multipotent cells (136) or in response to β-estradiol in MCF-7 cells (137).

Despite these fundamental differences, RhoU shares functional homology with its closest homolog Cdc42.

Subcellular targeting

As well as for the majority of Rho GTPases, post-translational modification of its CAAX box is necessary for RhoU membrane targeting. Although it is not prenylated like most Rho GTPases, palmitoylation of residue C256 and maybe C255 is critical for RhoU subcellular distribution, localizing RhoU to the plasma membrane and endomembranes that partially colocalize with endosomes (131). Subcellullar distribution of RhoU is also regulated by Src-dependent phosphorylation of Y254 residue adjacent to palmitoylation site (138). This C-terminal phosphorylation redistributes RhoU from plasma membrane to endosomes and leads to RhoU inactivation. Phosphorylation-dependent control of Rho GTPases function has also been reported for Cdc42 and Rac1 but in their case tyrosine phosphorylation occurs on the effector loop resulting in differential binding of Cdc42 and Rac1 to GDI, preventing Cdc42 association to the plasma membrane and relocalizing Rac1 from cytoplasm to focal adhesions (139 and 140). Post-translational modifications of RhoU have been proved critical for its function. Preventing RhoU palmitoylation or tyrosine phosphorylation have antagonistic effects on cells anchorage-independent growth by respectively reducing or increasing colonies formation in soft agar assays. In addition, RhoU is localized to cell adhesion structures like podosomes in osteoclasts and c-Src expressing cells (141), and focal adhesions in HeLa cells and fibroblasts (135 and 141). Although palmitoylation is not required to taget RhoU to adhesion structures, the C-terminal extension together with the effector binding loop is critical (141).

Effectors and binding partners

RhoU shares significant homology with Cdc42 and as such, shares common effectors and activates common signaling pathways. For example, RhoU and Cdc42 interact with PAK1 (129) and the polarity protein PAR6 (142). Cdc42 and RhoU promote activation of JNK (130 and 135), cell cycle progression in Swiss 3T3 cells (130) and control tight junction assembly in epithelial cells (142). However some differences exist since RhoU does not interact with WASP, a well-known effector of Cdc42 (88). It suggests that the effector loop of RhoU is somewhat different and effectors remain to be identified. In addition, the N-terminal extension confers specific properties by binding to SH3-containing proteins such as Grb2 or Nck and by acting as an inhibitory domain by preventing PAK1 binding. Interaction of the N-terminus with the Grb2 or Nckβ SH3 domains is sufficient to relieve the inhibition and promotes RhoU binding to PAK1 (129 and 143). As Nckβ

also interacts with the RhoU effector PAK1 through its N-terminal SH3 domain, it is therefore possible that a trimeric complex of RhoU, PAK1, and Nckβ exists in the cell (129). Though the functional advantage of such a complex has not been evaluated, it could be advantageous for more rapid PAK1 activation or may serve for a spatio-temporal organization of this process. Thus, for the activation of the effectors, RhoU has to be correctly localized in the cell and moreover, the efficiency of effector binding is controlled by the partners of the N-terminal proline-rich motifs.

Role in cell migration, adhesion and polarity

RhoU expression alters the cell morphology and induces rearrangements of the actin cytoskeleton which leads to cells rounding, disappearance of stress fibers, reduction of focal adhesions number and formation of filopodia (129; 135; 141 and 144). All these processes proceed simultaneously. Their exact mechanisms remain to be elucidated but it is currently known that RhoU is directly or indirectly connected to several proteins which are involved in the control of cell adhesion and migration. Thus RhoU induces myosin light chain phosphorylation (135), interacts with the nonreceptor tyrosine kinase Pyk2 and the focal adhesion kinase (FAK) (144), binds integrin β3 (145). In fact, RhoU was shown to regulate cell migration and adhesion of osteoclast precursors (132), fibroblasts (141), pancreatic cancer cells (146), T-cell acute lymphoblastic leukaemia (T-ALL) (134) and neural crest cells (147) presumably by altering adhesion structure turnover. Besides, RhoU, like Cdc42, is required to maintain epithelial cell polarity (142 and 148).

Role in the EGFR signaling

The epidermal growth receptor (EGFR) is a receptor tyrosine kinase which, upon activation by its ligand, dimerizes, autophosphorylates and triggers downstream signaling cascades to promote cell proliferation and/or migration (149). Phosphorylated sites are recognized by specific adaptor proteins (e.g. Grb2), which in turn recruit signal transducing complexes. Ligand binding and autophosphorylation induce receptor endocytosis with subsequent signaling.

It has been shown that RhoU is implicated in EGFR signaling through the SH3 and SH2 containing scaffold Grb2. Upon activation of the receptor, RhoU is recruited to the EGFR-Grb2 complex and as a consequence enhances EGF-dependent JNK/c-Jun/AP1 activation (146). Both N- and C-terminal extensions but not the GTP-loading of RhoU are required for RhoU recruitment to EGFR complex. RhoU has also been shown to be involved in EGF-dependent cell migration (146). Thus RhoU appears involved in the coupling between receptor tyrosine kinases signaling and migration.

Interaction with GAPs

Although the requirement for a GEF to activate RhoU is disputable due to its intrinsic exchange activity in vitro, inactivation of RhoU by GAP may be an effective way to locally inactivate or recruit RhoU to specific sites (138 and 141). Two RhoGAPs, the CdGAP and its recently described paralog ARHGAP30, are found in RhoU complex (150). Intriguingly, GAP activity towards RhoU has not been tested. ARHGAP30 inactivates RhoA and Rac1, but not Cdc42. The interaction with ARHGAP30 is independent of GTP-loading and requires N-terminal extension and intact effector loop of the GTPase. Interestingly, RhoU does not bind to the GAP domain of ARHGAP30, but to the C-terminal part of the protein, presuming that ARHGAP30 is unlikely to be a GAP for this GTPase. Overexpression of ARHGAP30 mimicked the RhoU overexpression in cells, namely stress fiber dissolution, loss of cell attachment and membrane blebbing (129). These effects required both C-terminal and GAP parts of the protein. CdGAP is also involved in the loss of cell adhesion downstream of RhoU but in contrast to ARHGAP30 its interaction with RhoU is GTP-dependent (150). But even if the described GAPs do not manifest GAP activity towards RhoU (which requires a direct proof), their interaction with RhoU may involve RhoU in the regulation and cross-talk of other Rho GTPases.

Tissue expression and a role in embryogenesis

RhoU is highly expressed in brain, skeletal muscles and placenta (130). It plays important role in embryogenesis of vertebrates and is spatially and temporally regulated during this process. In chicken embryogenesis it is detected by *in situ* hybridization in the primitive streak, the somites, the neural crest cells, and the gastrointestinal tract with distinct territories and/or temporal expression windows (151). RhoU is involved in cranial neural crest cells migration in vertebrates. Neural crest undergoes an epithelio-mesenchymal transition (EMT), i.e. loss of epithelial junctions and acquisition of pro-migratory properties, invades the entire embryo and differentiates into a wide diversity of terminal tissues. RhoU depletion impairs neural crest migration and the subsequent formation of craniofacial cartilages; whereas its moderate overexpression leads to the cell polarity loss thereby the migration is not directed (147). Moreover, RhoU can also rescue RhoV(Chp1) depletion in neural crest development but not vice versa (152). In mouse embryos RhoU is important for the development of the foregut endoderm, in particular for maintaining epithelial architecture and differentiation. RhoU knocked-down embryos have abnormally flattened foregut and impaired endoderm differentiation, which is accompanied by reduced expression of c-Jun/AP-1 target genes, consistent with a role for RhoU in regulating JNK activity. The epithelial architecture of RhoU silenced embryos endoderm is disrupted, the cells are depleted of microvilli and the F-actin content of their sub-apical cortical domain is reduced, however they are still capable to

maintain cell junctions. Downregulation of *Rhou* in individual endoderm cells results in a reduced ability of these cells to occupy the apical territory of the epithelium: they seem to be squeezed out from the monolayer (148). Together these data imply a crucial role of RhoU during embryogenesis, which may be associated with RhoU atypical properties, such as tight transcriptional regulation.

The summary of RhoU structure, interactions and functions, regulation, and available mutants is presented at Fig. 12.

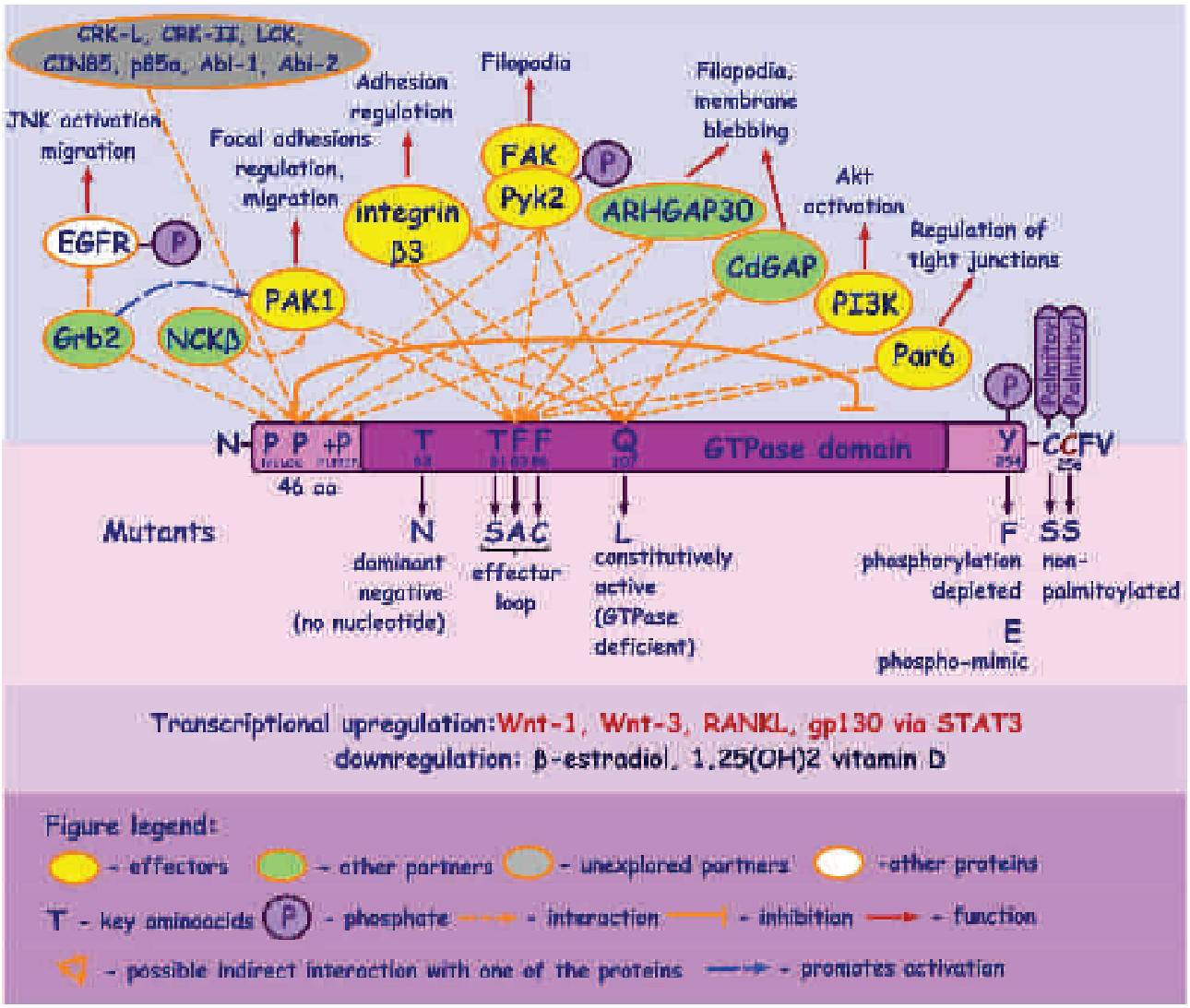

Fig. 12. RhoU structure, interactome, functions, mutants and regulation. Additional information about mutants (Tao et al., 2001): RhoU Q107L mutant is constitutively active and corresponds to RasL61 mutant, thereby lacking GTPase activity. RhoU T63N mutant is dominant negative and is identical to RasN17 mutant, thereby incapable of nucleotide binding and depleted of GTP or GDP.

One of RhoU atypical properties, namely proline-rich N-terminal extension, allows RhoU interaction with SH3-containing proteins, such as Nckβ and Grb2. Moreover, together with Grb2 RhoU is implicated in EGFR signaling, which is always accompanied by receptor internalization. It

was also shown that RhoU binds in vitro to other SH3-domain containing proteins, such as CRK-L, CRK-II, LCK, CIN85, p85α, Abi-1 and Abi-2, so RhoU might be recruited to many signal complexes (146). Together these findings support our presumption that RhoU may interact with ITSN1, which is also an SH3-scaffold implicated in receptor internalization, e.g. transferrin receptor and EGRF.

2. RhoU and its functional mutants co-precipitate with ITSN1-S, ITSN1-L in 293T cells.

In order to verify whether there is a potential interaction between RhoU and ITSN1 we performed a reciprocal co-immunoprecipitation (IP) with endogenous ITSN1-S or overexpressed ITSN1-L fused to the Omni-tag. ITSN1-S is expressed at relatively high level in 293T cells whereas ITSN1-L is expressed at low level. Interestingly, ITSN1-L cannot not be detected at all in 293 cell line, which is highly related to 293T (Fig. 13). RhoU transcript is also present in 293T cells (Fig. 28B). For this reason, we decided to choose 293T cells and to overexpress Omni-tagged ITSN1-L to obtain sufficient protein quantity to immunoprecipitate (as the amount of endogenous ITSN1-L was not enough for the IP). Moreover, we tried some α-RhoU antibodies but we could not make it work. Thus RhoU was also overexpressed as a Myc-tagged version. Anti-Omni or anti-Myc antibodies were used for IP and immunoblotting (IB) of the respective overexpressed proteins, polyclonal antibodies against the second EH domain (EH2) of ITSN1 were used for endogenous protein IP and IB.

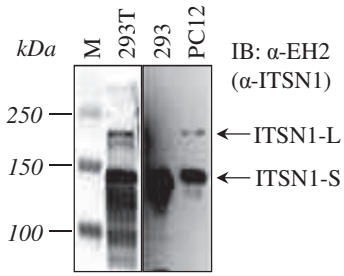

Fig. 13. Endogenous ITSN1 expression in model cell lines. Western blot of ITSN1-S and ITSN1-L levels in 293T, 293 and PC12 cell lines. Blots were probed with antibodies raised against the EH2 domain of ITSN1 (α-EH2 (α-ITSN1)).

Fig 14 shows that both endogenous ITSN1-S and overexpressed Omni-ITSN1–L co-precipitate with RhoU indicating a direct or indirect interaction between RhoU and both forms ofITSN1.

Next, we investigated whether this interaction depends on nucleotide loading of RhoU. To do so, we performed similar immunoprecipitations in cells expressing constitutively active (CA; Q107L) and dominant negative (DN; T63N) mutants of RhoU. As shown in Figure 14, both mutants co-precipitate with endogenous ITSN1-S and overexpressed ITSN1-L. Accordingly, both ITSN isoforms co-precipitate with the same efficiency with either RhoU CA or RhoU DN. These results suggest that RhoU-ITSN1 interaction occurs independently of the nucleotide-bound state of RhoU. However, it is interesting to note that upon the immunoprecipitation of ITSN1-S and -L the

level of co-precipitated RhoU DN mutant is higher than the level of co-precipitated RhoU CA or RhoU WT. We have no current explanation for that observation but one possibility might be that the dominant negative mutant of RhoU is folded in a way that enhances ITSN binding. This supposition can be proved only by a tertiary structure resolution.

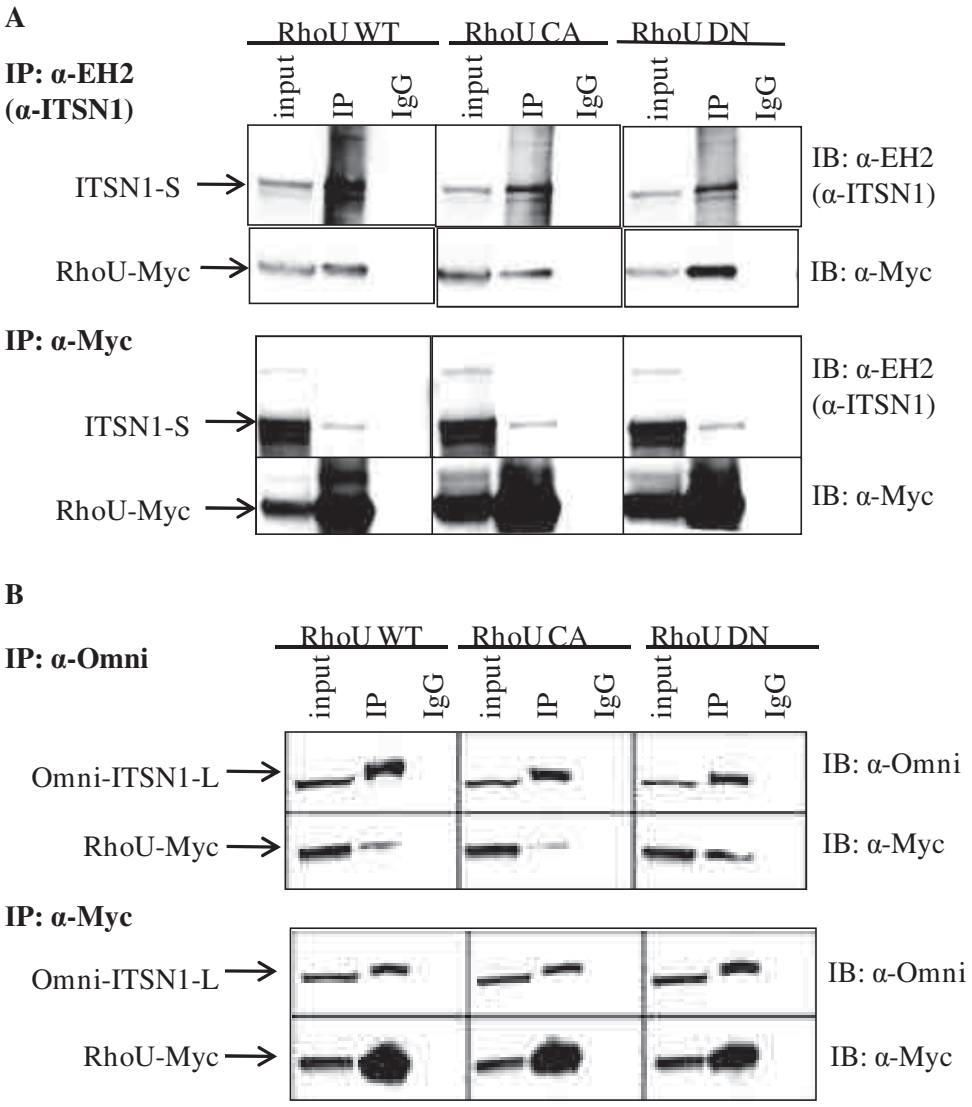

Fig. 14. ITSN1-S and Omni-ITSN1-L co-immunoprecipitate with RhoU WT as well as with its CA and DN mutants in 293T cells. A) Endogenous ITSN1 was immunoprecipitated with α-EH2 antibodies (upper panel) from 293T cells transfected with RhoU-Myc WT or its functional mutants: constitutively active (CA) and dominant negative (DN). Immunoprecipitated proteins were detected with α-ITSN1 and α-Myc antibodies. Endogenous ITSN1-S was reciprocally immunoprecipitated together with RhoU-Myc with α-Myc antibodies (lower panel). B) Omni-ITSN1-L was immunoprecipitated with α-Omni antibodies (upper panel) from 293T cells overexpressing Omni-ITSN1-L and RhoU-Myc WT, or CA, or DN. Immunoprecipitated proteins were detected with α-Omni and α-Myc antibodies. Omni-ITSN1-L was reciprocally immunoprecipitated together with RhoU-Myc with α-Myc antibodies (lower panel). IgG was used as a control for non-specific binding.

3. SH3 A, C and E domains of ITSN1 bind to the second proline-rich motif of RhoU.

Next, to define the motifs involved in ITSN1-RhoU interaction, we performed GST pull down experiments.

First, we determined which SH3 domains of ITSN1 could bind RhoU, as they are not identical and show different specificity for the ligands (alignment of ITSN1 SH3 domains at Fig. 15A). Thus we incubated GST-bound SH3 domains of ITSN1 (A, B, C, D or E) with the lysate of 293T cells, overexpressing RhoU-Myc WT (Fig. 15B). We used two alternatively spliced variants of the SH3A domain of ITSN1 (ubiquitously expressed and neuron-specific which has an additional exon 20 encoding 5 amino-acid insert) as it has been shown that these two isoforms have different specificity for proline-rich motifs of partner proteins (46). Only the SH3A (both variants), C and E domains were capable to bind to RhoU.

Secondly, to determine which proline-rich motif of RhoU is responsible for binding, we used 3 different forms of mouse RhoU containing point mutations in the proline-rich domain (146) (Fig. 15C). The mouse RhoU has only two proline-rich motifs (the human protein has three). In M1 mutant prolines 8 and 11 of the first proline-rich motif are substituted for alanines. In M2 mutant all four prolines (number 20, 21, 23 and 24) of the second proline-rich motif are substituted for alanines. Consequently, in double mutant (DM) all the six prolines are replaced. These substitutions lead to the loss of RhoU binding to its SH3 domain-containing partners (e.g. Grb2). We performed pull down assays with the GST-coupled tandem of the SH3(A-E) domains, incubated with the lysate of 293T cells overexpressing RhoU-Myc WT, M1, M2 or DM (Fig. 15D). Wild type RhoU and M1 mutant bound to GST-SH3(A-E) domains of ITSN1, whereas no interaction could be detected for M2 and the DM indicating that the second proline-rich motif of RhoU is responsible for the interaction with ITSN1 SH3A, C and E domains.

To verify if this specificity of interaction is maintained in the cell, we performed a reciprocal immunoprecipitation experiment in 293T cells expressing Omni-ITSN1-L along with the different RhoU proline-rich mutants. As shown in Fig. 16A and in agreement with pull down experiments, amount of ITSN1 precipitated with RhoU was dramatically reduced with M2 and DM mutants. Again, M1 mutations had a more modest effect. It was confirmed by a densitometric calculation of the amount of the precipitated protein with ImageJ software, based on three independent experiments (Fig. 16B). The results show fivefold decrease in interaction intensity with RhoU M2 and DM.

Even if we obtained such a drastic decrease in ITSN1 binding with RhoU DM mutant, we wanted to check whether this residual binding is mediated by the N-terminal extension. So we performed the reciprocal co-IP with RhoU ΔN, which lacks all 46 acids of N-terminal extension. The obtained results were in agreement with the previous: there was no binding at all in IP α-Omni and slightly detected bands in IP α-Myc (Fig. 16C). Thus I assumed that there is probably some additional indirect interaction between RhoU and ITSN1, which is mediated by a common partner (probably not yet identified). Another possibility is that the DH-PH domains of ITSN1-L are capable to bind RhoU. But this seems less likely for me, as the interaction of a GEF with a WT GTPase normally cannot be detected by IP.

Altogether these data indicate that proline-rich motifs of RhoU mediate the interaction with SH3 domains of ITSN1.

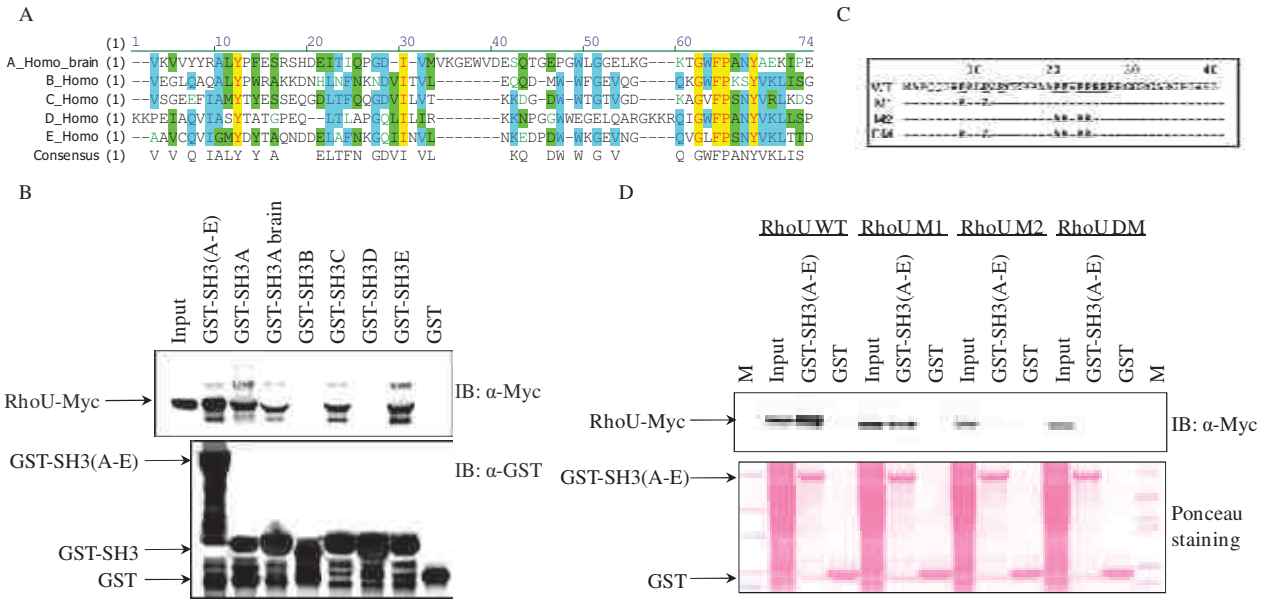

Fig. 15. SH3A, C and E domains of ITSN1 bind to the second proline-rich motif of RhoU. A) Alignment of SH3 domains of human ITSN1. The brain-specific SH3A isoform was used for the alignment. Yellow boxes denote identical amino acids (aa), blue boxes – conservative aa, green boxes – blocks of similar aa, green letters - weakly similar aa. B) Mapping of the region in the SH3 domains of ITSN1 required for binding to RhoU. Individual GST-SH3 domains (A, B, C, D and E) or all the five GST-fused SH3 domains (SH3(A-E)) were immobilized on the beads and incubated with the lysate of 293T cells, overexpressing RhoU-Myc. Empty GST was used as a control for non-specific binding. Precipitated proteins (upper panel) were detected with α-Myc antibody. GST-fusion proteins (lower panel) were visualized with α-GST antibodies. SH3A brain – neuron-specific isoform of SH3A domain of ITSN1. C) The scheme of mouse RhoU mutants for proline-rich motifs. The mutant constructions are a kind gift of Dr. Billadeau (Mayo Clinic, Rochester, MN, USA) (Zhang et al., 2011). D) Mapping of the motifs in the proline-rich N-terminal region of RhoU required for binding to ITSN1. The five GST-fused SH3 domains (SH3(A-E)) were immobilized on the beads and incubated with the lysate of 293T cells, overexpressing RhoU-Myc WT or different proline-rich mutants of RhoU-Myc (M1, M2 or DM). As in previous case, empty GST was used as a control for non-specific binding. Precipitated proteins (upper panel) were detected with α-Myc antibodies. GST-fusion proteins (lower panel) were visualized with Ponceau S staining.

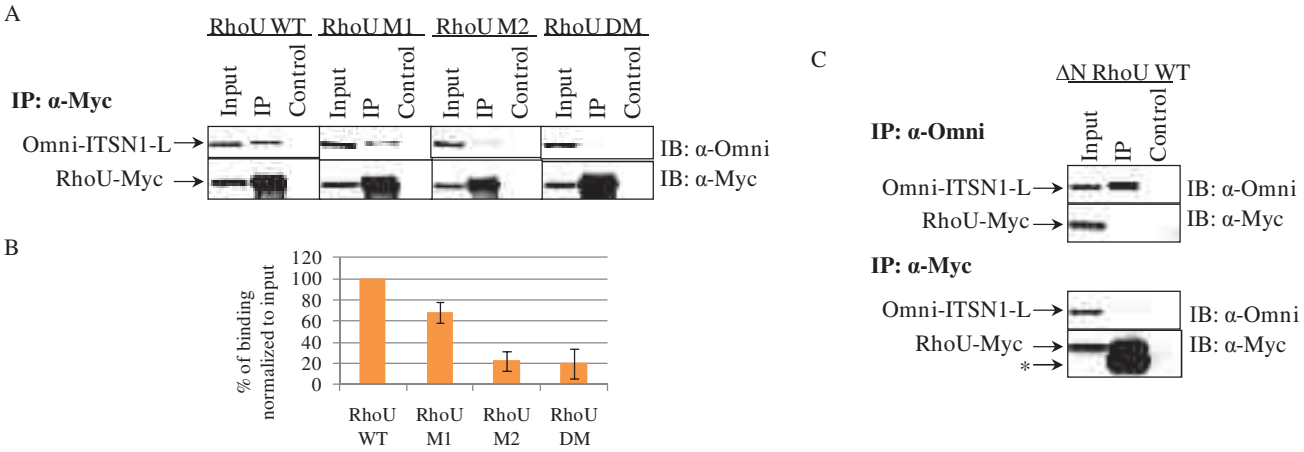

Fig. 16. ITSN1-L binds to RhoU in co-IP even in the absence of the N-terminal PxxP motifs. A) RhoU-Myc was immunoprecipitated with α-Myc antibody from 293T cells overexpressing RhoU-Myc WT or different proline-rich mutants of RhoU-Myc (M1, M2 or DM) together with Omni-ITSN1-L. IgG was used as a control for non-specific binding. Immunoprecipitated proteins were detected with α-Omni and α-Myc antibodies. B) Densitometric calculation of Omni-ITSN1-L co-immunoprecipitated with proline-rich mutants of RhoU. The results represent the average of three independent co-IPs. C) Omni-ITSN1-L was immunoprecipitated with α-Omni antibodies (upper panel) from 293T cells overexpressing Omni-ITSN1-L and ΔN (N-terminal truncated) RhoU-Myc. Immunoprecipitated proteins were detected with α-Omni and α-Myc antibodies. Omni-ITSN1-L was reciprocally immunoprecipitated together with ΔN RhoU-Myc with α-Myc antibodies (lower panel). IgG was used as a control for non-specific binding. The asterisk denotes the light chain of mouse IgG.

4. RhoU and ITSN1 colocalize in 293T cells.

To further investigate the functional relationship between RhoU and ITSN1, we analyzed the localization of RhoU-Myc and Omni-ITSN1-S or -L constructs into 293T cells (Fig. 17). It was previously described, that both isoforms of ITSN1 share similar localization at the plasma membrane, in the cytoplasm and in CCP (38; 62; 122 and 153). It was also shown that RhoU is localized at the plasma membrane, endomembranes (partially at endosomes) and in focal adhesions (131; 141 and 146). Our observations confirm previously reported subcellular distribution of both proteins (Fig. 17A-C). When coexpressed, both Omni-ITSN1-S and –L were nicely colocalized with RhoU at the plasma membrane and cytoplasmic vesicular structures (Fig. 17D, E). This observation is in agreement with our previous results, which demonstrated the interaction of two proteins.

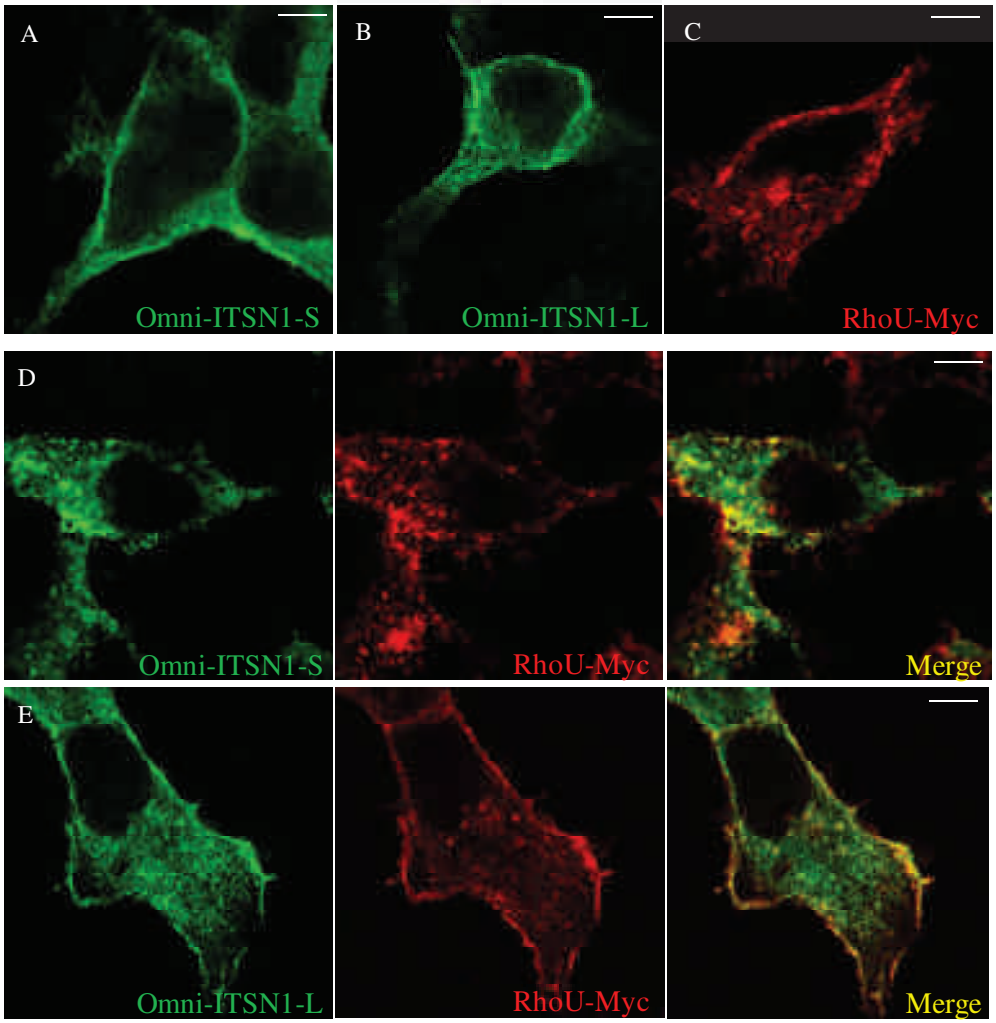

Fig. 17. RhoU-Myc and ITSN1 colocalize in 293T cells. The cells were transfected with constructions encoding Omni-ITSN1-S or Omni-ITSN1-L and/or RhoU-Myc. Omni-ITSN1-S or L were detected with rabbit α-Omni antibodies with subsequent visualization with Alexa 488-conjugated α-rabbit antibodies. RhoU-Myc was detected with mouse α-Myc antibody with subsequent visualization with Alexa 555-conjugated α-mouse antibodies. A) Subcellular distribution of Omni-ITSN1-S, B) Omni-ITSN1-L, C) RhoU-Myc expressed alone, D) Omni-ITSN1-S or E) Omni-ITSN1-L coexpressed with RhoU-Myc. Bars = 5µm. Images were taken by Leica SP5 confocal microscope.

5. Overexpression of RhoU rescues ITSN1-L induced inhibition of transferrin uptake.

What is the functional relevance of ITSN-RhoU interaction? ITSN1 has a modular structure composed of the EH and SH3 domains that are typical for scaffold endocytic proteins. Moreover, ITSN1 is known to be involved in endocytic processes as a scaffold (1; 19 and 41) and to interact with a plethora of endocytic proteins (reviewed in (48 and 154)).

Previous reports showed that ITSN1 overexpression inhibits clathrin-mediated endocytosis, presumably by sequestering different endocytic components (19 and 41). Thus I have decided to verify if the interaction with RhoU is able to compete with this process and to "rescue" this inhibitory effect on endocytosis. To do so, we assessed transferrin uptake in 293T cells expressing Omni-ITSN1-L and/or RhoU-Myc. After 30 min of internalization, we could easily detect transferrin concentrated in perinuclear vesicles known as recycling endosomes (155 and 156). We first controlled that ITSN1 overexpression was indeed inhibiting endocytosis. Unlike previous reports (19 and 41), we could not completely inhibit transferrin endocytosis. However, comparing to non-transfected cells, we observed that in 293T cells overexpressing ITSN1 (both S and L) transferrin did not accumulate efficiently in characteristic perinuclear recycling endosomes, but rather into dispersed endosomes (Fig. 18, data not shown for ITSN1-S). Possible explanations could be that ITSN1 overexpression impairs correct transferrin trafficking – or that it affects recycling endosome compartment, which should be further investigated.

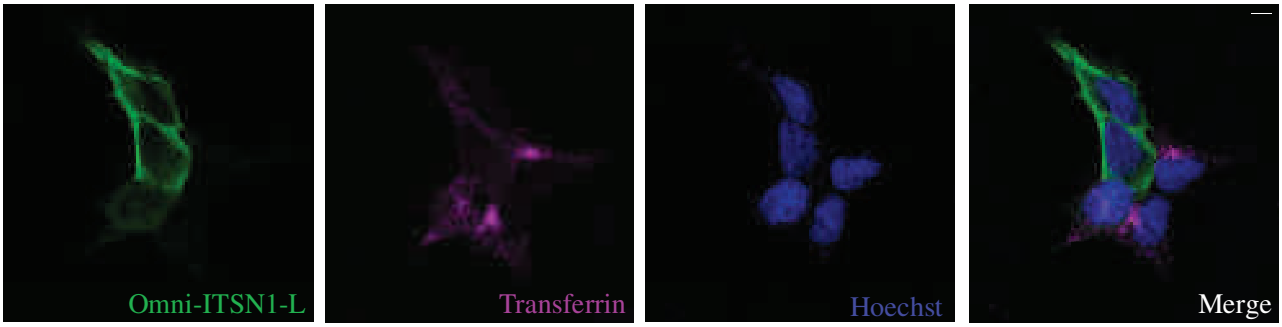

Fig. 18. ITSN1-L does not completely block transferrin uptake in 293T cells. The cells overexpressing Omni-ITSN1-L were serum starved 2h and incubated for 30 minutes in medium containing 50 mg/ml transferrin coupled to fluorescent Alexa-647. ITSN1-L was detected with rabbit α-Omni antibodies with subsequent visualization with Alexa 488 conjugated α-rabbit antibodies. Nuclei were probed with Hoechst 33258 stain. Bar = 5 µm. Images were taken by Leica SP5 confocal microscope.

In contrast, expression of RhoU together with ITSN1-L restored normal distribution of transferrin into perinuclear endosomes (Fig.19A). But, when DM mutant was expressed with ITSN1-L, transferrin kept a dispersed distribution suggesting that RhoU binding to ITSN1 was necessary to rescue transferrin uptake (Fig.19B). RhoU WT or DM expressed alone did not influence transferrin internalization (Fig. 19C, D).

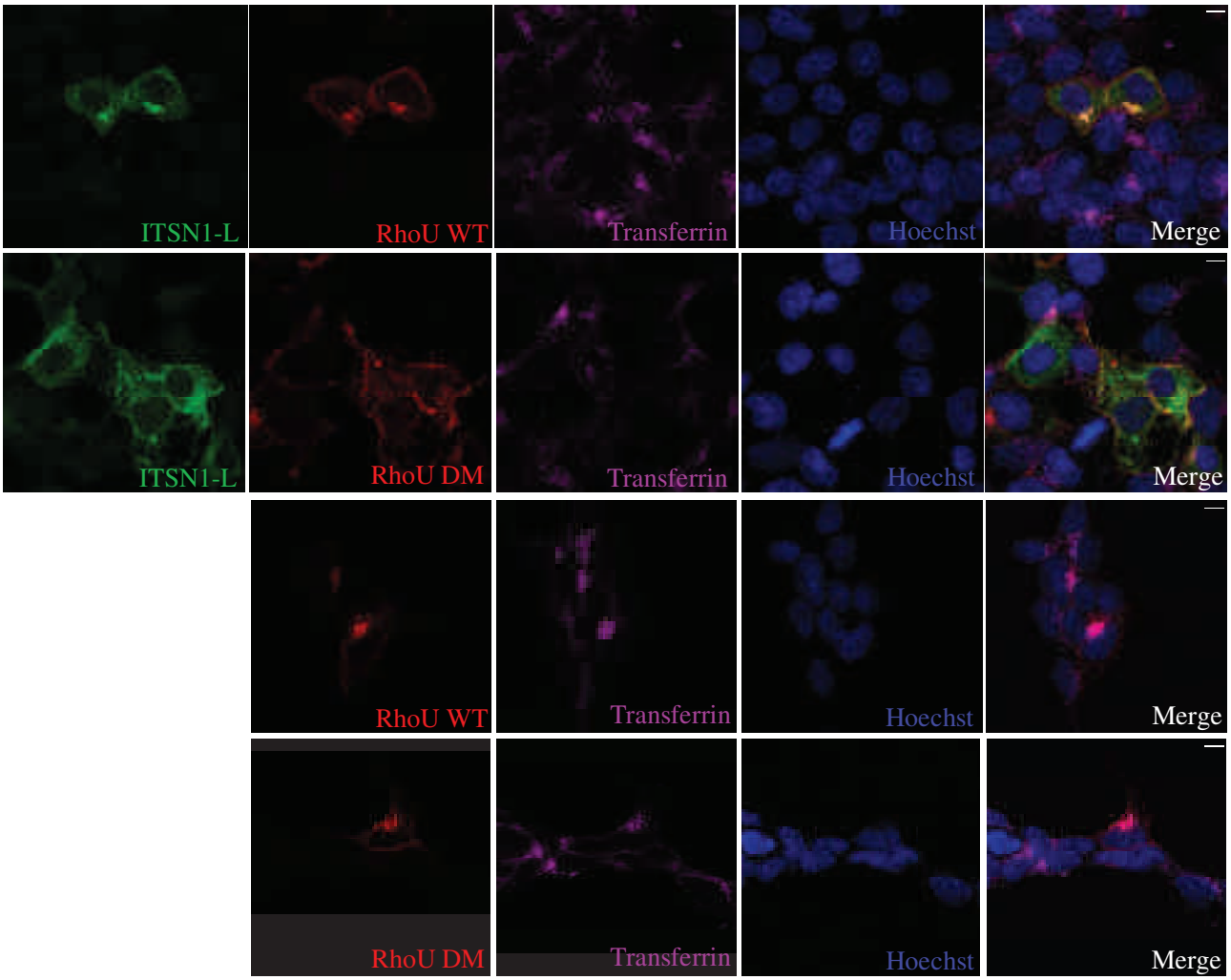

Fig. 19. RhoU WT, but not RhoU DM co-expression together with ITSN1-L rescues the inhibition of transferrin uptake. 293T overexpressing Omni-ITSN1-L and/or RhoU-Myc or RhoU-Myc DM were treated as at Fig. 17. ITSN1-L was detected with rabbit α-Omni antibodies with subsequent visualization with Alexa 488 conjugated α-rabbit antibodies. RhoU-Myc constructions were detected with mouse α-Myc antibodies with subsequent visualization with Alexa 555 conjugated α-mouse antibodies. Nuclei were probed with Hoechst 33258 stain. Bars = 5 μm. Images were taken by Leica SP5 confocal microscope.

Based on the subcellular distribution of transferrin-positive vesicles, we numbered cells with perinuclear versus dispersed transferrin (Fig. 20). Mock transfected cells showed around 60% of perinuclear transferrin positive vesicles whereas ITSN1-L reduced it to 20%. RhoU WT but not DM

RhoU restored normal distribution of transferrin. To determine whether binding to SH3 domains was sufficient to rescue transferrin uptake, we co-transfected 293T cells with ITSN1-L and CdGAP, the partner of the SH3D domain of ITSN1, and numbered cells with normal transferrin distribution. As shown in Fig. 20, CdGAP was not able to rescue transferrin accumulation in perinuclear endosomes suggesting that RhoU binding to ITSN1 SH3A, C and E domains has a specific function.

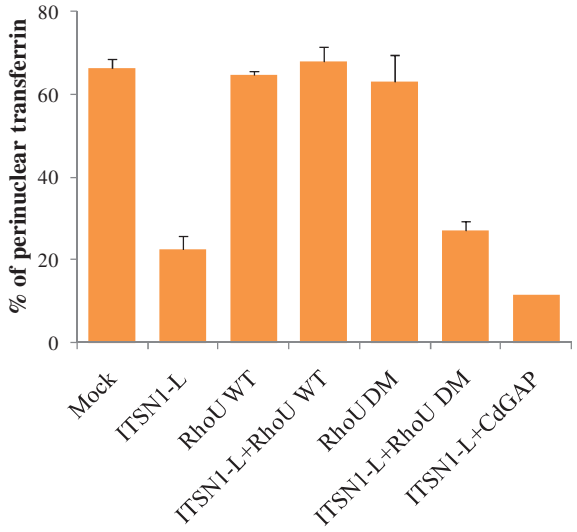

Fig. 20. Percentage of the perinuclear transferrin in 293T cells transfected with Omni-ITSN1-L and/or RhoU-Myc or RhoU-Myc DM or CdGAP. Mock-transfected cells were used as a control. At least 25 cells were probed for each condition.

In previous reports ITSN1 was shown to be localized in perinuclear Golgi-like structures but at the same time it did not colocalize with transferrin in the recycling endosomes (38 and 80), which is consistent with our observations. Interestingly, when ITSN1-L was coexpressed with RhoU WT, RhoU and ITSN1-L were found colocalized in perinuclear compartments positive for transferrin. These experiments support a model in which RhoU binds to ITSN1-L and interfere with its endocytic function; moreover, RhoU is also able to recruit ITSN1-L to the recycling endosomes.

IV. Part II: Characterization of alternatively spliced ITSN1 isoform ITSN1-LΔ35

ITSN1-LΔ35 is a minor isoform of ITSN1-L, which lacks exon 35 that encodes a part of DH domain and a part of the linker between DH and PH domains. This deletion leaves the core of the DH domain unaffected but its surrounding changes which can provoke the change in function. Thus it seemed interesting to characterize structure and biochemical properties of ITSN1-LΔ35 DH-PH domains (further called DH-PHΔ35).

1. Properties of ITSN1-LΔ35 DH-PH domains:

1.1. 3D-model of DH-PHΔ35 domains.

We have used bioinformatics to predict the most probable tertiary structure of the DH-PH domains of Δ35 isoform. This could provide us important information regarding its properties. Luckily, the group of Sondek has resolved X-ray crystal structure of the ITSN DH-PH domains (32) (Protein Data Bank (PDB) code 1ki1) (Fig. 22A, D). So we had a good and reliable structure of a very close protein to base our model on. The first step was to perform a classic ClustalW2 alignment of two sequences (Fig. 21). As templates we have used 1242 aa – 1573 aa from Snyder et al. model (GenBank Accession Number: NP 003015.2) and accordingly 1242 aa – 1405 aa of the putative ITSN1-LΔ35 protein (GenBank Accession Number: ABV24867). The gaps in the alignment correspond to the missing part encoded by exon 35 (in DH-PHΔ35 sequence) and to the unresolved residues in the DH-PH sequence. Second, we used this alignment in Modeller 9.8 program (salilab.org) (157), which is used for homology or comparative modeling of protein 3-D structures. In the resulting optimized 3D model (Fig. 22B) the core of the DH domain is unaffected (e.g. GTPase binding pocket). However, intriguingly α6 helix, which links DH and PH domain, completely disappears. In theory, since α6 helix of the full size ITSN does not interact with the GTPase (32), its deletion should not modify the nucleotide exchange activity properties of DH-PHΔ35.

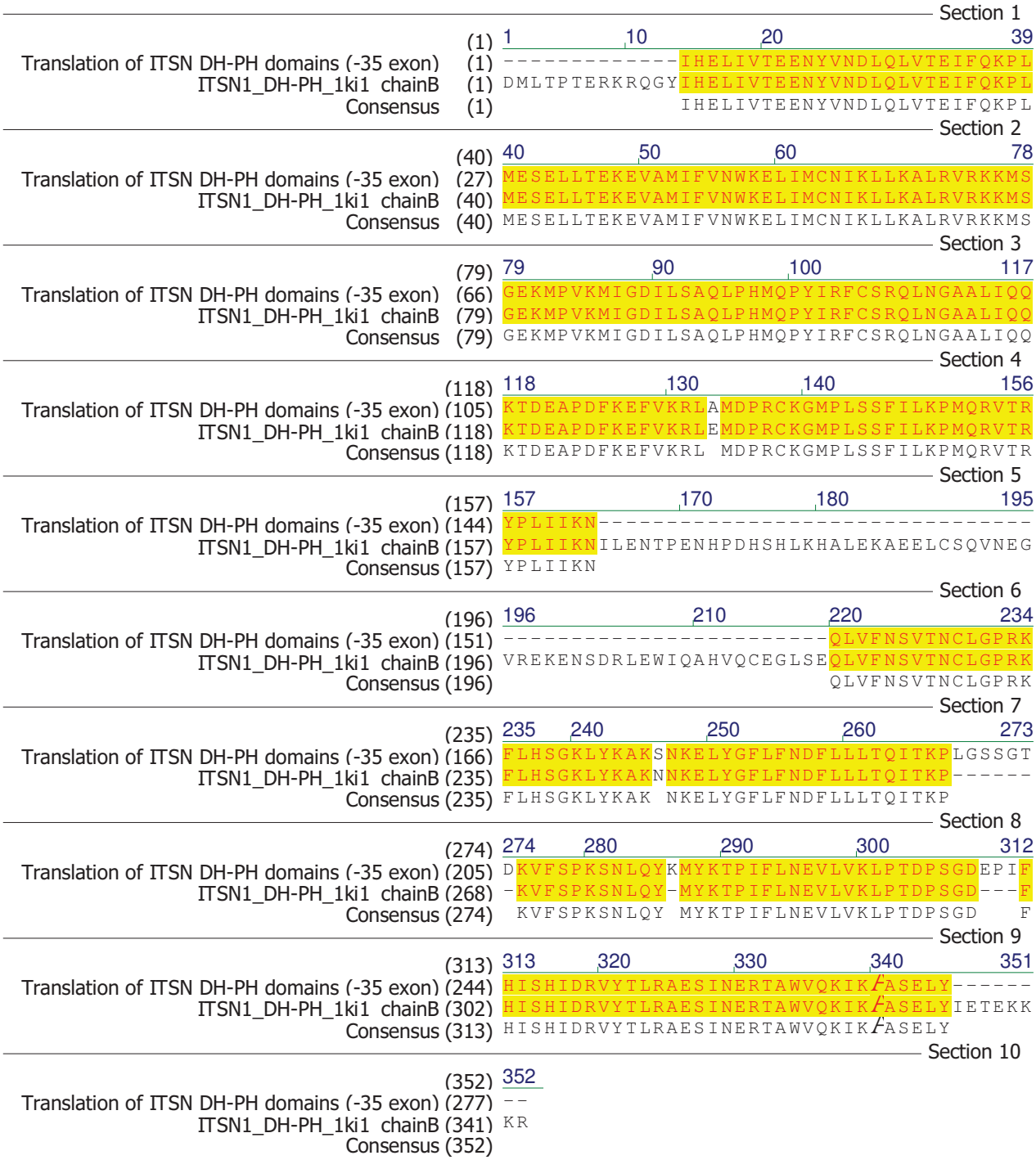

Fig. 21. ClustalW alignment of ITSN1 DH-PHΔ35 domains versus full-size DH-PH domains from X-ray crystal model (PDB entry 1ki1, chain B) from Snyder et al., 2002. The results of this alignment were used for building DH-PHΔ35 3D-model.

Interestingly, the deletion of α6 helix and a part of the DH-PH linker region results in the formation of a flexible and quite long linker between the DH and the PH domain. This may lead to

the contact of the PH domain of Δ35 with the GTPase which does not happen in full size DH-PH domains because of the long rigid α6 helix. We performed a 3D alignment in PyMol software to visualize the difference between the two structures (Fig. 22C). So we have noticed that the PH domain of Δ35 isoform could even approach to the GTPase binding pocket of the DH domain and hypothetically may even interact with DH domain. Consequently, this could interfere with the GTPase binding. But as was mentioned before, the linker between the DH and PH domains of Δ35 is so long and flexible, that if there would be no strong binding of PH and DH domains, in the solution the pH domain of Δ35 could be easily removed from the GTPase binding pocket.

Another possible consequence is that α6 helix deletion and the consequent approaching of the DH and PH domains will change the specificity of the ITSN1-LΔ35 as a GEF, as for many other GEFs PH domain play a key role in the recognition of the GTPase and its binding (32 and 158).

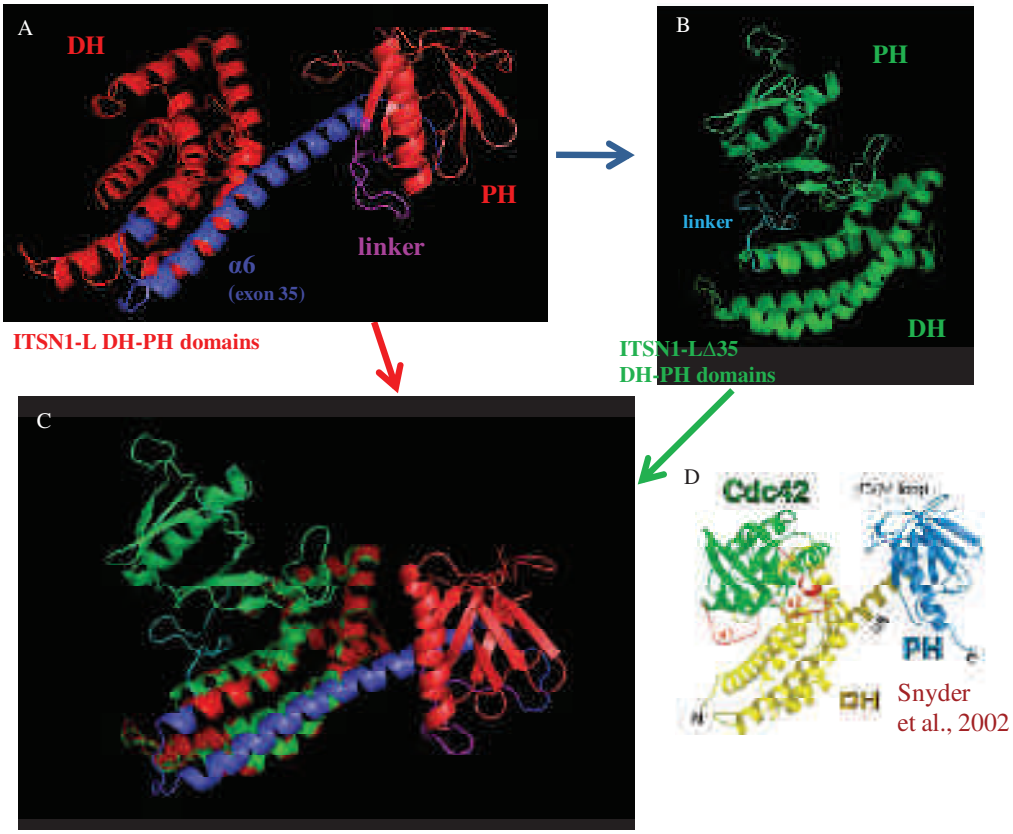

Fig. 22. 3D modeling of DH-PHΔ35 domains. A) Template DH-PH domains of ITSN1 from Snyder et al., 2002. PDB entry 1ki1. The part, encoded by exon 35, is marked blue. The linker between DH and PH domains is marked magenta. B) 3D model of DH-PHΔ35 domains. DH domain is light green, PH domain is green and the linker is blue. C) 3D alignment of DH-PH and DH-PHΔ35 domains. The colors are the same as in (A) and (B). D) DH-PH (yellow and blue) domains in association with Cdc42 (green, regions of switch 1 and 2 red) from the same article of Snyder et al., 2002.

1.2. DH-PHΔ35 domains still bind and activate Cdc42

Whether DH-PHΔ35 was still capable to bind and activate Cdc42 was an open question. To address it, we have performed a series of pull downs with GST-DH-PH or GST-DH-PHΔ35 domains and Omni-Cdc42 overexpressed in CHO cells (Fig. 23A). Both GST-coupled proteins were able to precipitate Cdc42. The Western blot densitometry calculation with ImageJ program showed that DH-PHΔ35 binds Cdc42 at a level comparable to those of DH-PH (Fig. 23B).

We have next evaluated the ability of DH-PHΔ35 to stimulate the nucleotide exchange in Cdc42. To do so, we have purified both variants of His-tagged DH-PH domains and performed Cdc42 GEF activity assay by measuring Mant-GTP incorporation (Fig. 24). When protein-bound, Mant moiety is highly fluorescent and its fluorescence fades out when it is free in the solution. It is a common mean for all present GTPase studies and it is used instead of hazardous radioactive labels. Figure 24A shows a time course of Cdc42 activation. A mix without GEF was considered as a background (a mix of buffer, Mant-GTP and 2 μM Cdc42), indicating intrinsic exchange of Cdc42. After 5 minutes of mix equilibration, appropriate GEF proteins were added (0.5 μM of Dbs DH-PH domains as a positive control; 0,62 μM of ITSN1-L or ITSN1-LΔ35 DH-PH domains). Addition of the GEF provoked an increase of fluorescence indicating the nucleotide exchange. Negative values before the GEF addition mean that in this point the fluorescence of background wells was higher than in the wells where GEF was going to be added.

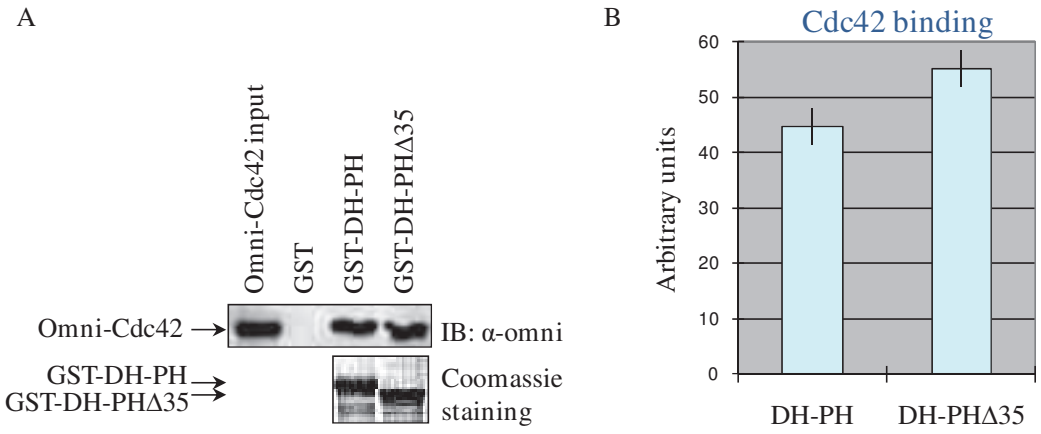

Fig. 23. DH-PHΔ35 is capable to bind Cdc42. A) GST-fused DH-PH and DH-PHΔ35 domains of ITSN1 were immobilized on the beads and incubated with the lysate of 293T cells, overexpressing Omni-Cdc42. Empty GST was used as a control for non-specific binding. Precipitated proteins (upper panel) were detected with α-Myc antibody. GST-fusion proteins (lower panel) were visualized with Coomassie staining. B) The amount of the precipitated Cdc42 was evaluated by densitometric analysis in ImageJ software and normalized to the relative quantity of GST-DH-PH domains. The graphic represents the data of three independent experiments.

As illustrated in figure 24A, no obvious differences in Cdc42 activation time course have been detected between the ITSN1-L and ITSN1-LΔ35. Moreover, by the mean fluorescence after 30 min of GEF incubation is similar for ITSN1-L and ITSN1-LΔ35 (Fig. 24B). Together our results clearly indicate that DH-PHΔ35 triggers Cdc42 activation as efficiently as the DH-PH domains of ITSN1-L, demonstrating that the deletion of α6 helix does not perturb the ITSN1-LΔ35 GEF activity towards Cdc42.

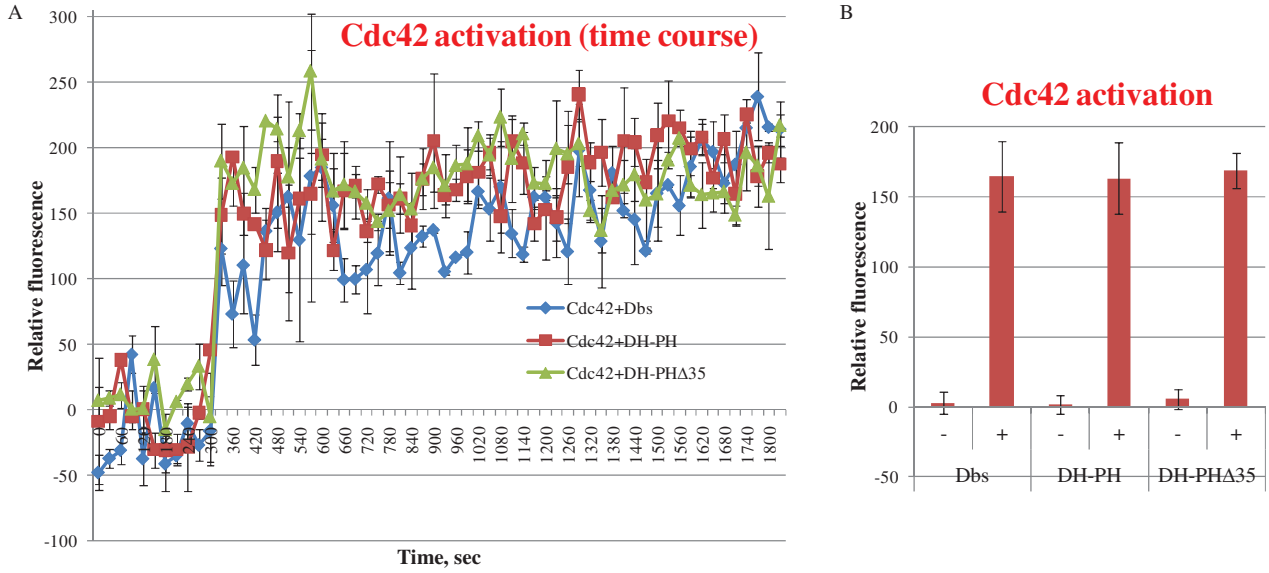

Fig. 24. DH-PHΔ35 is a GEF for Cdc42. A) Time course of Cdc42 activation by DH-PH domains of ITSN1-L (red squares) or ITSN1-LΔ35 (green triangles). As a positive control DH-PH domains of Dbs were used (blue diamonds). Intrinsic nucleotide exchange of Cdc42 was measured without addition of GEF proteins. The net contribution of GEF-stimulated nucleotidic exchange is then calculated by subtracting intrinsic nucleotide exchange values. Measurements were performed every 30 seconds during 30 minutes. Data are from four independent experiments each with three technical repeats for every condition. B) The mean fluorescence before (-) and after (+) the addition of the indicated GEF. The background without GEF is subtracted.

2. Small GTPase RhoU is a new partner of the alternatively spliced ITSN1 isoform ITSN1-LΔ35.

Next we verified our hypothesis about the change of the specificity of the DH-PHΔ35. As the core of the DH domain remained unaffected, we assumed that no drastic change in the GTPase preference should be expected (such as a preference for other Rho GTPases subfamily). Thus we decided to try Rho GTPases which are homologous to Cdc42 and not to RhoA or Rac1.

2.1. RhoU and DH-PH domains of ITSN1-LΔ35 co-precipitate in GST pull down experiments.

To check for differences in DH-PHΔ35 specificity, we have performed a reciprocal GST-pull-down assay in which purified GST-RhoU was incubated with the lysate of 293T cells expressing DH-PH or DH-PHΔ35 fused to Omni.

Interestingly, our results show that Omni-DH-PHΔ35 but not Omni-DH-PH is precipitated together with GST-RhoU (Fig. 25A). Similarly, only GST-DH-PHΔ35 and not GST-DH-PH precipitated RhoU from 293T cell lysate (Fig. 25B), confirming that DH-PH domains of ITSN1-LΔ35 specifically interact with RhoU. Further experiments are necessary to attest the GEF activity of DH-PHΔ35 towards RhoU, but the fact of interaction is however very encouraging.

To date, neither GEF nor GAP for RhoU has been identified. Because RhoU displays a high intrinsic nucleotide exchange rate, the involvement of a GEF in the activation/inactivation cycle of this GTPase has been questioned (129). However, it has been demonstrated that RhoU can be kept inactive after the translocation from the plasma membrane to the endosomal compartments (138). Moreover, expression of the constitutively active mutant RhoU Q107L clearly triggered distinguishable cellular effect from the wild type GTPase expression (142 and 143). Altogether, these observations suggest that pathways regulating RhoU activity should exist, although the cellular effect of RhoU Q107L could be also explained by probable difference in protein stability.

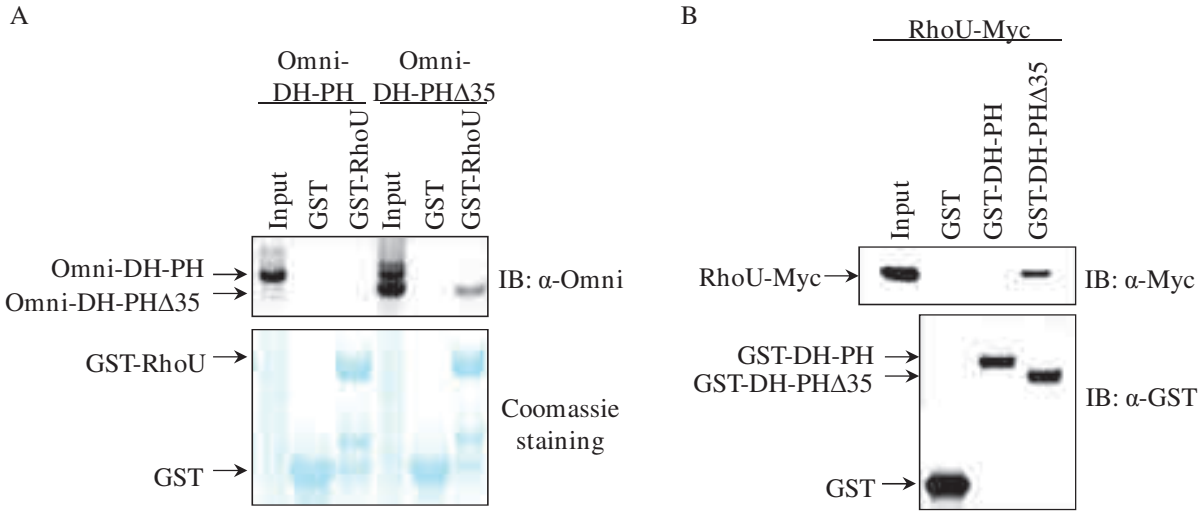

Fig. 25. RhoU specifically interacts with the DH-PH domains of the isoform ITSN1-LΔ35 (DH-PHΔ35) but not with the DH-PH domains of ITSN1-L. A) GST-fused RhoU was immobilized on the beads and incubated with the lysate of 293T cells, overexpressing Omni-DH-PH or DH-PHΔ35 domains of ITSN1. Empty GST was used as a control for non-specific binding. Precipitated proteins (upper panel) were detected with α-Omni antibody. GST-fusion proteins (lower panel) were visualized with Coomassie staining. B) GST-fused DH-PH and DH-PHΔ35 domains of ITSN1 were immobilized on the beads and incubated with the lysate of 293T cells, overexpressing RhoU-Myc. Empty GST was used as a control for non-specific binding. Precipitated proteins (upper panel) were detected with α-Myc antibody. GST-fusion proteins (lower panel) were visualized with α-GST antibodies.

2.2. RhoU co-immunoprecipitates with ITSN1-LΔ35 mostly via SH3-proline interaction

Next we wanted to verify if this interaction is strong enough to persist in the cell. So we performed an α-Myc co-IP in 293T cells overexpressing Omni-ITSN1-LΔ35 and RhoU-Myc WT or one of its proline-rich mutants (Fig. 26A) (the same as was described in section III chapter 3 for ITSN1-L). The results were exactly the same as with the main isoform ITSN1-L (Fig. 16A): Omni-ITSN1-LΔ35 readily co-precipitated with RhoU-Myc WT and even M1, but the quantity of co-precipitated protein drastically decreased in case of RhoU M2 and DM. Thus we have shown that in the cell the interaction of RhoU with ITSN1-LΔ35 is mediated by the RhoU binding to the SH3 domains of the isoform. As no significant difference of co-precipitated ITSN1-LΔ35 comparing to ITSN1-L was detected (data not shown), we supposed that these are not proper conditions to detect the DH-PHΔ35-RhoU interaction in the cell.

Though we have shown that SH3-proline interaction is dominant in RhoU-ITSN1-LΔ35 binding, however there was still a question whether the interaction of DH-PHΔ35 with RhoU is of

relevance in the cell. It is known that the GEFs have higher affinity to the depleted of nucleotide GTPase (159). Thus we performed another co-IP in 293T cells overexpressing Omni-ITSN1-LΔ35 or Omni-ITSN1-L with N-terminal-truncated (ΔN) (to exclude the binding to the SH3 domains) RhoU WT or its CA or DN functional mutants (Fig. 26B, C). The IP α-Myc results were consistent with previously obtained for ITSN1-L (Fig. 16C): in all cases a slight quantity of ITSN1 was co-precipitated probably due to some indirect interaction. Intriguingly, in α-Omni IP the ΔN RhoU DN is co-precipitated with ITSN1-LΔ35 (Fig. 26B), whereas no ΔN mutants co-precipitated with ITSN1-L (Fig. 26C). Thereby the fact that ΔN RhoU DN is precipitated with ITSN1-LΔ35 could be another evidence supporting our presumption that ITSN1-LΔ35 may appear a GEF for RhoU.

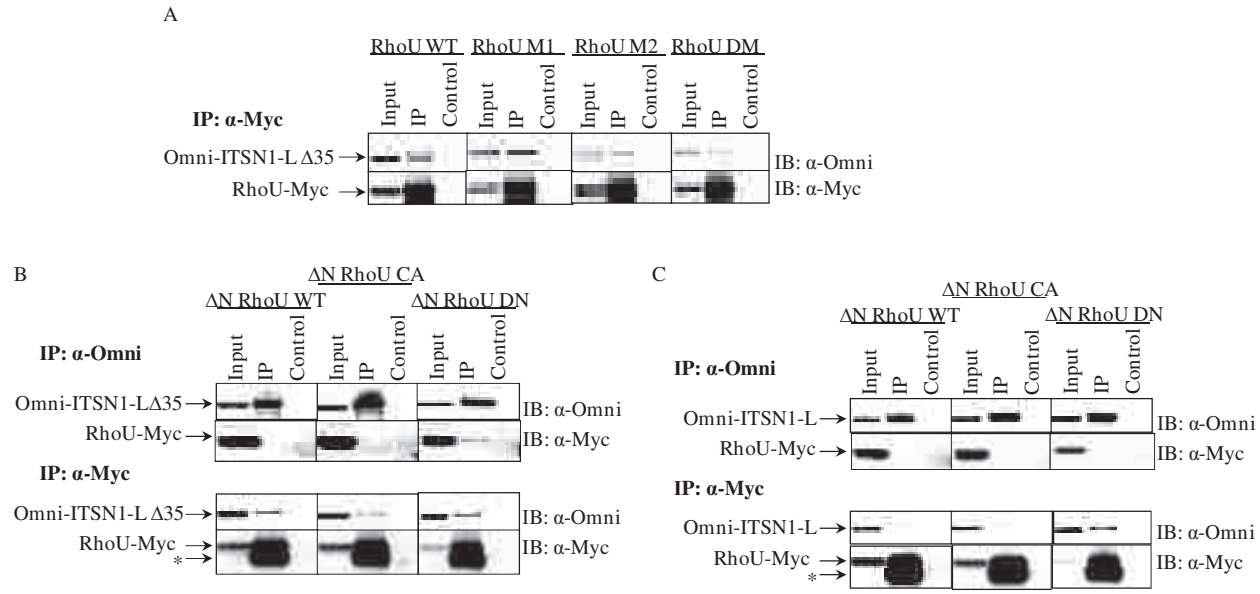

Fig. 26. ITSN1-LΔ35 binds to proline mutants of RhoU in the same way as ITSN1-L – except ΔN RhoU DN. A) RhoU-Myc was immunoprecipitated with α-Myc antibody from 293T cells overexpressing RhoU-Myc WT or different proline-rich mutants of RhoU-Myc (M1, M2 or DM) together with Omni-ITSN1-LΔ35. B) Omni-ITSN1-LΔ35 was immunoprecipitated with α-Omni antibodies (upper panel) from 293T cells overexpressing Omni-ITSN1-LΔ35 and ΔN (N-terminal truncated) RhoU-Myc WT or its functional mutants (CA and DN). Immunoprecipitated proteins were detected with α-Omni and α-Myc antibodies. Omni-ITSN1-LΔ35 was reciprocally non-specifically immunoprecipitated together with RhoU-Myc with α-Myc antibodies (lower panel). C) Omni-ITSN1-L was immunoprecipitated with α-Omni antibodies (upper panel) from 293T cells overexpressing Omni-ITSN1-L and ΔN RhoU-Myc WT, or CA, or DN. Immunoprecipitated proteins were detected with α-Omni and α-Myc antibodies. Omni-ITSN1-L was reciprocally non-specifically immunoprecipitated together with RhoU-Myc with α-Myc antibodies (lower panel). In all cases IgG was used as a control for non-specific binding. The asterisk denotes the light chain of mouse IgG.

2.3. RhoU and ITSN1-LΔ35 colocalize in 293T cells

Finally, we would like to verify if ITSN1-LΔ35 and RhoU display similar subcellular localization and whether it is different from previously observed colocalization of RhoU with ITSN1-L. To this end, we first expressed together RhoU-Myc and Omni-ITSN1-LΔ35 or Omni-ITSN1-L in 293T cells (Fig. 27 A, B). In both cases RhoU and ITSN1 colocalized near the plasma membrane and the cytoplasmic vesicular structures and we did not observe any significant difference in subcellular distribution of the proteins. Thereby the most probable is that the colocalization of all the ITSN1 isoforms (S, L and LΔ35) with RhoU is mediated by SH3 domains-proline-rich region interaction.

However we would like to determine whether the DH-PHΔ35-RhoU interaction makes any contribution to the localization of both proteins. So we performed the second colocalization assay with overexpressed GFP-RhoU and Omni-DH-PH or Omni-DH-PHΔ35 in the same cell line (Fig. 27 C, D). There was no striking difference in colocalization: in both cases we observed partial colocalization of DH-PH domains and RhoU near the membrane. There could be two possible explanations: whether *in vivo* DH-PH domain is capable to interact with RhoU or this partial colocalization is only due to a coincidence and general mistargeting of DH-PHΔ35 domains (in absence of EH domains and CCR present in full-size protein) (36) prevents DH-PHΔ35-RhoU interaction. A correctly performed GEF activity assay could answer this question.

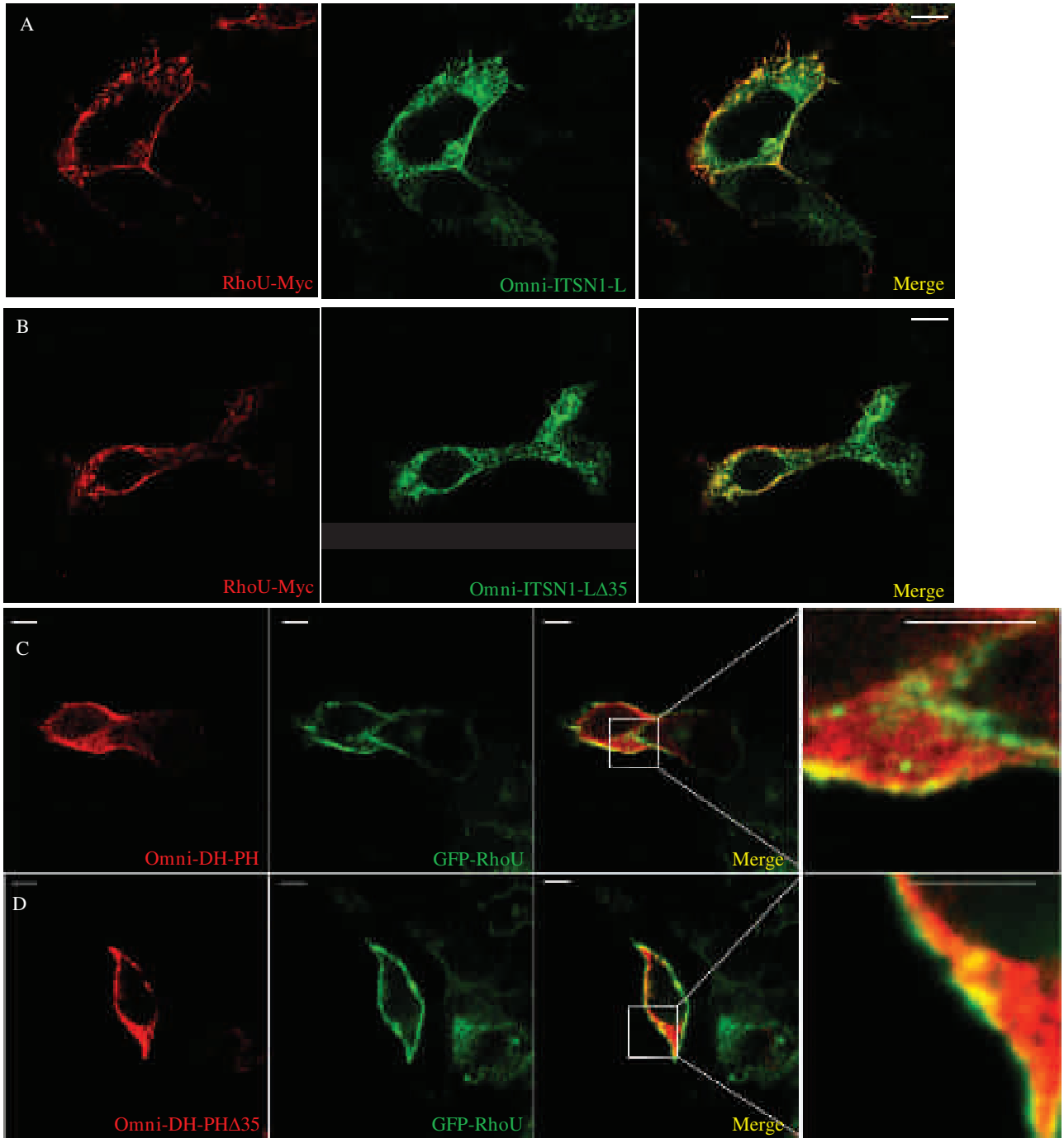

Fig. 27. The colocalization of ITSN1-LΔ35 and RhoU in 293T cells is mediated by SH3 domains-proline-rich motifs interaction. The cells were transfected with constructions encoding A) Omni-ITSN1-L or B) Omni-ITSN1-LΔ35 and RhoU-Myc. Omni-ITSN1-L and LΔ35 were detected with rabbit α-Omni antibodies with subsequent visualization with Alexa 488-conjugated rabbit antibodies. RhoU-Myc was detected with mouse α-Myc antibody with subsequent visualization with Alexa 555-conjugated α-mouse antibodies. 293T cells transfected with constructions encoding C) Omni-DH-PH or D) Omni-DH-PHΔ35 and GFP-RhoU. Omni-DH-PH and Omni-DH-PHΔ35 were detected with mouse α-Omni antibodies with subsequent visualization with Alexa 555 conjugated α-mouse antibodies. Bars = 5 μm. Images were taken by Leica SP5 or Zeiss LSM510 confocal microscope.

3. ITSN1-L, ITSN1-LΔ35 and RhoU mRNA expression in cell lines and oncogenic tumors.

Both RhoU and ITSN have been implicated in cancer-related processes. In particular, it has been shown that ITSN1 and RhoU are able to transform the cells (81; 131 and 160). Moreover, ITSN1 is implicated in maintaining glioma and neuroblastoma cells survival and migration (125; 126 and 127), whereas RhoU is involved in leukaemia cells migration (134). Therefore, we have decided to verify whether differential expression of ITSN1-L, ITSN1-LΔ35 and RhoU messengers occurs in tumor cell lines and human tumor samples.

First we have investigated the expression of RhoU and ITSN1 (both L and LΔ35) in different cell lines by reverse transcription PCR (RT-PCR). Because of low expression of ITSN1-LΔ35 we had mostly to use two additional rounds of nested PCR (three PCR rounds in total - Fig. 28A). The nested PCR allows amplifying and visualizing the target even if there are only a few copies of the transcript in the reaction mix. The inconvenience of this method is that after the third round of PCR the ratio between ITSN1-L and ITSN1-LΔ35 does not correspond to the initial ratio in the cell. All PCR products were sequenced to confirm their identity.

We have detected ITSN1-L transcripts in all tested cell lines, whereas ITSN1-LΔ35 has been found only in BON (human pancreatic cancer cell line), 293T (human embryonic kidney origin), MDA MB-231 (breast cancer) and HeLa (cervical cancer) (Fig. 28B), but not in PC12 (rat pheochromocytoma) or primary bovine chromaffin cells (BCC). Interestingly, in HeLa cells ITSN1-LΔ35 was detectable from the first round of PCR. RhoU transcript was also found in all tested cell lines: HEK293T, MDA MB-231 and HeLa.

Second, using the same RT-PCR approach, we have tested ITSN1-L and ITSN1-LΔ35 transcripts in breast, prostate or adrenal medulla (pheochromocytoma) tumor samples and RhoU transcripts in pheochromocytoma only. (Fig. 28C-E). The transcripts were present in all tested samples with no significant difference between tumor and healthy tissues. Interestingly, in most tumor samples ITSN1-LΔ35 was often detected in the first round of PCR.

However, it seems that ITSN1-L and ITSN1-LΔ35 expression levels as well as the ratio between these two transcripts (calculated when detected in the first round of PCR) could vary in cell lines and even in the same type of cancer samples. Usually, the ratio between these two isoforms of ITSN1-L in adult brain is about 10% of ITSN1-LΔ35 and 90% of ITSN1-L and seems decreasing in other tissues (42). According to our results the ratio between ITSN1-LΔ35 and ITSN1-L varies in tested samples (in tumor samples as well as in healthy tissue). However the quota of ITSN1-LΔ35 from total ITSN1-L transcripts is rarely below 10% and may reach up to 50% in certain cases (Fig.

28 C, D, E right graphics). This may indicate that ITSN1-LΔ35 transcript is more stable and resistant to nuclease degradation. Regarding RhoU transcript, its expression level in pheochromocytoma seems to be also variable (Fig. 28E). However RT-PCR is rather a qualitative method and a quantitative PCR should be performed to investigate if ITSN1 isoforms and RhoU are among genes whose expression varies in cancers.

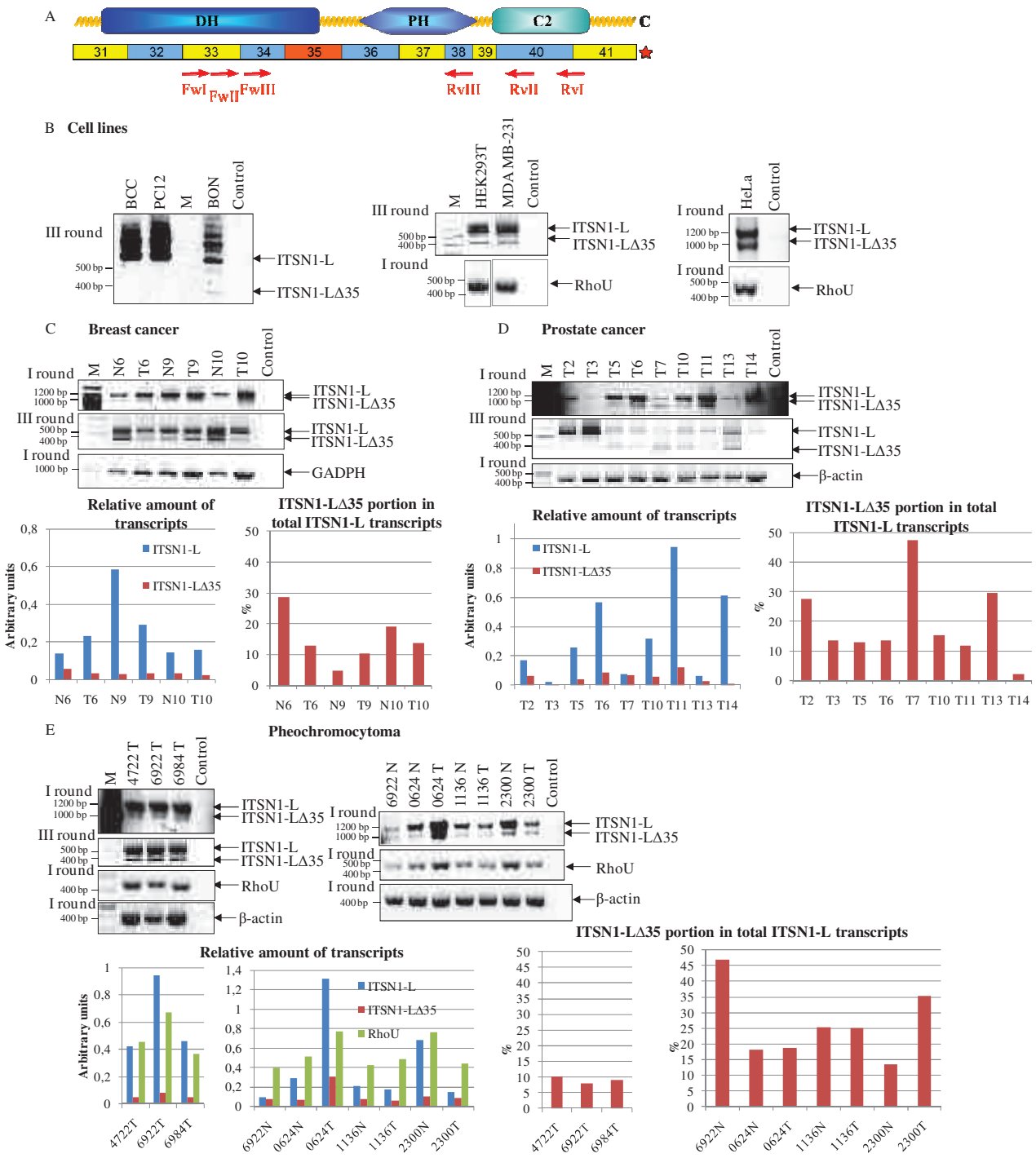

Fig. 28. ITSN1-L, ITSN1-LΔ35 and RhoU transcripts are detected in different cell lines and types of cancer tumors. A) The scheme of nested PCR for ITSN1-LΔ35 detection. Color boxes with numbers denote exons, an asterisk denotes the stop codon. Red arrows indicate forward (Fw) and reverse (Rv) primers positions for the three rounds of nested PCR (I-III). B)-E) RT-PCR (I round) and the last round of nested PCR (III round) in different cell lines (BCC – primary culture of bovine chromaffin cells) (B), breast cancer (C), prostate cancer (D) and pheochromocytoma (E). For the control of the initial material quality and reverse transcription efficiency we have used the reference mRNA of GADPH or actin. N – normal tissue, T – tumor. Controls correspond to the PCR-reaction without template added. The relative density of the bands of the I round of RT-PCR was calculated in ImageLab program. The graphics (left panels) represent relative levels of ITSN1-L, ITSN1-LΔ35 and RhoU transcripts in respective tumors normalized to the appropriate reference gene transcripts. Right panels represent a calculated portion of ITSN1-LΔ35 in total ITSN1-L transcripts.

V. Part III: GTPase activating protein oligophrenin1 (OPHN1) is a new partner of ITSN1.

This part of my work was published as a short communication in Ukrainian in the Biopolymers and Cell (See Annex-1).

1. Introduction: OPHN1 - a GTPase activating protein implicated in endocytosis and associated with X-linked mental retardation.

Oligophrenin1 (OPHN1) is a Rho GTPase Activating Protein (GAP) which was discovered as a gene associated with X-linked mental retardation (161). It consists of N-terminal BAR (Bin-Amphiphysin-Rvs) domain, PH domain, GAP domain and three C-terminal Pro-rich regions (Fig. 29A).

OPHN1 is expressed in developing spinal cord and in brain, particularly in neurons and glia cells (162). In neurons it is expressed both in pre- and postsynaptic sites (163).

As a GAP, OPHN1 effectively promotes GTP hydrolysis from RhoA, Rac1 and Cdc42 *in vitro* and *in vivo* (161 and 162). But *in vivo* its activity is regulated by the N-terminal part. OPHN1 has been largely involved in neuronal processes. For example, through its Rho-GAP activity, OPHN1 regulates dendritic spine morphogenesis (163). Besides, OPHN1 plays a critical role in the activity-dependent maturation and plasticity of excitatory synapses. It controls synapse structural and functional stability by forming a complex with postsynaptic AMPA receptors and stabilizing them. OPHN1 also selectively enhances AMPA-receptor-mediated synaptic transmission and spine size (164).

Interestingly, OPHN1 is also involved in compensatory endocytosis in neurosecretory cells. Indeed, OPHN1 regulates synaptic vesicle recycling at the presynaptic terminal by interacting with CIN85, amphiphysin II and endophilin. Endophilin targets OPHN1 to endocytic sites where the latter inactivates RhoA/ROCK pathway. Moreover, *Ophn1* knockout mice displays reduced synaptic vesicle recycling and receptor endocytosis, a phenotype that can be rescued by pharmacological inhibitors of ROCK (165 and 166). Finally, recent work obtained in our laboratory indicated that OPHN1 is also implicated in exocytosis and in compensatory endocytosis of large dense core granule membrane in neuroendocrine cells (S. Houy, unpublished).

According to the existence of possible interaction motifs in ITSN1 and OPHN1 (SH3 domains of ITSN1 and Pro rich motifs of OPHN1) and regarding their common function in exocytosis and endocytosis, it is tempting to imagine that ITSN1 and OPHN1 could interact. The

first indication of such potential interaction was given by a Scansite analysis in which the SH3A domain of ITSN1 is predicted as a binding motif for the Pro-rich regions of OPHN1. For all these reasons, we have decided to further explore this possible interaction.

2. C-terminal part of OPHN1 interacts with SH3A domain of ITSN1.

In vitro interactions were assessed by GST pull down. GST-coupled isoforms of ITSN1 SH3A domain were incubated with the lysate of PC12 cells overexpressing GFP-tagged full length or C-terminal part OPHN1 (Fig. 29A, B). We have taken two variants of the SH3A domain (ubiquitous and neuron-specific) because they are known to have different binding properties (46). Moreover, we have also used truncated mutant containing only the C-terminal region of OPHN1 (including RhoGAP and proline-rich motifs). As N-terminal part of OPHN1 is a negative regulator of the GAP activity, we speculated the possibility that the N-terminal part may interact directly with the C-terminal proline-rich domains thereby competing with the SH3 containing molecules (162).

C-terminal region of OPHN1 precipitated with both isoforms of the ITSN1 SH3A domain (Figure 29B) indicating a potential interaction between these two proteins. Full-size OPHN1 precipitated with the same efficiency demonstrating that N-terminal region of OPHN1 does not perturb SH3 domains binding to its proline-rich motifs.

Next we decided to check whether ITSN1-OPHN1 interaction is restricted only to SH3A domain of ITSN1. Thereby we repeated GST pull down with all five separate GST-coupled SH3 domains of ITSN1 (A, B, C, D, E) (Fig. 29C). Interestingly, only the two splice variants of SH3A domain precipitated full-size OPHN1 without any significant difference in binding (based on densitometry calculation, normalized to the quantity of GST protein, data not shown). Thus the SH3A domain of ITSN1 mediates its interaction in vitro with the C-terminal part of OPHN1.

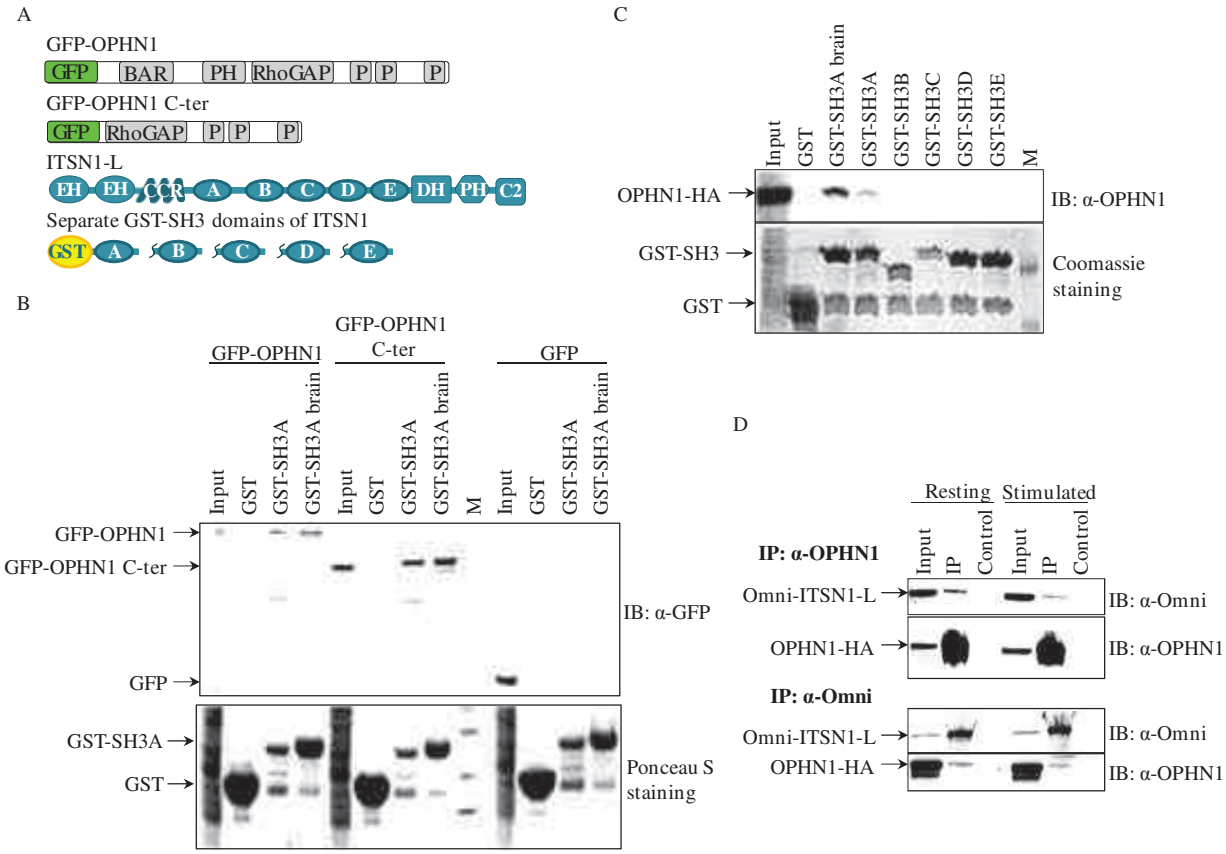

Fig. 29. ITSN1-L and OPHN1 interact *in vitro* and co-precipitate from cell lysates. A) Schematic structure of proteins used in the experiment. The constructions of OPHN1 were a kind gift of Dr. P. Billuart. B) GST-fused SH3A domains of ITSN1 were immobilized on the beads and incubated with the lysate of PC12 cells, overexpressing GFP-OPHN1 or its C-terminal part (GFP-OPHN1 C-ter). Empty GST and the lysate of PC12 cells, overexpressing empty GFP were used as controls for non-specific binding. Precipitated proteins (upper panel) were detected with α-GFP antibody. GST-fusion proteins (lower panel) were visualized with Ponceau S staining. SH3A brain – neuron-specific isoform of SH3A domain of ITSN1. C) . Individual GST-SH3 domains (A, B, C, D and E) were immobilized on the beads and incubated with the lysate of 293T cells, overexpressing OPHN1-HA. Empty GST was used as a control for non-specific binding. Precipitated proteins (upper panel) were detected with α-OPHN1 antibodies. GST-fusion proteins (lower panel) were visualized with Coomassie staining. D) PC12 cells were co-transfected with the constructions encoding Omni-ITSN1-L and OPHN1-HA. 48h after transfection cells were stimulated for exocytosis by 300 μM ATP in LOCKE for 5 minutes (Stimulated), or just rinsed with LOCKE for the same time (Resting). Immediately afterwards the cells were lysed on ice and obtained lysate was used for the reciprocal co-IP with either α-Omni or α-OPHN1 antibodies. Precipitated proteins were detected with appropriate antibodies. IgG was used as a control for non-specific binding.

3. ITSN1-L and OPHN1 co-precipitate and colocalize in PC12 cells independently of exocytosis stimulation.

Our next objective was to verify whether ITSN1 and OPHN1 form complexes in the cell. As we hypothesized that ITSN1 and OPHN1 may function together to regulate exo- and/or endocytosis in neurosecretoty cells, we have chosen PC12 rat pheochromocytoma cell line as it is a classic model of neuronal development and neurosecretion (167 and 168). We have also chosen ITSN1-L in preference of the short isoform of ITSN1, as ITSN1-L is prevailing in neurons. However, in PC12 both ITSN1-L and OPHN1 are expressed at very low level (Fig. 13, data not shown for OPHN1), so we had to overexpress both proteins.

Reciprocal co-immunoprecipitation of OPHN1 and ITSN1-L confirms that they form complexes in the cell (Fig. 29D). However there was no significant difference in quantity of precipitated protein in stimulated PC12 compared to resting condition. So it seems that ITSN1-L-OPHN1 complex formation does not depend on exocytotic activity of the cells.

A colocalization experiment performed in PC12 cells under the same conditions (resting or stimulated) was in agreement with previous results (Fig. 30). In both resting (Fig. 30A) and stimulated (Fig. 30B) cells only partial colocalization of ITSN1-L and OPHN1 was observed, mostly near the membrane or in the perinuclear region. We have also noticed that OPHN1 is relocalized to the membrane after the stimulation of the PC12, whereas ITSN1-L localization was not changed. Co-expression of the two proteins does not change their localization, as well as OPHN1 relocalization to the membrane upon exocytosis stimulation (Fig. 31).

Thus we have shown that ITSN1-L and OPHN1 interact and colocalize in neuroendocrine cells, though their common role remains to be elucidated. However, regarding known functions and partners of both proteins it seems probable that they may act together in compensatory endocytosis.

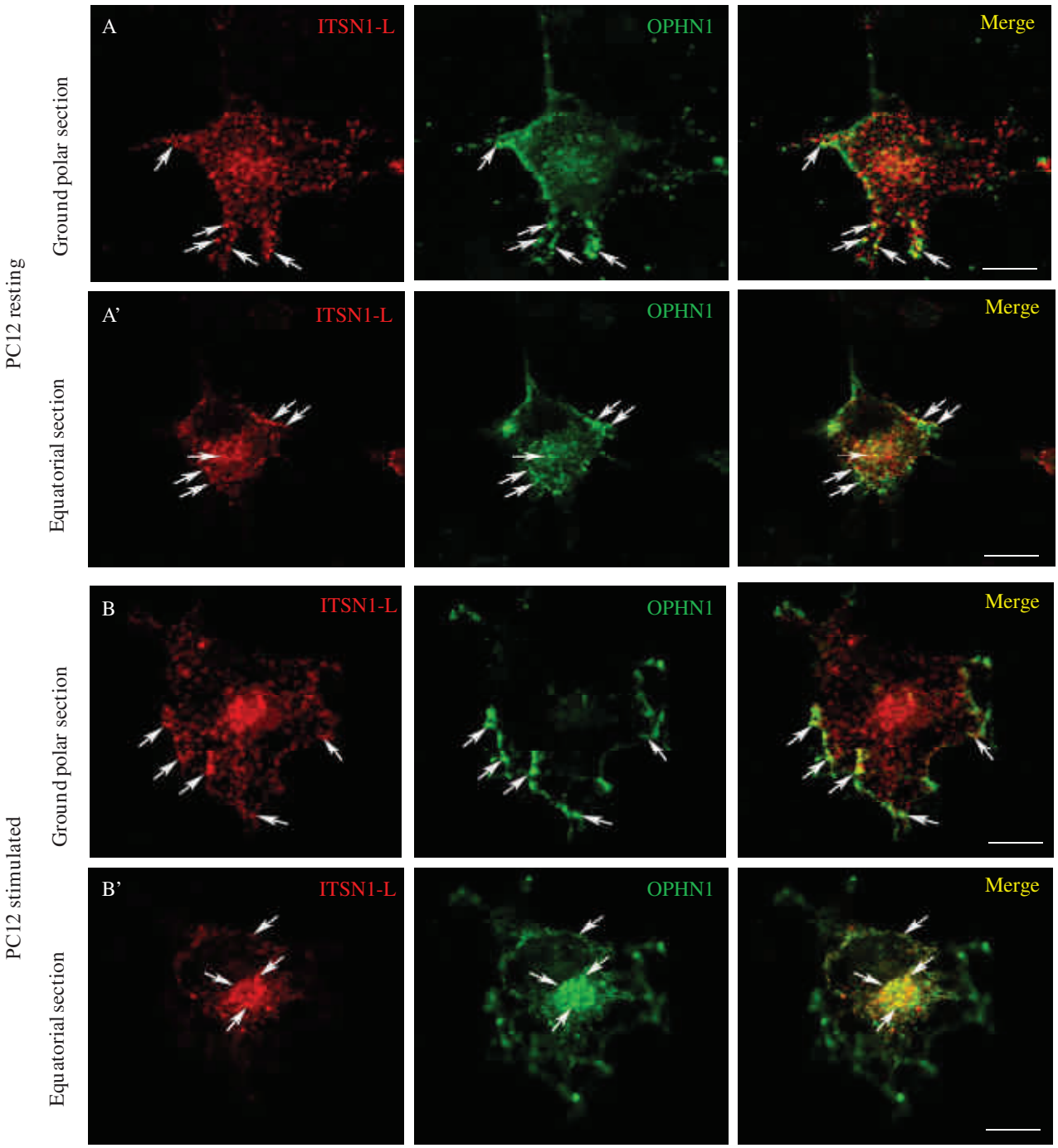

Fig. 30. ITSN1-L and OPHN1 colocalize in PC12 independently of exocytosis stimulation. The cells were co-transfected with Omni-ITSN1-L and OPHN1 and further stimulated to exocytosis (B, B') or kept resting (A, A') (as described at Fig. 28). The cells were fixed immediately after the stimulation. ITSN1-L was detected with mouse α-Omni antibody with subsequent visualization with Cy3 conjugated donkey α-mouse antibodies. OPHN1 was detected with goat α-OPHN1 antibodies with subsequent visualization with Alexa 488 conjugated donkey α-goat antibodies. Two confocal slices were taken for each cell: through the ground polar section (A, B) and through the equatorial section (A', B'). Bars = 5 μm. Images were taken by Leica SP5 confocal microscope.

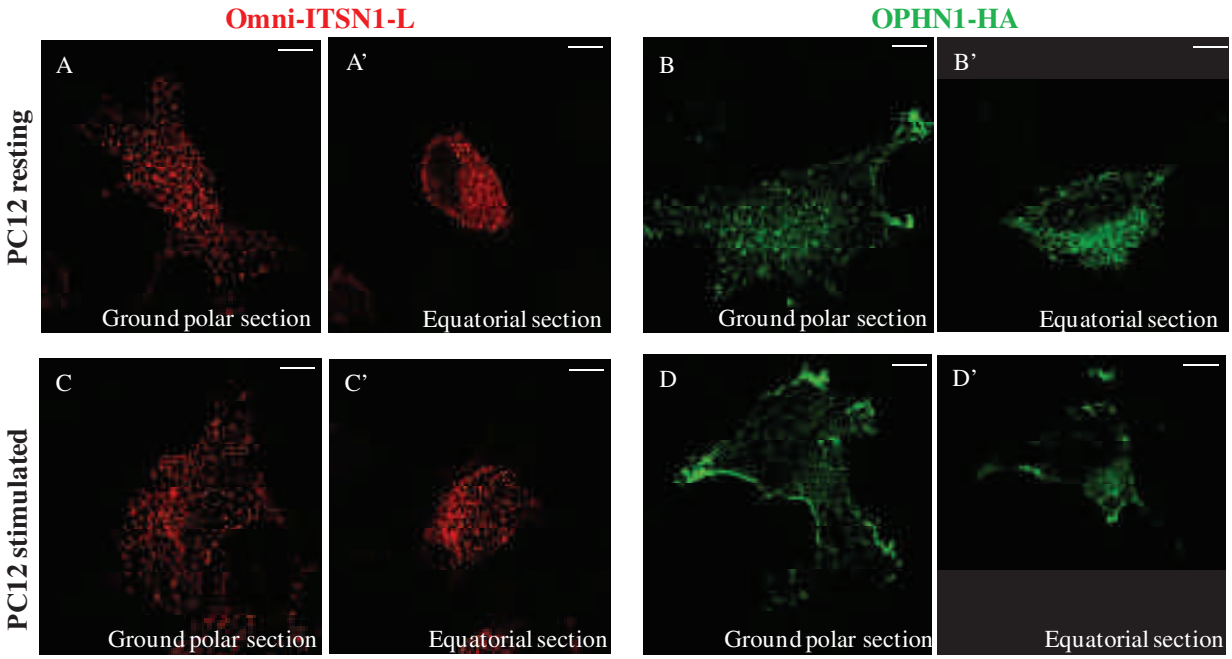

Fig. 31. Co-expression does not change the subcellular localization of Omni-ITSN1-L (A, A', C, C') or OPHN1-HA (B, B', D, D'). The cells were transfected with Omni-ITSN1-L or OPHN1-HA and further stimulated to exocytosis as described previously (C, C', D, D') or kept resting (A, A', B, B'). ITSN1-L was detected with mouse α-Omni antibody with subsequent visualization with Cy3 conjugated donkey α-mouse antibodies. OPHN1 was detected with goat α-OPHN1 antibodies with subsequent visualization with Alexa 488 conjugated donkey α-goat antibodies. Two confocal slices were taken for each cell: through the ground polar section (A, B, C, D) and through the equatorial section (A', B', C', D'). Bars = 5 μm. Images were taken by Leica SP5 confocal microscope.

VI. Part IV: ITSN1-S and L form complexes in cells.

This part was published in English as a short communication in Biopolymers and Cell (See Annex-2).

ITSN1 domain composition implies that it is a scaffold protein. These proteins possess multiple modular interaction domains and play a crucial role in spatial and temporal organization of cellular processes. Scaffolds regulate selectivity in signaling pathways using tethering mechanism and physically assembling chosen components of signaling pathway or network (18). It is known that some scaffold proteins dimerize or even oligomerize to perform their function (169 and 170). Our Ukrainian group has recently shown that ITSN1-S forms a heterodimer with its minor isoform 22a (47 and 171), so we presumed that probably other isoforms of ITSN1 are also able to interact with each other thereby assembling protein supercomplexes.

In order to prove the existence of ITSN1 macromolecular complexes in cells, we have performed a co-IP assay in different mammalian cell lines, transfected by Omni-ITSN1-L (Fig. 32). In all tested cell lines (PC12, 293 and 293T) endogenous ITSN1-S readily co-precipitated with recombinant Omni-ITSN1-L suggesting the existence of heterodimers. 293T cell line was of particular interest as it expressed endogenous ITSN1-L at relatively high level in contrast to 293 cell line (Fig. 13). In addition to full-size ITSN1-L protein in 293T cells a minor band with slightly lower molecular weight is present, which is presumably one of the possible alternatively spliced isoforms of ITSN1-L. The bands below the ITSN1-S can also refer to one of its minor isoforms, e.g. ITSN1-22a (47 and 171). Note that these minor isoforms also co-precipitate with overexpressed ITSN1-L suggesting that ITSN1 can form heterodimers of different isoform composition (Fig. 32B). However it requires direct evidence. Isoform-specific antibodies would be of use to prove this presumption.

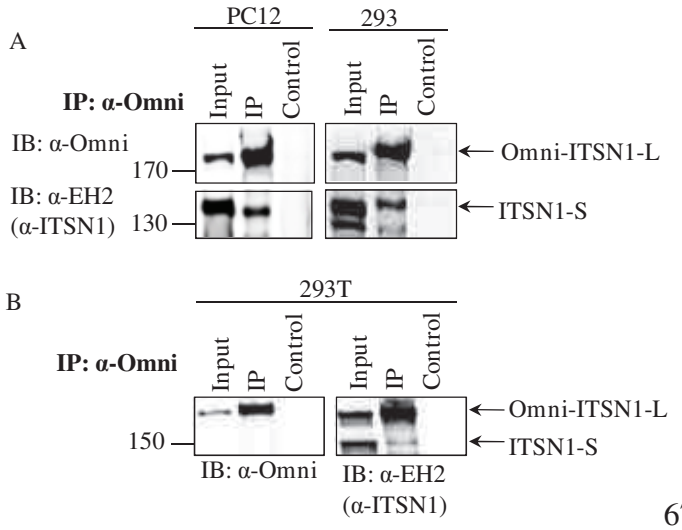

Fig. 32. ITSN1-L and ITSN1-S co-precipitate in mammalian cells. PC12 and 293 cells (A) or 293T cells (B) were transfected with Omni-ITSN1-L and subjected to α-Omni IP. Co-precipitated endogenous ITSN1-S was detected with α-EH2 (α-ITSN1) antibodies.

To explore whether ITSN1-S and ITSN1-L have similar subcellular localization, we overexpressed GFP-tagged ITSN1-L and mCherry-tagged ITSN1-S in 293T cells (Fig. 33A). Both ITSN1-S and ITSN1-L shared similar subcellular distribution and in agreement with previous observation were localized in cytoplasmic vesicular structures as well as in the cytoplasm. The distribution of the proteins was unchanged in the case of coexpression (Fig. 33B, C). Our data clearly indicate that both isoforms perfectly colocalize in 293T cells.

So we have demonstrated that ITSN1 isoforms interact in mammalian cells, together with their partners forming huge complexes of varying protein composition. ITSN1 complexes can function as large platforms in membrane trafficking as well as in cell signaling.

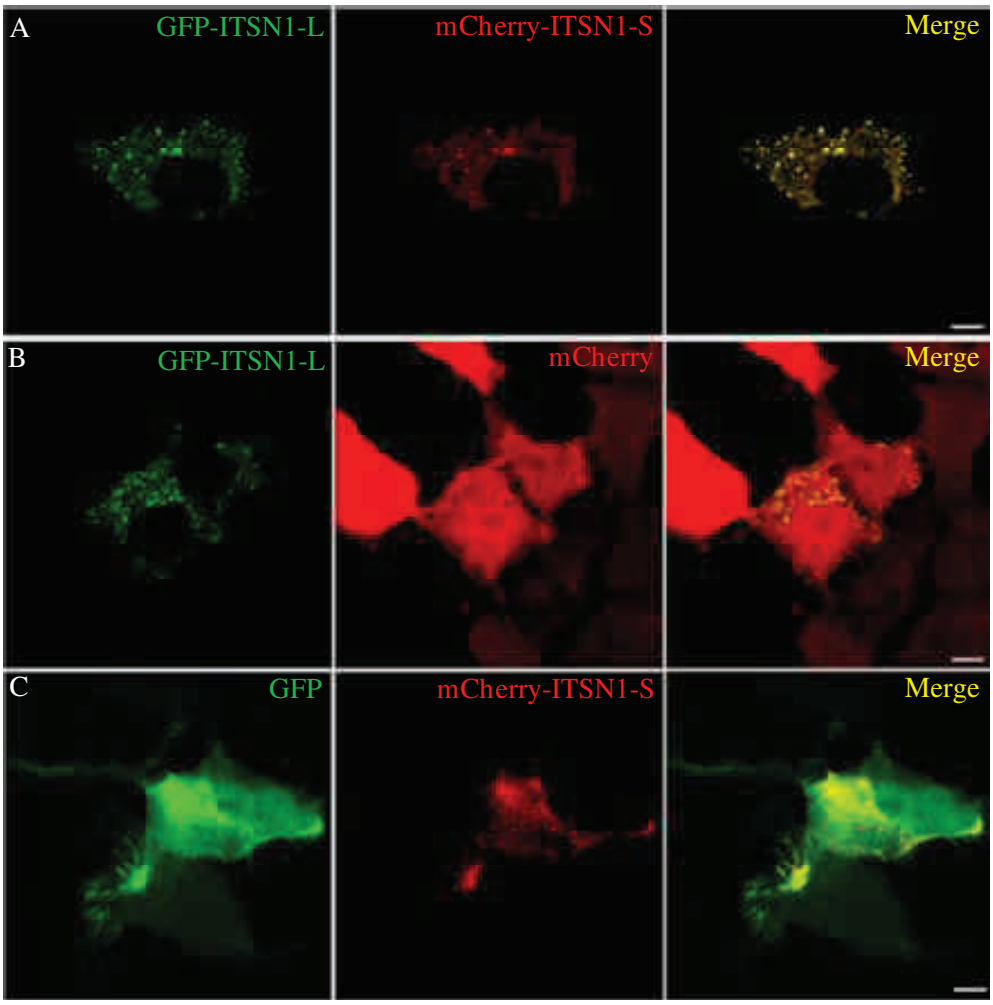

Fig. 33. Overexpressed GFP-ITSN1-L and mCherry-ITSN1-S colocalize in 293T cells. The cells were transfected with both GFP-ITSN1-L and mCherry-ITSN1-S (A) or with GFP-ITSN1-L alone and empty pmCherry vector (B) or with mCherry-ITSN1-S alone and empty pGFP-C1 vector (C). Bars = 5 µm. Images were taken by Leica SP5 confocal microscope.

VII. General discussion

In spite of apparent disconnectedness of the results, I allowed myself to make one common discussion instead of dividing in small separate discussions for the each section. At the end of cuurrent chapter I will try to combine all our findings in several hypothetical models, which will elucidate some possible cellular functions of investigated proteins' complexes.

1. ITSN1-LΔ35 is a possible GEF for RhoU.

In my opinion, one of our most intriguing discoveries is the binding of RhoU to the DH-PH domains of ITSN1-LΔ35 isoform. This presumes that ITSN1-LΔ35 might act as a GEF for RhoU. Notably, no GEF proteins for this GTPase have been reported so far. But this issue is quite controversial, and our data raised some questions and problems which I will try to discuss below.

1.1. The significance of binding of a GTPase to a GEF in GST pull down experiment.

I suppose the first question that would emerge from anyone who works with the GEFs and the GTPases is why we have performed a pull down assay to evaluate the specificity of a GEF to a GTPase? Indeed, an interaction between the GEF and the GTPase is commonly considered to be very transient, thereby it is difficult to detect by pull down or immunoprecipitation approaches. But in the case of RhoU we could not perform a classic *in vitro* GEF assay as RhoU has very high intrinsic exchange rate and under the standard conditions *in vitro* we will not see the difference upon the GEF addition. Thus we have used a GST pull down experiment to detect RhoU interaction with GEF domains. There is some evidence for detecting the interaction between the DH-PH domains of a GEF with a GTPase in the pull down experiment with dominant negative form of the GTPase (172 and 173) or bacterially expressed and non-loaded with nucleotide GTPase (174). The interaction was found to be specific and reflected the GEF activity in all described cases. This means that the binding in a pull down further was confirmed by a GEF activity *in vitro*. Even if it is known that the GEFs have the highest affinity to a nucleotide-depleted GTPase (159), this affinity remains specific to the type of GTPase, which that GEF activates. It was also confirmed by our results with Cdc42 binding and activation by the DH-PH domains of ITSN1-L or ITSN1-LΔ35 (Fig.17-18).

Moreover, the interaction of the ΔN-truncated form of the same RhoU DN mutant with ITSN1-LΔ35 (Fig. 26B, upper panel) supports our assumption that the interaction of DH-PH domain of ITSN1-LΔ35 with RhoU is GEF-specific.

Instead of dominant negative forms we have used the WT GTPases, overexpressed in mammalian cells, for the pull-down experiments of GST-coupled DH-PH or DH-PHΔ35 domains with Cdc42 or RhoU. But it is worth to mention that no nucleotide or Mg^{2+} (as it decreases intrinsic nucleotide dissociation and inhibits GEF activity (175)) was added to the lysis buffer during the harvesting of cells. Therefore the protein lysate was practically depleted of nucleotides and free magnesium ions, a condition favoring nucleotide-free GTPase, the form bound and stabilized by the GEF. Of course, this method probably would not work for an endogenous GTPase, as its amount in the cell is quite limited, but it seems to work well under overexpression conditions.

Thus we have shown the interaction of DH-PHΔ35 with RhoU, but the evidence of its GEF activity towards RhoU is still lacking, though the fact of interaction itself is very encouraging. The most convicting proof would be the classic *in vitro* nucleotide exchange assay with radioactive either fluorescent-labelled nucleotide (e.g. Mant-GTP in our case) (176). But, as it was mentioned above and would be detailed below, several obstacles had arisen in performing this test.

1.2. RhoU is not so fast exchanging as considered – but does it really need a GEF?

It has been stated by some authors, that a GEF for RhoU does not exist because of the high intrinsic nucleotide exchange rate of RhoU *in vitro*, as it exchanges 70% of nucleotide in 2 minutes (129) (Fig. 34). Therefore the implication of a GEF might not be required for promotion of the exchange in these conditions. But is the RhoU really so extremely fast exchanging? If looking more carefully at the graphic at Fig. 34, you can see, that above-stated 70% of exchange were obtained at 30°C. Whereas already at 20°C the exchange rate is not so far from that of Cdc42, which is known as a classic small GTPase regulated by GEFs and GAPs. Of course RhoU is exchanging faster than Cdc42 but not as instantly as it was claimed. As it will be discussed further, the *in vitro* GEF assay is extremely temperature sensitive, and the intrinsic exchange ability of a GTPase increases with the temperature.

Moreover, we should keep in mind that *in vitro* experiments do not take into account the additional regulatory pathways occurring *in vivo*. For example, the N-terminal extension of RhoU does not influence its intrinsic nucleotide exchange *in vitro*, but regulates its activity *in vivo*: the association with SH3 domains containing proteins promotes RhoU activation (129 and 143).

So the existence of a GEF for the RhoU seems more than probable to me. Even if it is considered that the regulation of RhoU activity is realized mostly by transcription activation, we can imagine that this process is not so fast. Moreover, it has been shown that under certain conditions the pool of RhoU in the cell may be inactivated (138). Thus in the situation of an immediate need the existing small pool of the GTPase may be rapidly activated by some GEF, for example. In our case the interaction with the SH3 domains of ITSN1 may withdraw the N-terminal inhibition, which could be sufficient for RhoU activation (as in case of adaptor proteins Grb2 and Nck2). This gives rise to a question: then what is the role of the interaction of RhoU with DH-PHΔ35? Presently we cannot answer this question. But as we do not know the exact mechanism of RhoU inactivation we can hypothesize that it may prevent the spontaneous nucleotide exchange. Thus following the GTP hydrolysis RhoU would be blocked in GDP-bound state. In this case a specific GEF might be of use for RhoU activation.

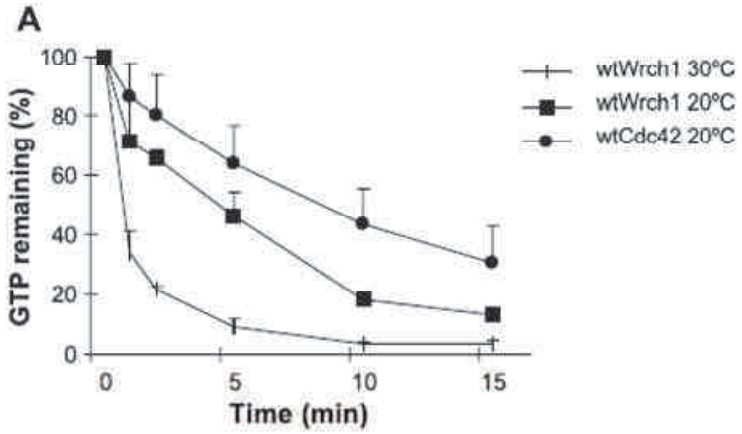

Fig. 34. Exchange rate of RhoU (Wrch1) (Saras et al., 2004). Recombinantly expressed Wrch1 was loaded with [3H]-GTP and the exchange rate in presence of an excess of cold GTP was determined. As a control, the exchange rate of Cdc42 was also determined. The experiments were conducted at temperatures indicated at the figure's legend.

As it was mentioned, the nucleotide exchange assay is extremely temperature-sensitive – at least it is true for Cdc42 and RhoU (Fig. 34 and 35). Thus in this case the studied GEF has to be very efficient exchanger of the GTPase; otherwise it will be very hard to see the difference. Such was our case in determining DH-PHΔ35 activity towards Cdc42, as our fluorimeter was not thermoregulated and luckily DH-PHΔ35 appeared to be an efficient GEF for Cdc42, so the difference was detectable and statistically significant. But at Fig. 35 you can compare two graphics of Cdc42 intrinsic exchange activity: the first one (A) represents data obtained at 20°C by Cytoskeleton, which show about 25% increase in fluorescence in 30 minutes; and the second (B) – reactions were carried out at 27°C, and the fluorescence is increased 5 to 6 fold in the same 30 minutes. So, in case of RhoU its activation cannot be assessed with classic *in vitro* GEF exchange assay without a strict temperature control.

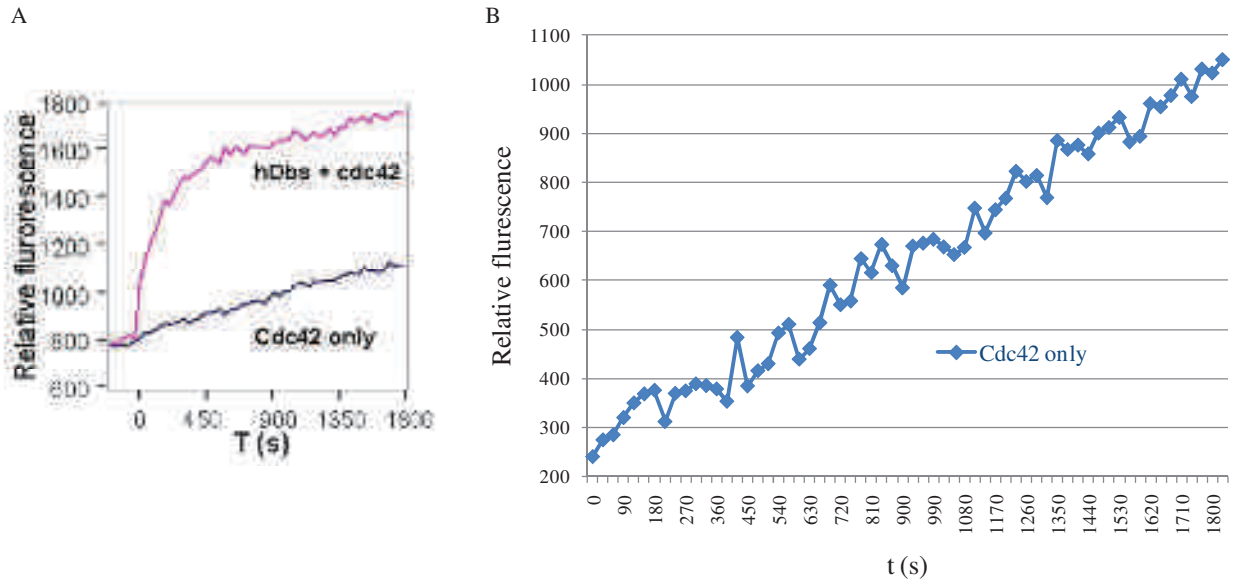

Fig. 35. Intrinsic nucleotide exchange rate of Cdc42 is temperature-dependent. A) Cdc42 exchange activity at 20°C (from Cytoskeleton BK100 kit manual v.2.4). B) Cdc42 exchange activity at 27-30°C (performed with the same BK100 kit as in A).

Another widely used method of evaluating GTPase activation *in vivo* is a quantification of the amount of activated GTPase by precipitation with its GST-coupled effector (PAK1 in case of Cdc42 or RhoU). However, we failed to determine the RhoU activation in this way (Fig. 36). The possible explanation is that under standard culturing conditions of 293T cells, which we have chosen as a model, RhoU is predominantly activated. Thus we have to find a method for RhoU activity attenuation to decrease the background. However, I think this *in vivo* GEF exchange assay is worth further trying.

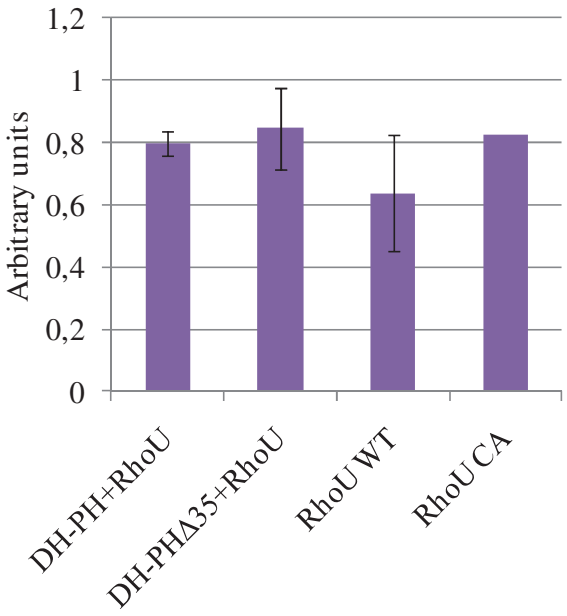

Fig. 36. The experiment for *in vivo* RhoU activation by coexpression with DH-PH domains of ITSN1-L or ITSN1-LΔ35 in 293T cells. 293T cells were transfected with RhoU-Myc and/or Omni-DH-PH or Omni-DH-PHΔ35 constructs. 24h posttransfection the cells were lysed and activated RhoU was precipitated to the GST-coupled CRIB domain of PAK1. The amount of the precipitate was evaluated by densitometric analysis in ImageJ software and normalized to the relative quantity of the total RhoU in the cell. The graphic represents the data of three independent experiments.

Thus our data neither confirm nor deny that ITSN1-LΔ35 is a probable GEF for RhoU, though their interaction is established. In my personal opinion the existence of a GEF for RhoU is probable, in spite of RhoU atypical features. In this case ITSN1-LΔ35 seems to be an ideal candidate. Besides, I assume a possible common role for RhoU and ITSN1-LΔ35 in central nervous system development. Previous data obtained in our laboratory in Ukraine demonstrated that ITSN1-LΔ35 is expressed at high level in embryonic brain and in a lesser extent in adult brain (42), and RhoU is implicated in embryonic development, particularly in neural crest cells migration (147; 151 and 152). Of course, more research and evidence are needed to confirm RhoU and ITSN1-LΔ35 common implication in the developmental processes..

2. ITSN1-RhoU interaction.

In my thesis, I have demonstrated the interaction between RhoU and ITSN1. However, the functional importance of this interaction is currently unknown. To date, we have no plausable explanation. But we have some conjectures which may shed light at the possible role of ITSN1-RhoU interaction in a cell and delineate possible consequences of their partnership.

2.1. Hypothetical model of ITSN1-L or ITSN1-LΔ35 and RhoU interaction.

We have shown that RhoU interacts with SH3A, C and E domains of ITSN1, and that this interaction is mediated mostly by the second proline-rich motif of the N-terminal extension of the GTPase. As RhoU is relatively small protein (37 kDa), theoretically there should be no steric hindrance for the binding of one molecule of RhoU to each of the interacting SH3A, C and E domains (Fig. 28A). However, it is possible, that in the cell only one of the SH3 domains will be accessible, first because of the large number of ITSN partners interacting with SH3 domains and second – because the conformation of the full-size ITSN1 is unknown, as the structure of the entire protein has not been resolved.

The situation seems even more complicated with ITSN1-LΔ35 for which 3 different scenarios could be considered (Fig. 28 B-D). Theoretically, four molecules of RhoU are capable to bind to the full-size ITSN1-LΔ35 protein (Fig. 28B). But I think it is unlikely because the interaction of RhoU with the DH-PHΔ35 domains appeared not very persistent. So, the second probable model would be the binding of RhoU to the SH3 domains with a subsequent binding and activation by DH-PHΔ35 (Fig. 28C). Finally, the third alternative scenario is the binding of RhoU to only one available SH3 domain and a subsequent activation by DH-PHΔ35 (Fig. 28D, SH3A domain was chosen for example, but with the equal probability it could be also SH3C or E domain).

To push further our hypothesis, it is worth recalling that the dominant negative RhoU mutant co-precipitates with ITSN1 at a higher level than the wild type RhoU, whereas the binding of constitutively active mutant seems slightly weaker (Fig. 14). So, an interesting mechanism would be that the inactive RhoU binds ITSN through the SH3 domains in order to be activated by DH-PHΔ35 and subsequently released as the affinity of the GTP-bound RhoU for ITSN1 SH3 domains decreases. Then another GDP-bound RhoU could interact with ITSN1-LΔ35 and be activated and so on and so forth, thereby allowing rapid activation of the available pool of RhoU.

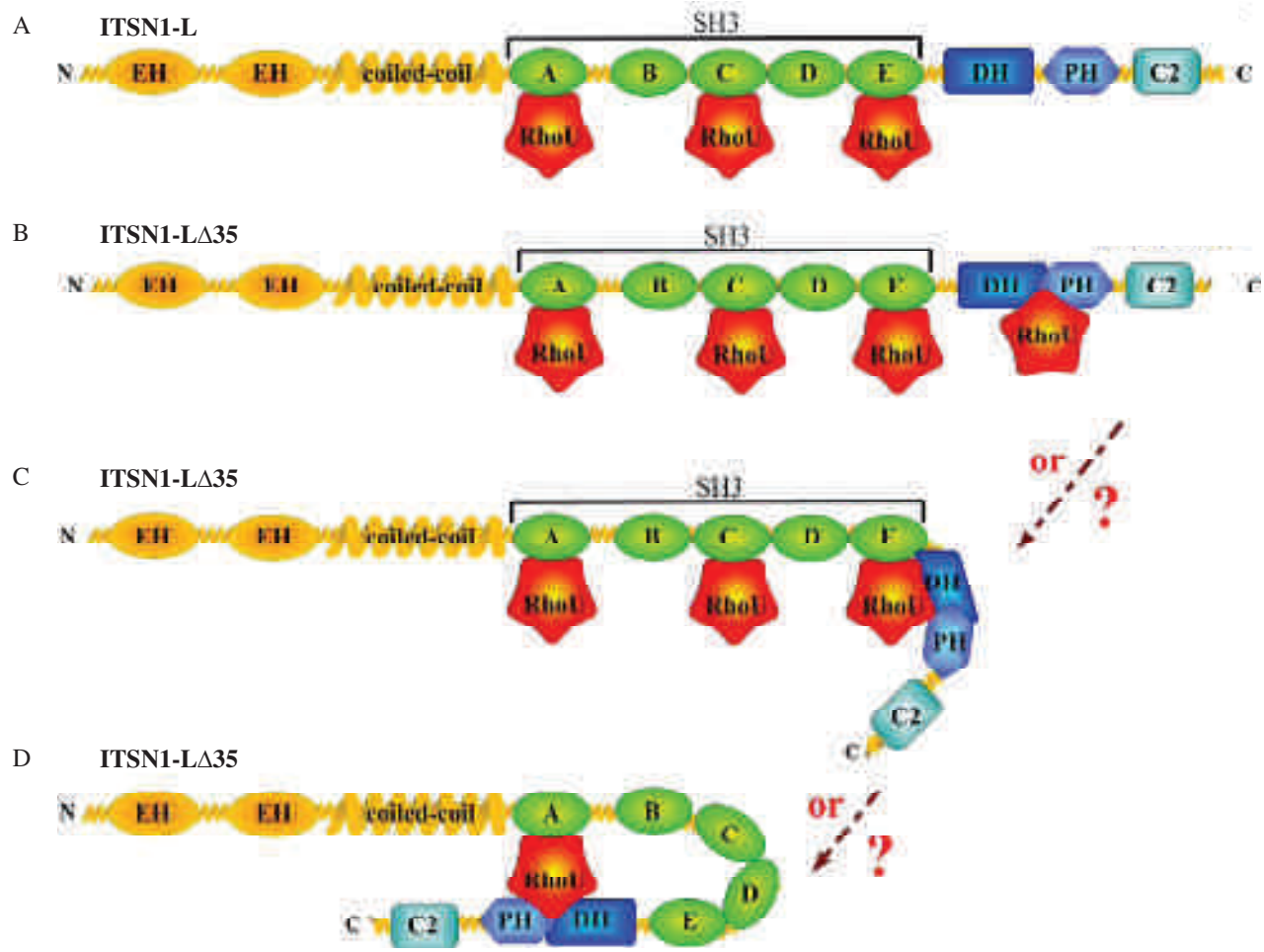

Fig. 37. Hypothetical models of RhoU interaction with ITSN1-L or ITSN1-LΔ35. A) Three molecules of RhoU may bind to ITSN1-L. B) ITSN1-LΔ35 may bind up to four RhoU molecules. C) However, as the interaction of RhoU with DH-PHΔ35 domains is not very stable, it is probable that the DH-PH domains of ITSN1-LΔ35 can interact with RhoU only after its binding to one of the SH3 domains. D) The most probable situation is that RhoU interacts with only one domain of ITSN1 (because of plethora of other SH3 domain partners in the cell), and in case of ITSN1-LΔ35 RhoU may subsequently interact with the DH-PHΔ35 domains.

2.2. Possible competition of RhoU with endocytic proteins for the binding to ITSN1.

Previously it has been shown that ITSN1 overexpression drastically inhibits clathrin-dependent endocytosis (e.g. transferrin uptake) (19 and 41). The common explanation is that ITSN1 functions as a scaffold, which assembles endocytic complex by interaction with various proteins such as Epsin, Eps15, AP2 and dynamin. Thus, overexpression of ITSN1 sequesters the components of endocytic machinery into non-productive complexes, leading to the endocytosis inhibition as it disrupts the formation of higher order complexes. This concept of “combinatorial

inhibition" as a consequence of the imbalance of a scaffold protein concentration was well highlighted by Ferrel J.E., 2000 (177).

The previous findings connected ITSN1-dependent endocytosis inhibition with the sequestration of dynamin, one of key endocytic proteins (Fig. 38A) (19). Intriguingly, dynamin also binds to the SH3A, C and E domains of ITSN1, the same as RhoU (21). This fact led us to the assumption that there may be a competition between dynamin and RhoU for the binding to ITSN1. Thus we performed a transferrin internalization assay, to verify whether RhoU interaction with ITSN1 is capable to rescue ITSN1-induced endocytosis inhibition. Indeed, the overexpression of RhoU together with ITSN1 did rescue the transferrin uptake in 293T cells (Fig. 19, 20). Moreover, this rescue was abolished by RhoU DM mutant that is not able to bind to ITSN1.

But surprisingly, in our hands, ITSN1 overexpression visibly affected transferrin uptake, but it did not cause the complete inhibition of transferrin endocytosis. Thus transferrin was still internalized, but the vesicles were dispersed in the cytoplasm and transferrin did not accumulate in perinuclear recycling endosome (Fig. 18), whereas other authors reported total impairment of endocytosis (19 and 41). First, we have supposed that this was because we used different cell type (293T comparing to COS1 cells). But I found unlikely that 293T cells would have their own mechanism of transferrin uptake. Moreover, Mohney et al., 2003, did not observe the inhibition of transferrin endocytosis upon ITSN1 overexpression in COS1 cells (80). Instead they observed the same vesicle dispersion, moreover, ITSN1 did not colocalize with transferrin. I think that this discrepancy could be explained by the level of the overexpression. Indeed, we observed a positive correlation between ITSN1 expression level and transferrin uptake inhibition (data not shown). Thus as we tried to work in low-expression conditions, only low level of endocytosis inhibition could be achieved.

These observations led to another hypothesis about the consequences of ITSN1 overexpression: as transferrin is still internalized but instead its localization to recycling endosome is impaired, it is more probable that the late stages of endocytosis and/or vesicle fusion with subsequent endosomal compartments are disrupted. Synaptojanin 1, as dynamin, binds SH3 A, C and E domains of ITSN1 (21). But dynamin and synaptojanin function at different stages of CME: dynamin is responsible for vesicle scission whereas synaptojanin dephosphorylates PIP_2 which leads to vesicle uncoating and fusion with endosomal compartments (178 and 179). Thus synaptojanin 1 seems to be a good candidate for an endocytic protein that is sequestered by the excess of ITSN1 (Fig. 38B). Besides, the impairment of synaptojanin function led to the accumulation of clathrin coated vesicles in the cytoplasm (178). Of course, this hypothesis requires further verification. As a first approach - an immunostaining, to examine whether endogenous

synaptojanin is relocalized upon ITSN1 overexpression, would be of use. Thus, as RhoU and synaptojanin bind the same ITSN1 SH3 A, C and E domains, RhoU could compete with synaptojanin 1 for ITSN1 binding and thus rescue the transferrin internalization (Fig. 38C). This is further supported by the observation that ITSN1 coexpression with CdGAP, which binds predominantly SH3D domain of ITSN1, did not influence the transferrin uptake inhibition (Fig. 20).

The fact that RhoU rescues ITSN1-induced transferrin uptake implies that the interaction of two proteins is of high relevance in the cell. Moreover, we have observed that together with RhoU ITSN1 is relocalized to the recycling endosome upon transferrin uptake, which presumes that they are together involved in some membrane trafficking processes.

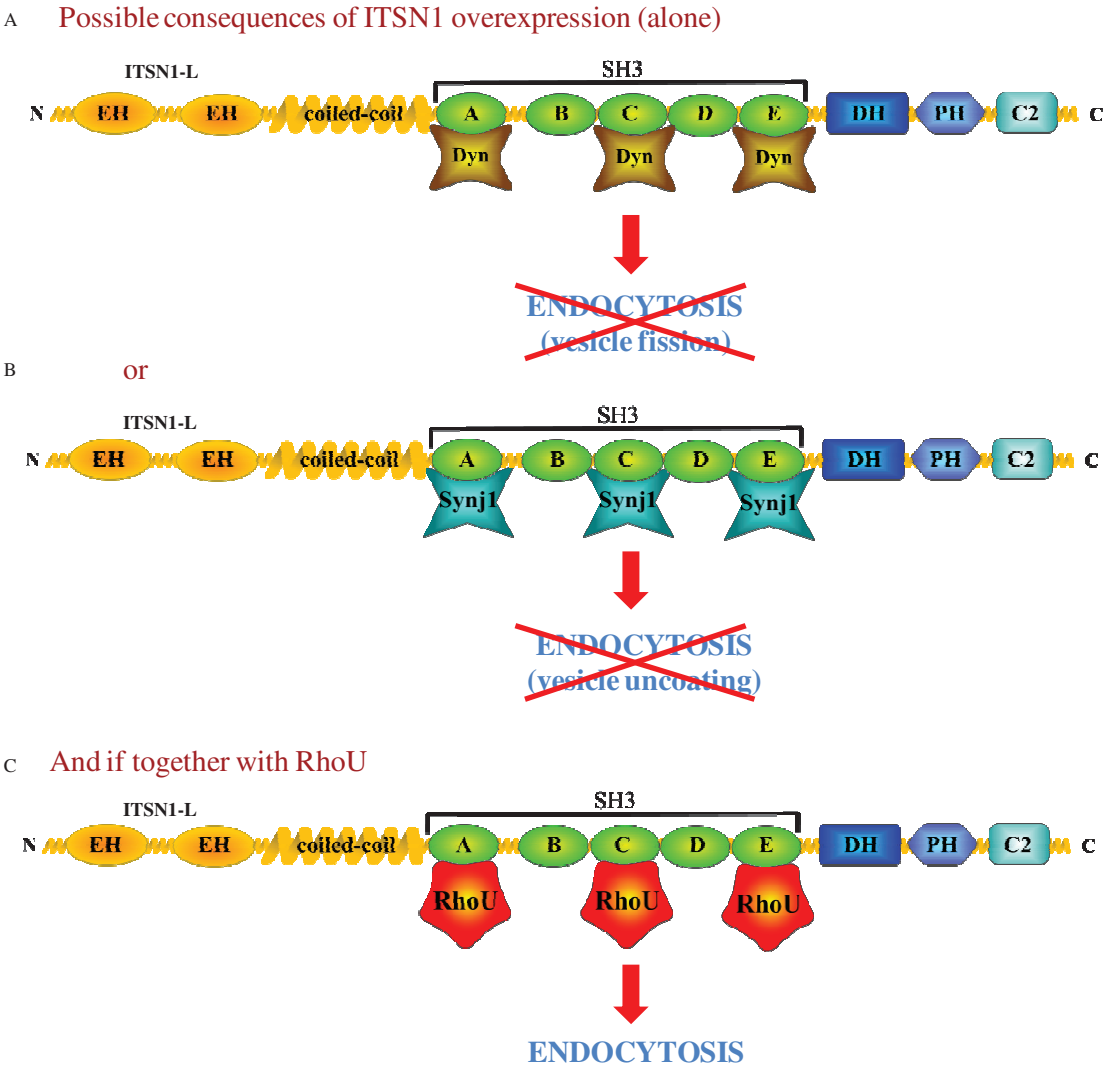

Fig. 38. Schematic representation of possible consequences of ITSN1-L overexpression alone or together with RhoU. A) ITSN1-L when overexpressed alone in the cell may sequester endogenous dynamin from the endocytic sites thereby blocking endocytosis at middle stages (e.g. vesicle fission) B) Another possible consequence of ITSN1 overexpression is sequestering synaptojanin 1 thereby blocking late stages of endocytosis (vesicle uncoating) and eventually vesicle fusion with endosomal compartments. C) If RhoU is overexpressed together with ITSN1-L, it forces out dynamin or synaptojanin 1 from SH3 domains of ITSN1 which leads to the restoring of the endocytosis. Dyn – dynamin, Synj1 - synaptojanin 1.

2.3. A common role for ITSN1 and RhoU in EGFR signaling.

Interestingly, both RhoU and ITSN have been involved in EGFR signaling and/or degradation (63 and 146). ITSN1 was shown to promote ubiquitylation and degradation of the activated EGFR through the ubiquitin-ligase Cbl, whereas RhoU was shown to be involved in EGFR signaling recruited by the adaptor protein Grb2.

Before we can hypothesize the common role of RhoU and ITSN1 in EGFR signaling, we should attempt to clarify the relations between ITSN1 and Grb2. Mysteriously, both ITSN1 and Grb2 were shown to have similar role in the fate of activated EGFR: they promote EGFR internalization and ubiquitylation by Cbl (63; 180-182). Surprisingly, there is not a single evidence about the relationship between these two proteins: whether they compete or work synergistically, or whether the preference of their interaction with EGFR is cell type specific or compartment-dependent. Theoretically they can both bind the activated EGFR, as Grb2 interacts with the phosphorylated EGFR directly binding to the phospho-tyrosine via its SH2-domain, whereas ITSN1 interaction with the receptor is indirect and may be mediated by AP-2 or Eps15, which are both common partners for ITSN1 and EGFR (19; 53; 61 and 183). As it has been shown that EGFR is internalized both via clathrin-mediated endocytosis (CME) and via alternative pathways (non-CME) (184 and 185), we may suppose that ITSN1 and Grb2 are involved in different pathways of receptor internalization. It could be an elegant way to demarcate EGFR interaction with Grb2 or ITSN1, but both proteins were shown to be implicated in both CME and non-CME (4; 66; 180; 181 and 184). However, non-CME pathways are very ill-defined and comprise several types of endocytosis, whose number and mechanisms remain unclear except a few key features (such as caveolin- or lipid rafts-association). So it may appear that ITSN1 and Grb2 take part just in different kinds of non-CME. However, unfortunately, analyzing available data we could not answer the question whether ITSN1 and Grb2 interact simultaneously with the activated EGFR and act complementary, or these interactions are mutually exclusive and regulated by some unknown mechanism. I think this question is a good challenge for further investigations. Thus meanwhile I will examine their function in parallel, as if they are competing in EGFR pathway. In my personal opinion this situation is more probable, than their synergistic functioning.

Another fact about ITSN1 and Grb2, which is even more intriguing for me, is that both ITSN1 and Grb2 interact with RhoU – by the same domains that interact with Cbl! This fact implies a competition between RhoU and Cbl. RhoU promotes EGFR signaling, whereas Cbl ubiquitinates the receptor which leads to EGFR sorting to the lysosomes and degradation, so their functions seem to be almost mutually exclusive. Our hypothetic model presumes both. According to our assumptions, if Cbl interacts with either ITSN1 or Grb2 it would lead to fast receptor degradation

and attenuation of the signaling (Fig. 39A). Whereas RhoU interaction with either of the proteins would lead to enforced signaling and probably EGFR may escape degradation and undergo recycling instead (Fig. 39B). In case of ITSN1-L the model may become even more complicated, if we presume that RhoU binding to SH3 domains may unmask the DH domain, as it was shown for WASP and Numb (31 and 35). Consequently it will lead to Cdc42 activation. Previously it was shown that activated Cdc42 together with Cool-1a/β-Pix sequesters Cbl from EGFR, which results in sustained EGFR signaling (186). So while RhoU binding to ITSN1 may prevent its interaction with Cbl, at the same time Cdc42, activated by ITSN1-L, together with Cool-1a/β-Pix may bind Cbl thereby further preventing the ubiquitylation and degradation of EGFR. Several assays with the EGFR-positive cells, overexpressing ITSN1-L and RhoU or depleted of both may help to verify these models.

Interestingly, it has been shown that CME-dependent EGFR internalization is associated with the receptor recycling whereas non-CME leads to the receptor degradation (185). Thereby we may hypothesize even further, assuming that ITSN1 interaction with RhoU is linked to clathrin-dependent EGFR internalization, whereas ITSN1 interaction with Cbl occurs in non-CME pathway. It would be very interesting to test this hypothesis. Selective inhibition of endocytic pathways together with quantitative assays and immunofluorescence could be used as approaches.

But we cannot exclude the alternative when both RhoU and Cbl can interact together with either ITSN1 or Grb2 (Fig. 39C). In this case it is quite hard to predict, which will be the fate of the activated EGFR. But it seems logic that this may combine enforced signaling with quick degradation. However the balance between these three described situations will determine cellular response to the EGFR stimulation and probably the fate of the EGFR itself.

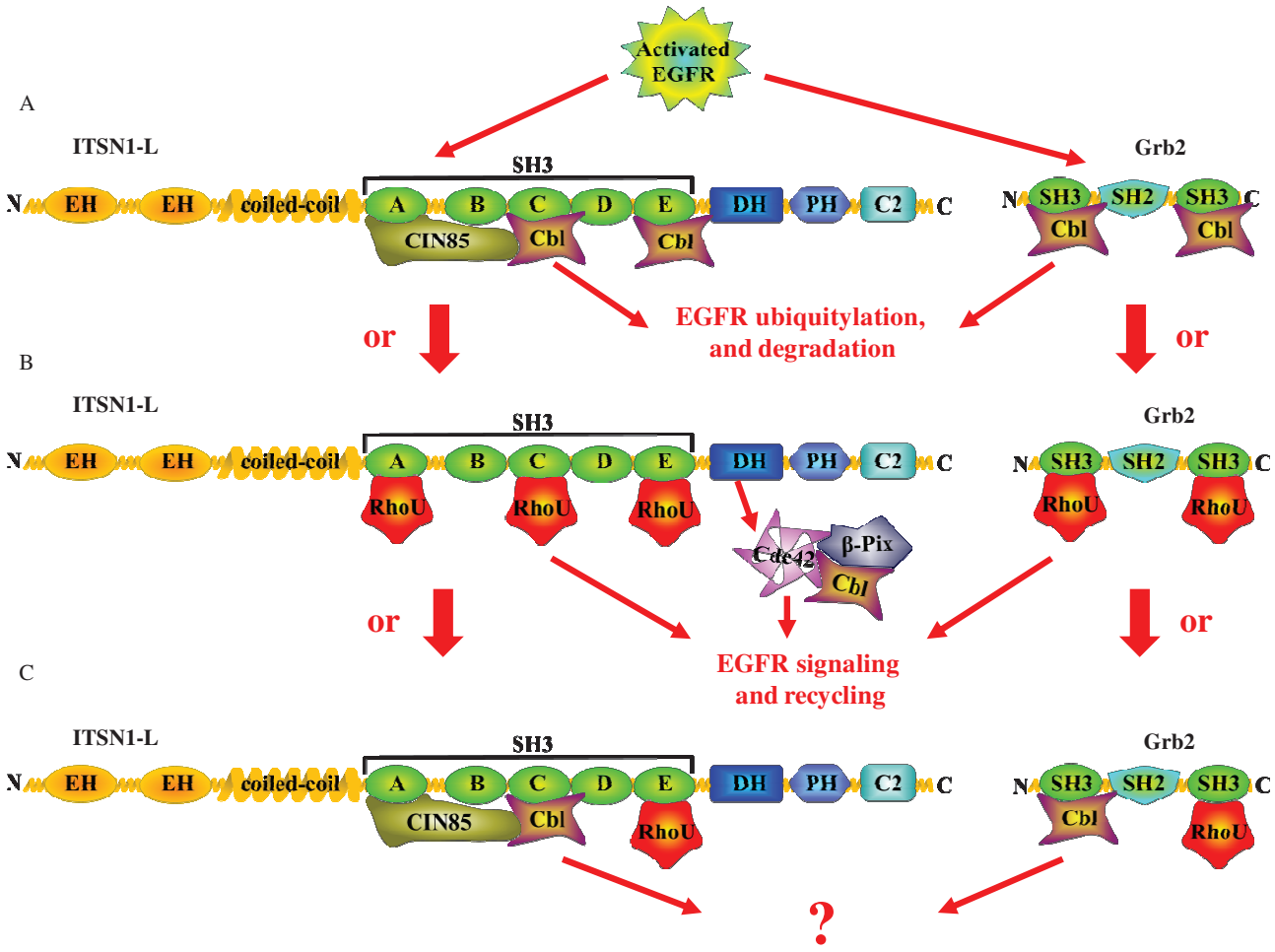

Fig. 39. Hypothetical model of ITSN1 or Grb2 interaction with Cbl or RhoU following the EGFR stimulation. A) ITSN1 interaction with ubiquitin-ligase Cbl and CIN85 (a scaffold that promotes Cbl activation) as well as Grb2 interaction with Cbl lead to the ubiquitylation and degradation of activated EGFR upon its internalization. B) RhoU interaction with either ITSN1 or Grb2 in place of Cbl may rescue EGFR from degradation and result in EGFR recycling. Grb2 interaction with RhoU promotes signaling from activated EGFR. ITSN1-L interaction with RhoU may serve the same signaling function. Moreover, the binding of RhoU to SH3 domains of ITSN1 may unmask the DH domain thus resulting in Cdc42 activation. The activated Cdc42 together with its effector β-Pix can sequester and inactivate Cbl further preventing EGFR ubiquitylation. C) The fate of EGFR is unknown in case of binding of both Cbl and RhoU to ITSN1 or Grb2.

2.4. ITSN1 and RhoU in cancer and other cellular pathologies.

Another interesting issue of ITSN1-RhoU interaction could be their common implication in cellular transformation and cancerogenesis. Indeed, both of them were shown to induce cellular transformation (81; 131 and 143), though the mechanisms remain unclear. It has been shown that RhoU needs to be palmitoylated to transform NIH3T3 fibroblasts. Moreover, deletion of the N-terminal region of RhoU increases the transformation rate. It is not surprising if we remember that the proline-rich N-terminal extension inhibits RhoU activity and that interaction with the SH3 domain-containing partners relieves this autoinhibition (143). Although it needs a proof, we can suppose that ITSN1 may also be one of these SH3 domain-containing partners.

As for the role of ITSN1 in cellular transformation, the situation is even less clear than with RhoU. It was shown that ITSN1-L overexpression leads to transforming activity through Cdc42 and Ras pathways, namely JNK activation (160). Indeed, ITSN1 is a binding partner for Sos1, a GEF for the GTPase Ras (28). Interestingly, Sos1 interacts with the same domains of ITSN1 as RhoU (SH3A, C and E), which implies a potential competition. However there is no evidence that RhoU prevents Ras activation, but it was shown that RhoU overexpression also leads to JNK activation (130 and 135).

Although ITSN1-induced transformation is not very well studied, it has been shown recently that ITSN1 is able to regulate other important steps of cancer development like cell survival or cell migration and invasion (125-127). ITSN1 induces cancer cells survival through PI3K-C2β, in the same way as neuron survival (84). Of particular interest, PI3K-C2β also binds the SH3A and C domains of ITSN1, whereas RhoU has been shown to interact with the regulatory p85 subunit of another class of PI3K and to promote Akt activation (135). Thus we might imagine that ITSN1 and RhoU can cooperate in some way to induce PI3K-Akt-survival pathway. Another role for ITSN1 and RhoU in cellular transformation may be in sustaining EGFR signaling, as it was hypothesized in previous chapter. It is well-established that excess of EGFR signaling has been involved in the progression of many cancers (187).

The presumption that ITSN1 together with RhoU are involved in cancer cell migration seems more prominent. Indeed, ITSN1 has been shown to participate in migration and invasion of glioma cell line through the regulation of FAK (focal adhesion kinase), PAK1 and integrin β1 (125 and 126). RhoU is also implicated in cancer cell migration (134), and is able to bind integrin (but β3) (145) and FAK (144), whereas PAK1 is its effector (129). It has also been shown that RhoU negatively regulates focal adhesions and induces filopodia formation (129 and 144). Thus it seems very probable that ITSN1 together with RhoU regulate focal adhesions downstream of integrins and promote cellular migration, namely in cancer cells.

However our analysis of ITSN1-L and RhoU transcripts in different cancers show that their expression is rather variable even in the same tumor type. This may be explained by intratumoral heterogeneity and intratumoral clonal evolution (188-190). The new insights at cancerogenesis include evolutional theory of cancer. Cancer progression represents a macro-evolutionary process where karyotype change or genome replacement plays the key role. These changes in karyotype cause differential gene expression. As each spontaneous tumor evolves in its own way, especially in response to different stress factors such as drugs, uses different survival pathways and acquire new properties (e.g. migration and invasiveness), thus naturally the levels of protein expression vary among different tumors and even among different cellular subclones of the same tumor (191-193). Thus the variable expression of ITSN1-L and RhoU in different cancer samples may be sequent to variable tumor adaptations. This may indicate that RhoU and ITSN1 are dispensable for cancer progression in general, though may be crucial for some tumor subclones. For example, for those who acquire migratory and invasive properties.

Besides cancer progression, RhoU misregulation was also shown to disrupt epithelium cyst formation (138; 142 and 148), thus presuming its implication in cell polarity determination. But it seems that ITSN1 is not implicated in this process, as its silencing does not lead to the significant diminishing in cyst number (194). However, ITSN1 has been shown to be implicated in polarity determination in drosophila neuroblasts (195). Interestingly, another protein from ITSN family, ITSN2-L, was shown to regulate the mitotic spindle orientation during epithelium lumen formation together with Cdc42 (196). As was mentioned previously, the results of Dr. S. Ory (unpublished) indicate that RhoU interacts with ITSN2 as well. Thus ITSN2-L, RhoU and Cdc42 may be connected in epithelium cell polarity determination.

3. Possible role of ITSN1-OPHN1 interaction in coupling exo- and endocytosis.

Both ITSN1 and OPHN1 play important role in various membrane trafficking processes especially in exo- and endocytosis. There are more evidence concerning their role in endocytosis (19; 41; 61; 165 and 166) and less for their role in exocytosis (122 and 123). For example, both proteins are involved in synaptic vesicle (SV) retrieval (53; 54; 61; 71; 165 and 166). We uncovered a specific interaction between ITSN1 and OPHN1 and have shown that the complexes of ITSN1-L-OPHN1 persist independently of exocytosis stimulation. This fact that does not imply a common implication in exocytosis but neither denies it completely.

According to their dual importance in both exo- and endocytosis, it is tempting to consider that ITSN1 and OPHN1 might regulate the coupling between these two processes especially in neurosecretory cells like neurons and neuroendocrine cells. Indeed, ITSN1 regulates synaptic vesicle recycling at the periactive zone of the synapse (197). Though there is no direct evidence for OPHN1 implication in exocytosis, this GTPase has been shown to act as RhoA inhibitor in neurons (165). According to the results obtained in our laboratory in Strasbourg, RhoA is inactivated during regulated exocytosis in the neuroendocrine cells (100; 101; 104), whereas Cdc42 is activated (103 and 122). To date, the mechanism by which RhoA is inactivated remained unsolved. These facts inspired me an elegant model hypothesizing a function for ITSN1-L-OPHN1 interaction (Fig. 40A). During exocytosis, ITSN1-L is recruited to the exocytotic sites where it activates Cdc42/N-WASP pathway to stimulate actin remodeling required for late phase of exocytosis (122). By interacting with ITSN1-L, OPHN1 will be targeted directly at the exocytotic sites where it can trigger (concomitantly to ITSN-induced activation of Cdc42) the inactivation of RhoA which is bound to the secretory granule membrane (198) Similar local differential regulation of Rho GTPases has been proposed for wound healing (by Abr, a protein bearing GEF and GAP activity (199)) or during mitosis (Pebble GEF and p50RacGap in Drosophila (200)).

As both ITSN1 and OPHN1 function in endocytosis, the potential implication of OPHN1-ITSN1 complex in compensatory endocytosis of secretory granule membrane also appears as a plausible scenario (Fig. 40B) (14). Thus ITSN1 may recruit OPHN1 to endocytic complexes, where OPHN1 was shown to inactivate RhoA/ROCK pathway (165). Given the presence of a BAR domain in OPHN1, the latter could cooperate in the sensing and generation of membrane curvature at endocytic sites. Indeed, the BAR + PH domains of OPHN1 have been shown to tubulate liposomes (201).

Thus OPHN1 might be a versatile player in neurons, whose role is highly context-dependent. In different surroundings it may promote endocytosis or exocytosis. Thus the interaction

with ITSN1 could serve in performing at these tasks. But the common function of two proteins may also lie beyond the membrane trafficking, which will be discussed in the last section.

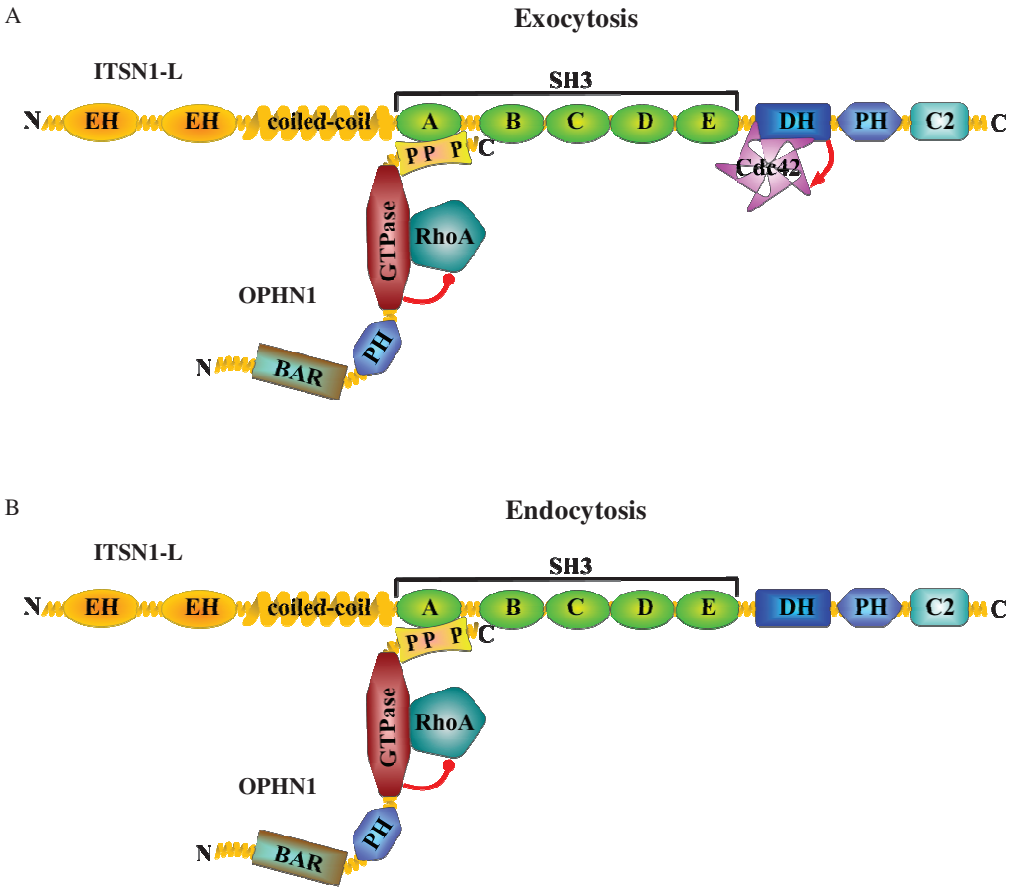

Fig. 40. A schematic representation of ITSN1-L-OPHN1 interaction in exo- and endocytosis. A) The interaction of OPHN1 with ITSN1-L may promote GTPase regulation in exocytosis: RhoA inactivation by OPHN1 simultaneously with Cdc42 activation by ITSN1. B) In endocytosis ITSN1-L may recruit OPHN1 to endocytic sites where OPHN1 inactivates RhoA. Red arrow means activation, red arrow with a blunt end – inactivation.

4. Intersectins form huge scaffold supercomplexes in cells.

Additionally, I have demonstrated that short and long isoforms of ITSN1 interact together in mammalian cells, thereby allowing the formation of large complexes with varying protein composition (202). Here I'd like to discuss the molecular mechanism controlling the formation of these huge super-scaffolding platforms as well as their possible functions. Such ITSN1-ITSN1 interaction could be mediated by coiled-coil region (CCR) as it is the case for Eps15, another CCR and EH domain containing protein (203). This assumption is supported by the work of Wong et al., 2012 (204). They have used a high throughput yeast two hybrid screening to characterize the possible partners of the ITSN scaffolds and have identified ITSN1 as a target of a prey containing its EH2 domain and a half of CCR (204-522 aa). They have further shown the existence of ITSN1 homo- and heteromeric complexes by bimolecular fluorescence complementation.

ITSN1 SH3 domains may also contribute to this interaction as it has already been shown that some other proteins dimerize via their SH3 domains (205 and 206). In our laboratory in Ukraine it was shown that the SH3 domains of ITSN1 can precipitate the full-size endogenous ITSN1-S from 293 cells lysate. One more open question is whether ITSN1 forms binary complexes or there are more interacting units?

Interestingly, Wong et al., 2012, have shown that not only short and long isoforms of ITSN1 interact with each other, but also ITSN1 and ITSN2 form complexes as well as their different isoforms (204). Thereby if we will count only principal isoforms of each protein (short and long) in case of binary complex formation, there could be 10 different combinations. If we will also take into consideration a multitude of minor isoforms (only for ITSN1 there are about 34) – the number of possible complexes will reach several hundred! For example it has already been shown the dimer formation between ITSN1-S and its minor isoform 22a (47 and 171), and we have also co-precipitated ITSN1-S with ITSN1-LΔ35 (data not shown). The functional relevance of ITSN1 isoform complexes is in assembling a specific pattern of their partners which are required for certain process. For example, 22a isoform of ITSN1 specifically interacts with amphiphysin 1 and may recruit it to the complex with ITSN1-S, which is not capable to interact with amphiphysin (47). Together these data presume ITSN1 complexes create subtly and precisely regulated interacting platforms with a large number of interaction combinations.

5. ITSN1, OPHN1 and RhoU may have a common function in neurons.

In previous sections I have discussed possible implications of ITSN1 together with its new partners mainly in membrane trafficking. It was logic as all abovementioned proteins are involved in exo- and/or endocytosis. But there is no doubt that their functionality is not limited only to membrane transport. All of them have appeared versatile players in different processes and in different cellular systems. However, I'd like to discuss here their common features in order to build several molecular models integrating ITSN1, RhoU and OPHN1 (Fig. 41).

The model presented in figure 41A proposes a simultaneous interaction of ITSN1-L with OPHN1, RhoU, Cdc42 and N-WASP. N-WASP can bind to SH3A, C, D or E domains of ITSN1 (31), but I have presumed in that model a preferential interaction with the SH3E domain, as it is the closest domain from the DH-PH tandem.. Hussain and collaborators (31) have proposed an elegant model in which the binding of N-WASP to SH3 domain of ITSN unmasks the DH domain, which in turn activates Cdc42 leading to N-WASP-induced actin polymerization. Accordingly, it has been shown recently that the linker between the SH3E and DH domains inhibits the DH domain activity (33 and 34).

At the same time, OPHN1 could interact with the SH3A domain whereas RhoU could bind to the SH3C domain. In this case OPHN1 could act as a GAP for RhoA. Even if OPHN1 was shown to be a GAP for RhoA, Cdc42 and Rac1 *in vitro* and *in vivo* (161 and 162), to date its functioning in cells is mostly connected to RhoA inactivation (163 and 165).

As for RhoU, though it has been shown that RhoU seems to indirectly induce RhoA activation, which is manifested in membrane blebbing (129), it has not been directly proven. Moreover, later findings show that RhoU binds to ARHGAP30, a new GAP for RhoA, whose expression alone induces the same membrane blebbing as together with RhoU or RhoU alone (150). It also should be reminded that RhoA is responsible for the focal adhesion and stress fiber formation, whereas RhoU was shown to disrupt these structures (86 and 88). Together these data indicate that the observed membrane blebbing may occur from another origin. We can also presume that RhoU seems to work rather antagonistically to RhoA than synergistically (I have marked it at the scheme as indirect inhibition relationship).

Thus it seems possible that ITSN1-L may serve as a regulator of the switching between Rho pathways, promoting inactivation of RhoA with simultaneous activation of Cdc42 and probably RhoU (if the interaction with ITSN1 SH3 domains relieves RhoU N-terminal inhibition).

If we replace ITSN1-L by ITSN1-LΔ35, which might appear a GEF for RhoU, it may substantially change our model (Fig. 41B and C). Evidently, this should not change the interaction

with OPHN1 and subsequent inactivation of RhoA. But as for RhoU and Cdc42, the situation may change drastically. One of the possibilities is that RhoU binds to the SH3 domains (for example, SH3E, as it is the closest to the DH-PH tandem, and as we have already discussed the binding to it with high probability relieves the autoinhibition of the DH domain). Thus N-WASP would be no more present in this complex. The RhoU interaction with ITSN1-LΔ35 may mediate its sustained activation by DH-PHΔ35 domains.

Another interesting fact is that among common ITSN1 and RhoU partners is CdGAP, a GAP for Cdc42 and Rac1 that is inhibited when bound to ITSN1 (118 and 150). It is not sure that in this case activated RhoU could interact with CdGAP, but what if this interaction persists and thus RhoU may relieve this inhibition (Fig. 40B)? This may lead to the inactivation of Cdc42 by the released CdGAP, thus leading to the switching from RhoA and Cdc42 to RhoU-activated pathways.

Though we cannot exclude the abovementioned combination, it is more probable that the interaction of RhoU with CdGAP should lead to the further inhibition of CdGAP or should not persist at all (Fig. 40C). Just because, as it was also discussed above, an inactivation of Cdc42 close by its GEF is at least useless (and ITSN1-LΔ35 is as efficient GEF for Cdc42 as ITSN1-L). Moreover, both Cdc42 and RhoU have similar effects on actin cytoskeleton rearrangement, such as filopodia formation. Whereas for Cdc42 the mechanism is well-established: it induces filopodia through N-WASP and Arp2/3 (207), RhoU does not interact with N-WASP or any other protein, directly involved in filopodia formation (88). Thus it is possible that to induce filopodia, RhoU may promote Cdc42 activation in yet unknown way. So it seems probable that RhoU and Cdc42 work synergistically when bound to ITSN1-L.

Finally, the most complete model would include also a complex between two molecules of ITSN1 (Fig. 41D). There we may have some new players, such as Numb, which is implicated together with ITSN1-L in dendritic spine development and was shown to induce ITSN1-L-dependent Cdc42 activation in this process (35). Thus ITSN1-L in complex with Numb may activate Cdc42, which will immediately activate its effector N-WASP, whereas ITSN1-LΔ35 may keep CdGAP inhibited while activating RhoU. OPHN1 may interact with one of the ITSN1 molecules for choice and inactivate RhoA. The resulting complex would provide a really effective mean for the switching from RhoA-regulated pathways to RhoU and/or Cdc42-activated processes.

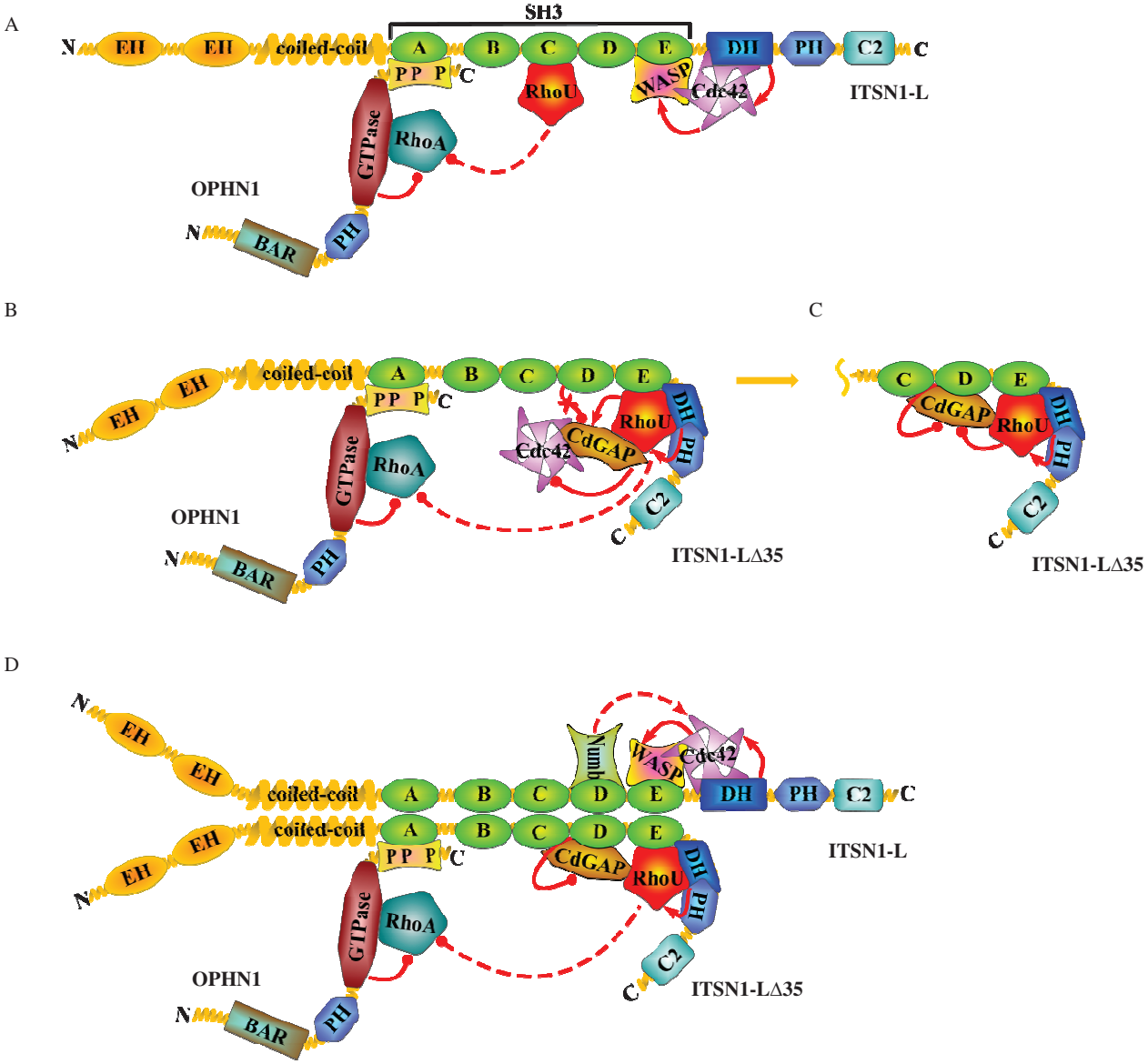

Fig. 41. Hypothetical models for ITSN1-L, ITSN1-LΔ35, OPHN1, RhoU and Cdc42 interaction and relationship. A) ITSN1-L interaction with OPHN1, RhoU and Cdc42 could promote switching from RhoA to Cdc42/RhoU-regulated pathways. SH3 domains of ITSN1 could simultaneously interact with OPHN1, RhoU and N-WASP. In this case N-WASP promotes Cdc42 activation by the DH domain of ITSN1 and is subsequently activated by Cdc42. At the same time OPHN1 inactivates RhoA. RhoU may also indirectly promote RhoA inactivation. B) ITSN1-LΔ35 interaction with OPHN1, RhoU, CdGAP and Cdc42 could promote inactivation of both RhoA and Cdc42 with subsequent RhoU activation. OPHN1 (and probably indirectly RhoU) promotes RhoA inactivation, whereas RhoU binding to the SH3 and DH domains of ITSN1-LΔ35 may result in sustained RhoU activation. Moreover, activated RhoU may interact with CdGAP and relieve its inhibition by SH3D domain of ITSN1, which would result in Cdc42 inactivation. C) Another scenario of RhoU and CdGAP interaction with ITSN1-LΔ35. Activated RhoU together with SH3D domain of ITSN1 may maintain CdGAP inhibition. In this case Cdc42 will not be inactivated. D) A complex of ITSN1-L and ITSN1-LΔ35 may promote effective switching between Rho GTPase-regulated processes in neurons. In complex with either of ITSN1 isoforms OPHN1 inactivates RhoA, whereas ITSN1-L together with N-WASP and Numb, ITSN1-L neuron-specific partner, activates Cdc42. At the same time ITSN1-LΔ35 may promote sustained RhoU activation together with simultaneous inhibition of CdGAP to prevent Cdc42 inactivation. Red arrow means activation, red arrow with a blunt end – inactivation. Dashed line means indirect influence.

We have very good players, but what is the stage they are playing at? After a thorough examination of all available literature, I have discovered that the described complex may exist in neurons. Not at the presynaptic membrane, as we have previously supposed, but rather in dendritic spines during neuron development. Indeed, ITSN1-L is implicated in dendritic spine formation together with Numb and Cdc42 (35 and 64) and in neuron survival during the development (84). ITSN1-LΔ35 is expressed in embryonic brain and in adult brain (42). OPHN1 is also implicated in dendritic spine morphogenesis, where it inactivates RhoA pathway (163). RhoU is highly expressed in brain (130) and is involved in nervous system development (147 and 151), though its role in single neuron development is still unexplored. RhoA is known to be activated during a restricted period during dendritic spine development to stop the growth stage (primary filopodia) and to lance maturation (head and neck formation). It has been shown that RhoA hyperactivation negatively controls spine density and length. So its activity should be vigorously controlled, otherwise spine formation will be abolished (208). Moreover, it has been shown that RhoA activation leads to the destruction of the existing spines in the brain (209). So the described complex may be indispensable at the stage(s) of switching off RhoA and activating Cdc42 and/or RhoU pathways. Thus, summarizing all available evidence, it seems that neurons in general and developing dendritic spines in particular are the most probable sites for the functioning of the described complex(es).

VIII. Conclusions

In current work I have shown for the first time the interaction of endocytic scaffold protein ITSN1 with RhoU, a small atypical GTPase from the Rho family. Moreover, I have shown that the SH3A, C and E domains of ITSN1 and the second N-terminal proline-rich motif of RhoU mediate ITSN1/RhoU interaction. The following co-immunoprecipitation of both recombinant and endogenous proteins confirmed that this interaction exists in the cell. In addition, by immunolocalization experiment we have shown that RhoU expression changes the subcellular localization of ITSN1, triggering it to the plasma membrane and endosomes. In regard to the functional consequence of ITSN1/RhoU complex formation, we have shown that RhoU expression rescues the ITSN1-induced inhibition of transferrin endocytosis. This rescue effect is abolished when proline-rich motifs RhoU mutant (unable to bind ITSN) is expressed. Though the main function for ITSN1 and RhoU awaits to be discovered, we presume that they may be implicated in the membrane trafficking, EGFR signaling and cell migration (including in cancer cells) as both proteins were shown to be involved in these processes.

I have also characterized one of the minor isoforms of ITSN1-L - Δ35. This isoform lacks exon 35, which encodes a part of DH domain and a part of the linker between the DH and PH domains. We have designed a homology-based 3D model of the DH-PHΔ35 domains which clearly demonstrates that the excision of the exon 35 leads to the disappearance of α6 helix in the DH domain. This motif in the full-size DH-PH domains of ITSN1 is responsible for the distance between the DH and PH domains thereby preventing the PH domain from the interaction with the GTPase. In our 3D model the PH domain appeared very close to the DH domain and could even approach to the GTPase-binding pocket of the latter. Thus we have assumed that this spicing event may lead to the change of the specificity towards the GTPase. On the other hand, because of the approaching of the DH and PH domains of ITSN1-LΔ35 there was also a possibility of the PH domain interfering in the DH domain interaction with the target GTPase. However, it was not very likely as the new linker between the two domains appeared long and flexible. We have tested the binding and exchange abilities of the DH-PHΔ35 domains towards Cdc42, a well-known specific partner of ITSN1-L. Obtained results clearly demonstrated that DH-PHΔ35 activate Cdc42 as efficiently as the main isoform DH-PH domains. Besides, we have demonstrated that the DH-PHΔ35 domains specifically interact with RhoU, whereas the full size DH-PH domains do not. This fact confirmed the assumption that the excision of exon 35 leads to the change of specificity of the DH-PH domains. This finding is of particular interest as it demonstrates for the first time the interaction of RhoU with a GEF. However, additional experiments should be performed to test whether ITSN1-LΔ35 can act as a GEF for RhoU or their interaction has another functional consequence. There is a discussion whether RhoU needs protein regulators as classic Rho family

members or transcriptional regulation is enough to control RhoU functions. On the one hand it has high intrinsic nucleotide exchange level; on the other one it has been shown that RhoU may be inactivated in the cell upon serum stimulation, which presumes that it may need some activators. Answering the question about the role of RhoU/DH-PHΔ35 interaction will shed light at both the ways of regulation of atypical Rho GTPases and the purpose of alternative splicing.

Taking into account that both ITSN1 and RhoU are implicated in cellular transformation and cancer pathogenesis, we decided to verify whether they are expressed in different cancer tumor specimens. We have tested a panel of human breast and prostate cancer samples for the presence of ITSN1-L and ITSN1-LΔ35 transcripts, whereas the pheochromocytoma samples were tested for all the three investigated mRNAs (ITSN1-L, ITSN1-LΔ35 and RhoU). The target transcripts were identified in all tested tumors as well as normal tissues but their levels were varied. Thus it appears that ITSN1 and RhoU belong to genes whose expression is varied in cancer. Noteworthy, we observed a higher level of Δ35 transcript than was previously observed in tissue samples, which could imply that ITSN1-LΔ35 is more resistant to nuclease degradation.

Additionally we have identified OPHN1, a Rho GAP for RhoA, Rac1 and Cdc42 as ITSN1 binding partner. We have demonstrated the importance of SH3A domain of ITSN1 in mediating this interaction. OPHN1 is implicated in synaptic endocytosis and the maturation of synapses. We have shown that OPHN1 and ITSN1-L co-precipitate and colocalize in PC12, a cell line of rat neuroendocrine cells. Moreover, OPHN1 is relocalized to the plasma membrane upon the stimulation of the exocytosis. We suggest that OPHN1 and ITSN1-L could function together in the compensatory endocytosis in neurons and neuroendocrine cells. But this assumption remains unexplored and is a subject for future studies.

Finally we have shown that ITSN1-S and ITSN1-L interact in mammalian cells. As both of them act as high-capacity scaffolds, we speculate their interaction might lead to the assembling of multimolecular protein complexes. Previously the interaction of the other isoforms of ITSN1 as well as ITSN2 isoforms has been described. Regarding the number of ITSN1 partners, these complexes may have various protein composition and serve as platforms for the assembling of other components implicated in membrane trafficking and cellular signaling. However, the exact mechanism of ITSN1-ITSN1 interaction remains to be elucidated, as well as the precise complex compositions.

I have also elaborated several hypothetical models for the ITSN1 interaction with its new partners, as well as their possible implication in cellular processes. I suggest the interaction of ITSN1 with its partners – RhoU and OPHN1 occurs most likely in neurons, particularly during the

dendritic spine development. Intriguingly, ITSN1 seems to be an important node of the GTPase crosstalk network. It was shown that ITSN1 indirectly regulates Rac1- and Ras-depending signaling pathways, whereas ITSN1-L directly activates Cdc42. The identified new partners are the GAP for RhoA (predominantly) and an atypical GTPase from Rho family. Moreover, RhoU itself seems to be implicated in RhoA inactivation and/or activation of Rac1. But this issue remains to be investigated. Undoubtedly a further understanding of mechanism of small GTPases regulation by ITSN1 would bring at a new level our knowledge about small GTPases cross-talk.

Taken together our findings complete the present knowledge about ITSN1 interactome in mammalian cells as well as ITSN1 implication in various cellular processes, e.g. membrane trafficking. However, a plethora of questions about precise mechanisms of these interactions and functional relevance remains to be addressed.

IX. Materials and methods.

Antibodies

Mouse monoclonal α-Omni (D-8), polyclonal rabbit α-Omni (M-19) and polyclonal goat α-OPHN1 (C-19) antibodies were from Santa Cruz Biotechnology (USA). Mouse monoclonal α-Myc antibody was from Molecular Probes (USA). Rabbit polyclonal α-GFP and α-GST antibodies were from CliniSciences (France). Rabbit polyclonal antibodies against the EH2 domain of ITSN1 were described previously (82). HRP-conjugated goat α-mouse and α-rabbit antibodies, donkey α-goat antibodies as well as fluorescent Alexa 488-conjugated goat α-rabbit antibodies, Alexa 555-conjugated goat α-mouse antibodies, Alexa 488-conjugated donkey α-goat antibodies and Cy3-conjugated donkey α-mouse antibodies were purchased from Invitrogen (USA).

DNA plasmid constructs

ITSN1-L and ITSN1-LΔ35 Omni-tagged constructs were cloned by Dr. S. Kropyvko as indicated below. The coding sequence of ITSN1-L was amplified from human embryonic brain cDNA and cloned into the pcDNA4His/Max C vector (Invitrogen). The fragment of ITSN1-LΔ35 was amplified from embryonic brain cDNA with following primers: F.3188 (GCCCATAAGGAAGTCTACAAGC) and R.5254 (CCCTCGAGCTACGGCTCATCAAACAAC) (the reverse primer had a site for XhoI). Obtained fragment was subcloned in ITSN1-S construct in pBlueScript using unique HindIII and XhoI sites with following sequencing in search of ITSN1-LΔ35 clone. Finally ITSN1-LΔ35 was cloned into the pcDNA4His/Max C vector (Invitrogen). Plasmids encoding GFP-ITSN1-L, mCherry-ITSN1-S and Omni-ITSN1-S were described previously (57; 77; 82). The coding sequences of DH-PH and DH-PHΔ35 fragments were amplified from human embryonic brain cDNA with following primers: F.3723 (AGGAATTCATCCACGAGCTCATTGTCACC) with a site for EcoRI and R.4719 (TGCTCGAGGTAGAGTTCAGAAGCAGCTT) with a site for XhoI. Obtained PCR products were cloned in pGEX4T1 vector to generate GST-fusion constructs as well as in pcDNA4His/Max C vector (Invitrogen) for the Omni-tagged constructs. His-tagged DH-PH and DH-PHΔ35 fragments were generated from GST-fusion constructs by EcoRI and XhoI digestion with a following subcloning in pET30a vector. The identity of all obtained constructs was confirmed by sequencing. GST-fusion ITSN1 SH3 constructions were described previously (46 and 82).

The DNA constructs encoding GFP-RhoU and ΔN RhoU mutants were also described previously (141). Myc-tagged RhoU and its point-mutants (CA – Q107L and DN – T63N) were kindly provided by Dr. P. Aspenstrom (129). Myc-tagged mouse RhoU WT and mutants of proline-rich motifs (M1, M2 and DM) were a kind gift of Dr. D. Billadeau (146). GST-RhoU was generated

from RhoU-Myc by EcoRI and BamHI digestion with following cloning in pGEX4T1 vector. DNA constructs of hemagglutinin (HA)- or GFP-tagged OPHN1 were a kind gift of Dr. P. Billuart (162).

Cell culture and transfection

All cell lines were obtained from the American Type Culture Collection. CHO, 293 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (Sigma), 50 U/ml penicillin and 100 mg/ml streptomycin. The cells were transiently transfected using JetPEI transfection reagent (Polyplus Transfection) according to manufacturer recommendations and further processed 24 h after transfection.

PC12 cells were maintained in DMEM supplemented with 5% fetal calf serum (Sigma), 10% horse serum (Sigma), 50 U/ml penicillin and 100 mg/ml streptomycin. The cells were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer recommendations and further processed 48 h after transfection.

Recombinant GST-fusion and His-tagged protein expression and pull down experiment

GST-fusion ITSN1 SH3 domains were expressed in *Escherichia coli* BL21(DE3) strain and purified with glutathione-sepharose beads (GE Healthcare) according to the manufacturer's instructions. For pull-down experiments 5-10 μg of unfused GST or GST-fusion proteins bound to glutathione-sepharose were incubated for 1 h +4°C with the precleared cell lysates (2 mg of proteins). The beads were washed three times and eluted with Laemmli sample buffer.

GST-fusion ITSN1 DH-PH and DH-PHΔ35 domains were expressed in *Escherichia coli* BL21(DE3)pLys strain and purified with glutathione-sepharose beads (GE Healthcare) according to the manufacturer instructions with slight modifications: the lysis and binding buffers additionally contained 0,25% sarcosyl (sodium lauroyl sarcosinate). GST-RhoU was also expressed in *Escherichia coli* BL21 strain and purified with glutathione-sepharose beads (GE Healthcare) according to the manufacturer instructions but 2 mM $MgCl_2$ and 1 μM GDP were added to all buffers.

His-tagged ITSN1 DH-PH and DH-PHΔ35 domains were expressed in *Escherichia coli* BL21(DE3)pLys strain and purified with HisTrap Ni sepharose columns (GE Healthcare) according according to the manufacturer instructions with slight modifications: the lysis and binding buffers additionally contained 0,25% sarcosyl. The elutions were performed with 250 mM imidazole and directly used for subsequent applications.

Immunoprecipitation

The cells were lysed in IP buffer (150 mM NaCl, 20 mM Tris, pH7.5, 10% glycerol, 0,5% NP40, protease inhibitors cocktail (Sigma) and centrifuged for 15 min at 16,000g. Supernatant (2 mg of proteins) was incubated with 2 µg of α-Omni antibodies and 20 µl Protein A/G Plus Ultralink Resin (Thermo Scientific) for 4 h at +4°C. Then beads were washed four times with IP buffer without inhibitors. Immunoprecipitated complexes were eluted with Laemmli buffer, resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad).

Western blot analysis

The membranes were blocked with 5 % non-fat milk in TBS-T (1xTBS (Euromedex), 0,1% Tween-20) for 1 h, incubated with indicated primary antibodies and washed. Detection was performed by HRP-conjugated appropriate secondary antibodies.

Densitometric analysis of obtained results was carried out using the public domain ImageJ program, developed at the U.S. National Institutes of Health (http://rsb.info.nih.gov/ij/).

Immunofluorescence and confocal microscopy

The cells were plated on coverslips and transfected with the appropriate plasmid DNA. 24h or 48h post-transfected cells were washed with ice-cold 1xPBS and then fixed in 4% paraformaldehyde for 15 min at room temperature. Then cells were permeabilized in 0,1% Triton X-100 for 10 min, blocked with 3% BSA and 5% of appropriate serum in 1xPBS for 30 min at room temperature (or overnight at +4°C). The following incubations with the appropriate primary antibodies diluted in 3% BSA in 1xPBS were performed (1h at room temperature). For immunofluorescence respective secondary antibodies were used. Cell nuclei were stained with Hoechst 33258 (Sigma Aldrich). The slides were mounted in Mowiol (Sigma Aldrich) and further analyzed using Leica SP5 or Zeiss LSM510 confocal microscope.

Transferrin internalization assay

The transfected 293T cells were maintained for 2 h in serum-free DMEM, and then incubated for 30 min in DMEM containing 50 mg/ml Alexa 647-conjugated transferrin (Sigma Aldrich) at +37°C. After a wash with serum-free DMEM the cells were fixed at room temperature, permeabilized and incubated with appropriate antibodies as described above.

Exocytosis stimulation assay

The PC12 cells were plated on coverslips or in 10 mm culture plates and transfected with the respective plasmid DNA. 48h post-transfection cells were washed three times in LOCKE buffer

(140 mM NaCl; 4,7 mM KCl; 2,5 mM $CaCl_2$; 1,2 mM KH_2PO_4; 1,2 mM $MgSO_4$; 10 µM EDTA; 15 mM HEPES; 11 mM glucose) at +37°C. Exocytosis was stimulated by adding of 300 µM ATP in LOCKE buffer for 5 min. The control cells were incubated without adding of ATP. Afterwards cells were immediately fixed in 4% paraformaldehyde and proceeded to immunofluorescence or lysed in IP buffer and proceeded to IP as described above.

RhoGEF exchange assay

RhoGEF (guanine nucleotide exchange factor) activity assay was performed with His-tagged DH-PH domains using BK100 kit (Cytoskeleton). The kit allow to quantify the uptake of the fluorescent nucleotide analog N-methylanthraniloyl-GTP (Mant-GTP) by GTPases. As Mant-GTP gets bound in the nucleotide binding pocket of a GTPase, its fluorescence (ex: 360 nm, em: 440 nm) increases dramatically. Therefore, the enhancement of Mant-GTP fluorescent intensity in the presence of a small GTPase indicates a nucleotide uptake (or exchange for already bound nucleotide) by the GTPase. If an appropriate GEF is added to the reaction mix it provokes a drastic increase of Mant-GTP fluorescence as a result of quick nucleotide exchange. The assay was carried out in 384-well plate according to manufacturer recommendations with slight modifications. Thus no temperature control was possible during the assay performance. Eluates of His-tagged proteins in PBS with 250 mM imidazole were used. Correspondingly, PBS with 250 mM imidazole was added to the control samples. According to our data, neither PBS nor imidazole influence the nucleotide exchange dynamics. We have used two different controls which were substracted from the results: the exchange buffer without any proteins (used as a background control) and the only Cdc42 without GEF (as a control of intrinsic nucleotide exchange level). Fluorescent signals were analysed using Mithras LB 940 Multimode Microplate Reader from Berthold technologies.

RT-PCR

Human embryonic tissues were supplied by the Center of Embryonic Tissues (Kyiv, Ukraine). Breast and prostate tumor samples were from the collection of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology. (Kyiv, Ukraine). Pheochromocytoma samples were from Centre Hospitalier Universitaire de Nancy Brabois (Dr L. Brunaud), Nancy, France. Total RNA from 20-40 mg of tissue samples was isolated using the TRI Reagent (Sigma) or RNeasy Mini Kit from QIAGEN according to manufacturer recommendations. Total RNA from the cell lines was extracted from confluent 10 cm plates using RNeasy Mini Kit from QIAGEN according to manufacturer recommendations. cDNA was synthesized from 1 to 5 µg of total RNA using oligo(dT) primers and Expand Reverse Transcriptase (Roche) or M-MLV reverse

transcriptase (Invitrogen). 10% of the obtained cDNA was used as a template for PCR (5% for reference genes amplification). cDNA

The following primers were used for the detection of target transcripts in RT-PCR and nested PCR in cell lines and tumor samples:

- for DH-PH and DH-PHΔ35 detection:
    - I round: F.4160 (GCGCTGAGAGTCCGCAAGAAGAT), R.5307 (ATCTCCGTCCGACCCAAAAAATC);
    - II round: F.4320 (CAGACTTCAAGGAGTTCGTCAAA), R.5142 (ATGGTCACCTCACAGTACGGGTT);
    - III round: F.4415 (ACAAGATACCCACTGATCATTAA), R.4967 (TGATTTTCTGCACCCAGGCAGTC);
- for RhoU detection: F.252 (CAACTTCTCCGCGGTGGTGTCTG), R.582 (TCGATGTAGGAGGCGGCTTTGATT);
- for β-actin: F.442 (CCACTGGCATCGTGATGGAC), R.851 (GCGGATGTCCACGTCACACT);
- for GADPH: F.113 (TGAAGGTCGGAGTCAACGGATTTGGT), R.1072 (CATGTGGGCCATGAGGTCCACCAC).

We did not have to use the control without reverse transcriptase as forward and reverse primers were selected in different exons; thereby the size of the product is itself the control of a correct RT reaction and DNA absence in the sample. Instead we have used no template control for the PCR.

Densitometric analysis of bands was performed using the ImageLab software (Biorad).

3D modeling of DH-PHΔ35 domains

To obtain DH-PHΔ35 three-dimensional (3D) model, the crystal structure of ITSN1 DH-PH domains in complex with Cdc42 (PDB code: 1ki1) (32) was used as a template on the basis of BLAST search against PDB database. Pairwise alignment of DH-PH and DH-PHΔ35 sequences was created using ClustalW2 and formatted manually according to the syntax of Modeller *.ali files. The program Modeller (157) was used to generate initial homology models. Modeller generates protein 3D structures by satisfying spatial restraints imposed by the sequence alignment with the template structure and applying the terms of the CHARMM-22 force-field (210). A 3D protein model is obtained by optimizing the molecular probability density function while simultaneously minimizing input restraint violations. To guarantee sufficient conformational

sampling of each active site residue, several homology models are generated in this step. Usually, a number between 10 and 100 models provides a satisfactory sampling. Assessment of homology models is usually performed with two methods: statistical potentials or physics-based energy calculations. Both methods produce an estimate of the energy for the model or models being assessed.

Physics-based energy calculations aim to capture the interatomic interactions that are physically responsible for protein stability in solution, especially van der Waals and electrostatic interactions. These calculations are performed using a molecular mechanics force field; proteins are normally too large even for semi-empirical quantum mechanics-based calculations. Modeller objective function is an example of physics-based energy score.

Statistical potentials are empirical methods based on observed residue-residue contact frequencies among proteins of known structure in the PDB. They assign a probability or energy score to each possible pairwise interaction between amino acids and combine these pairwise interaction scores into a single score for the entire model. Some of such methods can also produce a residue-by-residue assessment that identifies poorly scoring regions within the model, though the model may have a reasonable score overall. Modeller Discrete Optimized Molecule Energy (DOPE) score is an example of statistical potential. The best model of DH-PHΔ35 domains was selected by the smallest value of normalized DOPE score (211).

To check Modeller scores independently, we used MolProbity suite (212), which allows performing of comprehensive analysis of protein structure residue by residue and calculates the score using statistical potential and physics-based approaches. After thorough examination of the best model with MolProbity suite, 5 regions for refinement were selected and the model ensembles for each refinement region were generated using Modeller loop refinement procedure. The model with the best energy score and best Molprobity score was selected for further analysis and visualization.

Superposition and visualization of three-dimensional structures was performed by PyMOL v.1.2 (213). Quantitative data were analyzed with the help of Microsoft Office XP Excel software.

X. References.

1. **McMahon H.T., Boucrot E.** Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol.* 2011, Vol. 12, 8, pp. 517-33.

2. **Jarousse N., Kelly R.B.** Endocytotic mechanisms in synapses. *Curr Opin Cell Biol.* 2001, Vol. 13, 4, pp. 461-9.

3. **Seto E.S., Bellen H.J., Lloyd T.E.** When cell biology meets development: endocytic regulation of signaling pathways. *Genes Dev.* 2002, Vol. 16, 11, pp. 1314-36.

4. **Goh L.K., Huang F., Kim W., Gygi S., Sorkin A.** Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. *J Cell Biol.* 2010, Vol. 189, 5, pp. 871-83.

5. **McPherson P.S., Kay B.K., Hussain N.K.** Signaling on the endocytic pathway. *Traffic.* 2001, Vol. 2, 6, pp. 375-84.

6. **Mayor S., Pagano R.E.** Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol.* 2007, Vol. 8, 8, pp. 603-12.

7. **Gundelfinger E.D., Kessels M.M., Qualmann B.** Temporal and spatial coordination of exocytosis and endocytosis. *Nat Rev Mol Cell Biol.* 2003, Vol. 4, 2, pp. 127-39.

8. **Zhai R.G., Bellen H.J.** The architecture of the active zone in the presynaptic nerve terminal. *Physiology (Bethesda).* 2004, Vol. 19, pp. 262-70.

9. **Rizo J., Rosenmund C.** Synaptic vesicle fusion. *Nat Struct Mol Biol.* 2008, Vol. 15, 7, pp. 665-74.

10. **Pang Z.P., Südhof T.C.** Cell biology of Ca2+-triggered exocytosis. *Curr Opin Cell Biol.* 2010, Vol. 22, 4, pp. 496-505.

11. **Trikha S., Lee E.C., Jeremic A.M.** Cell secretion: current structural and biochemical insights. *ScientificWorldJournal.* 2010, Vol. 10, pp. 2054-69.

12. **Kennedy M.J., Ehlers M.D.** Mechanisms and function of dendritic exocytosis. *Neuron.* 2011, Vol. 69, 5, pp. 856-75.

13. **Smith S.M., Renden R., von Gersdorff H.** Synaptic vesicle endocytosis: fast and slow modes of membrane retrieval. *Trends Neurosci.* 2008, Vol. 31, 11, pp. 559-68.

14. **Ceridono M., Ory S., Momboisse F., Chasserot-Golaz S., Houy S., Calco V., Haeberlé A.M., Demais V., Bailly Y., Bader M.F., Gasman S.** Selective recapture of secretory granule components after full collapse exocytosis in neuroendocrine chromaffin cells. *Traffic.* 2011, Vol. 12, 1, pp. 72-88.

15. **Barg S., Machado J.D.** Compensatory endocytosis in chromaffin cells. *Acta Physiol (Oxf).* 2008, Vol. 192, 2, pp. 195-201.

16. **Zeke A., Lukács M., Lim W.A., Reményi A.** Scaffolds: interaction platforms for cellular signalling circuits. *Trends Cell Biol.* 2009, Vol. 19, 8, pp. 364-74.

17. **Szymkiewicz I., Shupliakov O., Dikic I.** Cargo- and compartment-selective endocytic scaffold proteins. *Biochem J.* 2004, 383, pp. 1-11.

18. **Good M.C., Zalatan J.G., Lim W.A.** Scaffold proteins: hubs for controlling the flow of cellular information. *Science.* 2011, Vol. 332, 6030, pp. 680-6.

19. **Sengar A.S., Wang W., Bishay J., Cohen S., Egan S.E.** The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. *EMBO J.* 1999, Vol. 18, 5, pp. 1159-71.

20. **Okamoto M., Schoch S., Südhof T.C.** EHSH1/intersectin, a protein that contains EH and SH3 domains and binds to dynamin and SNAP-25. A protein connection between exocytosis and endocytosis? *J Biol Chem.* 1999, Vol. 274, 26, pp. 18446-54.

21. **Yamabhai M., Hoffman N.G., Hardison N.L., McPherson P.S., Castagnoli L., Cesareni G., Kay B.K.** Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J Biol Chem.* 1998, Vol. 273, 47, pp. 31401-7.

22. **Roos J., Kelly R.B.** Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with Drosophila dynamin. *J Biol Chem.* 1998, Vol. 273, 30, pp. 19108-19.

23. **Rose S., Malabarba M.G., Krag C., Schultz A., Tsushima H., Di Fiore P.P., Salcini A.E.** Caenorhabditis elegans intersectin: a synaptic protein regulating neurotransmission. *Mol Biol Cell.* 2007, Vol. 18, 12, pp. 5091-9.

24. **Guipponi M., Scott H.S., Chen H., Schebesta A., Rossier C., Antonarakis S.E.** Two isoforms of a human intersectin (ITSN) protein are produced by brain-specific alternative splicing in a stop codon. *Genomics.* 1998, Vol. 53, 3, pp. 369-76.

25. **Pucharcós C., Fuentes J.J., Casas C., de la Luna S., Alcántara S., Arbonés M.L., Soriano E., Estivill X., Pritchard M.** Alu-splice cloning of human Intersectin (ITSN), a putative multivalent binding protein expressed in proliferating and differentiating neurons and overexpressed in Down syndrome. *Eur J Hum Genet.* 1999, Vol. 7, 6, pp. 704-12.

26. **Guipponi M., Scott H.S., Hattori M., Ishii K., Sakaki Y., Antonarakis S.E.** Genomic structure, sequence, and refined mapping of the human intersectin gene (ITSN), which encompasses 250 kb on chromosome 21q22.1-->q22.2. *Cytogenet Cell Genet.* 1998, Vol. 83, 3-4, pp. 218-20.

27. **Confalonieri S., Di Fiore P.P.** The Eps15 homology (EH) domain. *FEBS Lett.* 2002, Vol. 513, 1, pp. 24-9.

28. **Tong X.K., Hussain N.K., de Heuvel E., Kurakin A., Abi-Jaoude E., Quinn C.C., Olson M.F., Marais R., Baranes D., Kay B.K., McPherson P.S.** The endocytic protein intersectin is a major binding partner for the Ras exchange factor mSos1 in rat brain. *EMBO J.* 2000, Vol. 19, 6, pp. 1263-71.

29. **Li S.S.** Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. *Biochem J.* 2005, Vol. 390, pp. 641-53.

30. **Rossman KL, Der CJ, Sondek J.** GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol.* 2005, Vol. 6, 2, pp. 167-80.

31. **Hussain N.K., Jenna S., Glogauer M., Quinn C.C., Wasiak S., Guipponi M., Antonarakis S.E., Kay B.K., Stossel T.P., Lamarche-Vane N., McPherson P.S.** Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP. *Nat Cell Biol.* 2001, Vol. 3, 10, pp. 927-32.

32. **Snyder J.T., Worthylake D.K., Rossman K.L., Betts L., Pruitt W.M., Siderovski D.P., Der C.J., Sondek J.** Structural basis for the selective activation of Rho GTPases by Dbl exchange factors. *Nat Struct Biol.* 2002, Vol. 9, 6, pp. 468-75.

33. **Ahmad K.F., Lim W.A.** The minimal autoinhibited unit of the guanine nucleotide exchange factor intersectin. *PLoS One.* 2010, Vol. 5, 6, p. e11291.

34. **Kintscher C., Wuertenberger S., Eylenstein R., Uhlendorf T., Groemping Y.** Autoinhibition of GEF activity in Intersectin 1 is mediated by the short SH3-DH domain linker. *Protein Sci.* 2010, Vol. 19, 11, pp. 2164-74.

35. **Nishimura T., Yamaguchi T., Tokunaga A., Hara A., Hamaguchi T., Kato K., Iwamatsu A., Okano H., Kaibuchi K.** Role of numb in dendritic spine development with a Cdc42 GEF intersectin and EphB2. *Mol Biol Cell.* 2006, Vol. 17, 3, pp. 1273-85.

36. **Pruitt W.M., Karnoub A.E., Rakauskas A.C., Guipponi M., Antonarakis S.E., Kurakin A., Kay B.K., Sondek J., Siderovski D.P., Der C.J.** Role of the pleckstrin homology domain in intersectin-L Dbl homology domain activation of Cdc42 and signaling. *Biochim Biophys Acta.* 2003, Vol. 1640, 1, pp. 61-8.

37. **Snyder J.T., Rossman K.L., Baumeister M.A., Pruitt W.M., Siderovski D.P., Der C.J., Lemmon M.A., Sondek J.** Quantitative analysis of the effect of phosphoinositide interactions on the function of Dbl family proteins.

38. **Hussain N.K., Yamabhai M., Ramjaun A.R., Guy A.M., Baranes D., O'Bryan J.P., Der C.J., Kay B.K., McPherson P.S.** Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells. *J Biol Chem.* 1999, Vol. 274, 22, pp. 15671-7.

39. **Scappini E., Koh T.W., Martin N.P., O'Bryan J.P.** Intersectin enhances huntingtin aggregation and neurodegeneration through activation of c-Jun-NH2-terminal kinase. *Hum Mol Genet.* 2007, Vol. 16, 15, pp. 1862-71.

40. **Rizo J., Südhof T.C.** C2-domains, Structure and Function of a Universal Ca2+-binding Domain. *J Biol Chem.* 1998, Vol. 273, 26, pp. 15879-82.

41. **Pucharcos C., Estivill X., de la Luna S.** Intersectin 2, a new multimodular protein involved in clathrin-mediated endocytosis. *FEBS Lett.* 2000, Vol. 478, 1-2, pp. 43-51.

42. **Kropyvko S., Gerasymchuk D., Skrypkina I., Dergai M., Dergai O., Nikolaienko O., Rynditch A., Tsyba L.** Structural diversity and differential expression of novel human intersectin 1 isoforms. *Mol Biol Rep.* 2010, Vol. 37, 6, pp. 2789-96.

43. **Tsyba L., Skrypkina I., Rynditch A., Nikolaienko O., Ferenets G., Fortna A., Gardiner K.** Alternative splicing of mammalian Intersectin 1: domain associations and tissue specificities. *Genomics.* 2004, Vol. 84, 1, pp. 106-13.

44. **McGlincy N.J., Smith C.W.** Alternative splicing resulting in nonsense-mediated mRNA decay: what is the meaning of nonsense? *Trends Biochem Sci.* 2008, Vol. 33, 8, pp. 385-93.

45. **Dergai M., Tsyba L., Dergai O., Zlatskii I., Skrypkina I., Kovalenko V., Rynditch A.** Microexon-based regulation of ITSN1 and Src SH3 domains specificity relies on introduction of

charged amino acids into the interaction interface. *Biochem Biophys Res Commun.* 2010, Vol. 399, 2, pp. 307-12.

46. **Tsyba L., Gryaznova T., Dergai O., Dergai M., Skrypkina I., Kropyvko S., Boldyryev O., Nikolaienko O., Novokhatska O., Rynditch A.** Alternative splicing affecting the SH3A domain controls the binding properties of intersectin 1 in neurons. *Biochem Biophys Res Commun.* 2008, Vol. 372, 4, pp. 929-34.

47. **Dergai M, Skrypkina I, Dergai O, Tsyba L, Novokhatska O, Filonenko V, Drobot L, Rynditch A.** Identification and characterization of a novel mammalian isoform of the endocytic adaptor ITSN1. *Gene.* 2011, Vol. 485, 2, pp. 120-129.

48. **Tsyba L., Nikolaienko O., Dergai O., Dergai M., Novokhatska O., Skrypkina I., Rynditch A.** Intersectin multidomain adaptor proteins: regulation of functional diversity. *Gene.* 2011, Vol. 473, 2, pp. 67-75.

49. **Horvath C.A., Vanden Broeck D., Boulet G.A., Bogers J., De Wolf M.J.** Epsin: inducing membrane curvature. *Int J Biochem Cell Biol.* 2007, Vol. 39, 10, pp. 1765-70.

50. **Martina J.A., Bonangelino C.J., Aguilar R.C., Bonifacino J.S.** Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. *J Cell Biol.* 2001, Vol. 153, 5, pp. 1111-20.

51. **Kelly L.E., Phillips A.M.** Molecular and genetic characterization of the interactions between the Drosophila stoned-B protein and DAP-160 (intersectin). *Biochem J.* 2005, Vol. 388, pp. 195-204.

52. **Maritzen T., Podufall J., Haucke V.** Stonins--specialized adaptors for synaptic vesicle recycling and beyond? *Traffic.* 2010, Vol. 11, 1, pp. 8-15.

53. **Koh T.W., Korolchuk V.I., Wairkar Y.P., Jiao W., Evergren E., Pan H., Zhou Y., Venken K.J., Shupliakov O., Robinson I.M., O'Kane C.J., Bellen H.J.** Eps15 and Dap160 control synaptic vesicle membrane retrieval and synapse development. *J Cell Biol.* 2007, Vol. 178, 2, pp. 309-22.

54. **Evergren E., Gad H., Walther K., Sundborger A., Tomilin N., Shupliakov O.** Intersectin is a negative regulator of dynamin recruitment to the synaptic endocytic zone in the central synapse. *J Neurosci.* 2007, Vol. 27, 2, pp. 379-90.

55. **Xie J., Vandenbroere I., Pirson I.** SHIP2 associates with intersectin and recruits it to the plasma membrane in response to EGF. *FEBS Lett.* 2008, Vol. 582, 20, pp. 3011-7.

56. **Nakatsu F., Perera R.M., Lucast L., Zoncu R., Domin J., Gertler F.B., Toomre D., De Camilli P.** The inositol 5-phosphatase SHIP2 regulates endocytic clathrin-coated pit dynamics. *J Cell Biol.* 2010, Vol. 190, 3, pp. 307-15.

57. **Dergai O., Novokhatska O., Dergai M., Skrypkina I., Tsyba L., Moreau J., Rynditch A.** Intersectin 1 forms complexes with SGIP1 and Reps1 in clathrin-coated pits. *Biochem Biophys Res Commun.* 2010, Vol. 402, 2, pp. 408-13.

58. **Allaire P.D., Ritter B., Thomas S., Burman J.L., Denisov A.Y., Legendre-Guillemin V., Harper S.Q., Davidson B.L., Gehring K., McPherson P.S.** Connecdenn, a novel DENN domain-containing protein of neuronal clathrin-coated vesicles functioning in synaptic vesicle endocytosis. *J Neurosci.* 2006, Vol. 26, 51, pp. 13202-12.

59. **Allaire P.D., Marat A.L., Dall'Armi C., Di Paolo G., McPherson P.S., Ritter B.** The Connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. *Mol Cell.* 2010, Vol. 37, 3, pp. 370-82.

60. **He G., Wang H.R., Huang S.K., Huang C.L.** Intersectin links WNK kinases to endocytosis of ROMK1. *J Clin Invest.* 2007, Vol. 117, 4, pp. 1078-87.

61. **Pechstein A., Bacetic J., Vahedi-Faridi A., Gromova K., Sundborger A., Tomlin N., Krainer G., Vorontsova O., Schäfer J.G., Owe S.G., Cousin M.A., Saenger W., Shupliakov O., Haucke V.** Regulation of synaptic vesicle recycling by complex formation between intersectin 1 and the clathrin adaptor complex AP2. *Proc Natl Acad Sci U S A.* 2010, Vol. 107, 9, pp. 4206-11.

62. **Henne W.M., Boucrot E., Meinecke M., Evergren E., Vallis Y., Mittal R., McMahon H.T.** FCHo proteins are nucleators of clathrin-mediated endocytosis. *Science.* 2010, Vol. 328, 5983, pp. 1281-4.

63. **Martin N.P., Mohney R.P., Dunn S., Das M., Scappini E., O'Bryan J.P.** Intersectin regulates epidermal growth factor receptor endocytosis, ubiquitylation, and signaling. *Mol Pharmacol.* 2006, Vol. 70, 5, pp. 1643-53.

64. **Thomas S., Ritter B., Verbich D., Sanson C., Bourbonnière L., McKinney R.A., McPherson P.S.** Intersectin regulates dendritic spine development and somatodendritic endocytosis but not synaptic vesicle recycling in hippocampal neurons. *J Biol Chem.* 2009, Vol. 284, 18, pp. 12410-9.

65. **Simpson F., Hussain N.K., Qualmann B., Kelly R.B., Kay B.K., McPherson P.S., Schmid S.L.** SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat Cell Biol.* 1999, Vol. 1, 2, pp. 119-24.

66. **Predescu S.A., Predescu D.N., Timblin B.K., Stan R.V., Malik A.B.** Intersectin regulates fission and internalization of caveolae in endothelial cells. *Mol Biol Cell.* 2003, Vol. 14, 12, pp. 4997-5010.

67. **Fernández-Chacón R., Achiriloaie M., Janz R., Albanesi J.P., Südhof T.C.** SCAMP1 function in endocytosis. *J Biol Chem.* 2000, Vol. 275, 17, pp. 12752-6.

68. **Liao H., Zhang J., Shestopal S., Szabo G., Castle A., Castle D.** Nonredundant function of secretory carrier membrane protein isoforms in dense core vesicle exocytosis. *Am J Physiol Cell Physiol.* 2008, Vol. 294, 3, pp. C797-809.

69. **Zhang J., Castle D.** Regulation of fusion pore closure and compound exocytosis in neuroendocrine PC12 cells by SCAMP1. *Traffic.* 2011, Vol. 12, 5, pp. 600-14.

70. **Bykhovskaia M.** Synapsin regulation of vesicle organization and functional pools. *Semin Cell Dev Biol.* 2011, Vol. 22, 4, pp. 387-92.

71. **Koh T.W., Verstreken P., Bellen H.J.** Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. *Neuron.* 2004, Vol. 43, 2, pp. 193-205.

72. **Marie B., Sweeney S.T., Poskanzer K.E., Roos J., Kelly R.B., Davis G.W.** Dap160/intersectin scaffolds the periactive zone to achieve high-fidelity endocytosis and normal synaptic growth. *Neuron.* 2004, Vol. 43, 2, pp. 207-19.

73. **Wang W., Bouhours M., Gracheva E.O., Liao E.H., Xu K., Sengar A.S., Xin X., Roder J., Boone C., Richmond J.E., Zhen M., Egan S.E.** ITSN-1 controls vesicle recycling at the neuromuscular junction and functions in parallel with DAB-1. *Traffic.* 2008, Vol. 9, 5, pp. 742-54.

74. **Yu Y, Chu PY, Bowser D.N., Keating D.J., Dubach D., Harper I., Tkalcevic J., Finkelstein D.I., Pritchard M.A.** Mice deficient for the chromosome 21 ortholog Itsn1 exhibit vesicle-trafficking abnormalities. *Hum Mol Genet.* 2008, Vol. 17, 21, pp. 3281-90.

75. **Sengar A.S., Ellegood J., Yiu A.P., Wang H., Wang W., Juneja S.C., Lerch J.P., Josselyn S.A., Henkelman R.M., Salter M.W., Egan S.E.** Vertebrate intersectin1 is repurposed to facilitate cortical midline connectivity and higher order cognition. *J Neurosci.* 2013, Vol. 33, 9, pp. 4055-65.

76. **Khanna R., Li Q., Stanley E.F.** 'Fractional recovery' analysis of a presynaptic synaptotagmin 1-anchored endocytic protein complex. *PLoS One.* 2006, 1, p. e67.

77. **Morderer D., Nikolaienko O., Skrypkina I., Cherkas V., Tsyba L., Belan P., Rynditch A.** Endocytic adaptor protein intersectin 1 forms a complex with microtubule stabilizer STOP in neurons. *Gene.* 2012, Vol. 505, 2, pp. 360-4.

78. **Glodowski D.R., Chen C.C., Schaefer H., Grant B.D., Rongo C.** RAB-10 regulates glutamate receptor recycling in a cholesterol-dependent endocytosis pathway. *Mol Biol Cell.* 2007, Vol. 18, 11, pp. 4387-96.

79. **Tong X.K., Hussain N.K., Adams A.G., O'Bryan J.P., McPherson P.S.** Intersectin can regulate the Ras/MAP kinase pathway independent of its role in endocytosis. *J Biol Chem.* 2000, Vol. 275, 38, pp. 29894-9.

80. **Mohney RP, Das M, Bivona TG, Hanes R, Adams AG, Philips MR, O'Bryan JP.** Intersectin activates Ras but stimulates transcription through an independent pathway involving JNK. *J Biol Chem.* 2003, Vol. 278, 47, pp. 47038-45.

81. **Adams A., Thorn J.M., Yamabhai M., Kay B.K., O'Bryan J.P.** Intersectin, an adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways. *J Biol Chem.* 2000, Vol. 275, 35, pp. 27414-20.

82. **Nikolaienko O., Skrypkina I., Tsyba L., Fedyshyn Y., Morderer D., Buchman V., de la Luna S., Drobot L., Rynditch A.** Intersectin 1 forms a complex with adaptor protein Ruk/CIN85 in vivo independently of epidermal growth factor stimulation. *Cell Signal.* 2009, Vol. 21, 5, pp. 753-9.

83. **Predescu S.A., Predescu D.N., Knezevic I., Klein I.K., Malik A.B.** Intersectin-1s regulates the mitochondrial apoptotic pathway in endothelial cells. *J Biol Chem.* 2007, Vol. 282, 23, pp. 17166-78.

84. **Das M., Scappini E., Martin N.P., Wong K.A., Dunn S., Chen Y.J., Miller S.L., Domin J., O'Bryan J.P.** Regulation of neuron survival through an intersectin-phosphoinositide 3'-kinase C2beta-AKT pathway. *Mol Cell Biol.* 2007, Vol. 27, 22, pp. 7906-17.

85. **Wennerberg K., Der C.** Rho-family GTPases: it’s not only Rac and Rho (and I like it). *Journal of Cell Science.* 2004, 117, pp. 1301-1312.

86. **Burridge K., Wennerberg K.** Rho and Rac take center stage. *Cell.* 167-79., 2004, Vol. 116, 2.

87. **Boureux A., Vignal E., Faure S., Fort P.** Evolution of the Rho family of ras-like GTPases in eukaryotes. *Mol Biol Evol.* 2007, Vol. 24, 1, pp. 203-16.

88. **Aspenstrom P., Fransson A. and Saras J.** Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem. J.* 2004, 377, pp. 327–337.

89. **Raftopoulou M., Hall A.** Cell migration: Rho GTPases lead the way. *Dev Biol.* 2004, Vol. 265, 1, pp. 23-32.

90. **Govek E.E., Newey S.E., Van Aelst L.** The role of the Rho GTPases in neuronal development. *Genes Dev.* 2005, Vol. 19, 1, pp. 1-49.

91. **Govek E.E., Hatten M.E., Van Aelst L.** The role of Rho GTPase proteins in CNS neuronal migration. *Dev Neurobiol.* 2011, Vol. 71, 6, pp. 528-53.

92. **Koh C.G.** Rho GTPases and their regulators in neuronal functions and development. *Neurosignals.* 2006-2007, Vol. 15, 5, pp. 228-37.

93. **Ramakers G.J.** Rho proteins, mental retardation and the cellular basis of cognition. *Trends Neurosci.* 2002, Vol. 25, 4, pp. 191-9.

94. **Aspenström P., Ruusala A., Pacholsky D.** Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. *Exp Cell Res.* 2007, Vol. 313, 17, pp. 3673-9.

95. **Qualmann B., Mellor H.** Regulation of endocytic traffic by Rho GTPases. *Biochem J.* 2003, Vol. 371, pp. 233-41.

96. **de Toledo M., Senic-Matuglia F., Salamero J., Uze G., Comunale F., Fort P., Blangy A.** The GTP/GDP cycling of rho GTPase TCL is an essential regulator of the early endocytic pathway. *Mol Biol Cell.* 2003, Vol. 14, 12, pp. 4846-56.

97. **Bu W., Lim K.B., Yu Y.H., Chou A.M., Sudhaharan T., Ahmed S.** Cdc42 interaction with N-WASP and Toca-1 regulates membrane tubulation, vesicle formation and vesicle motility: implications for endocytosis. *PLoS One.* 2010, Vol. 5, 8.

98. **Wang Z., Thurmond D.C.** Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J Cell Sci.* 2009, Vol. 122, pp. 893-903.

99. **Williams J.A., Chen X., Sabbatini M.E.** Small G proteins as key regulators of pancreatic digestive enzyme secretion. *Am J Physiol Endocrinol Metab.* 2009, Vol. 296, 3, pp. 405-14.

100. **Momboisse F., Houy S., Ory S., Calco V., Bader M.F., Gasman S.** How important are Rho GTPases in neurosecretion? *J Neurochem.* 2011, Vol. 117, 4, pp. 623-31.

101. **Ory S., Gasman S.** Rho GTPases and exocytosis: What are the molecular links? *Semin Cell Dev Biol.* 2011, Vol. 22, 1, pp. 27-32.

102. **Gasman S., Chasserot-Golaz S., Bader M.F., Vitale N.** Regulation of exocytosis in adrenal chromaffin cells: focus on ARF and Rho GTPases. *Cell Signal.* 2003, Vol. 15, 10, pp. 893-9.

103. **Gasman S., Chasserot-Golaz S., Malacombe M., Way M., Bader M.F.** Regulated exocytosis in neuroendocrine cells: a role for subplasmalemmal Cdc42/N-WASP-induced actin filaments. *Mol Biol Cell.* 2004, Vol. 15, 2, pp. 520-31.

104. **Bader M.F., Doussau F., Chasserot-Golaz S., Vitale N., Gasman S.** Coupling actin and membrane dynamics during calcium-regulated exocytosis: a role for Rho and ARF GTPases. *Biochim Biophys Acta.* 2004, Vol. 1742, 1-3, pp. 37-49.

105. **Malacombe M., Bader M.F., Gasman S.** Exocytosis in neuroendocrine cells: new tasks for actin. *Biochim Biophys Acta.* 2006, Vol. 1763, 11, pp. 1175-83.

106. **Boettner B., Van Aelst L.** The role of Rho GTPases in disease development. *Gene.* 2002, Vol. 286, pp. 155–174.

107. **Price L.S., Collard J.G.** Regulation of the cytoskeleton by Rho-family GTPases: implications for tumour cell invasion. *Semin Cancer Biol.* 2001, Vol. 11, 2, pp. 167-173.

108. **Stengel K., Zheng Y.** Cdc42 in oncogenic transformation, invasion, and tumorigenesis. *Cell Signal.* 2011, Vol. 23, 9, pp. 1415–1423.

109. **Parri M., Chiarugi P.** Rac and Rho GTPases in cancer cell motility control. *Cell Commun Signal.* 2010, Vol. 8, 23.

110. **Bar-Sagi D., Hall A.** Ras and Rho GTPases: a family reunion. *Cell.* 2000, Vol. 103, 2, pp. 227-38.

111. **Matozaki T., Nakanishi H., Takai Y.** Small G-protein networks: their crosstalk and signal cascades. *Cell Signal.* 2000, Vol. 12, 8, pp. 515-24.

112. **Guilluy C., Garcia-Mata R., and Burridge K.** Rho protein crosstalk: another social network? *Trends Cell Biol.* 2011, Vol. 21, 12, pp. 718–726.

113. **Moon S.Y., Zheng Y.** Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol.* 2003, Vol. 13, 1, pp. 13-22.

114. **Tcherkezian J., Lamarche-Vane N.** Current knowledge of the large RhoGAP family of proteins. *Biol Cell.* 2007, Vol. 99, 2, pp. 67-86.

115. **Garcia-Mata R., Boulter E., Burridge K.** The 'invisible hand': regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol.* 2011, Vol. 12, 8, pp. 493-504.

116. **Mulinari S., Häcker U.** Rho-guanine nucleotide exchange factors during development: Force is nothing without control. *Small GTPases.* 2010, Vol. 1, 1, pp. 28-43.

117. **Orchard R.C., Alto N.M.** Mimicking GEFs: a common theme for bacterial pathogens. *Cell Microbiol.* 2012, Vol. 18, 1, pp. 10-8.

118. **Jenna S., Hussain N.K., Danek E.I., Triki I., Wasiak S., McPherson P.S., Lamarche-Vane N.** The activity of the GTPase-activating protein CdGAP is regulated by the endocytic protein intersectin. *J Biol Chem.* 2002, Vol. 277, 8, pp. 6366-73.

119. **Primeau M., Ben Djoudi Ouadda A., Lamarche-Vane N.** Cdc42 GTPase-activating protein (CdGAP) interacts with the SH3D domain of Intersectin through a novel basic-rich motif. *FEBS Lett.* 2011, Vol. 585, 6, pp. 847-53.

120. **Yang G., Pan F., Gan W.B.** Stably maintained dendritic spines are associated with lifelong memories. *Nature.* 2009, Vol. 462, 7275, pp. 920-4.

121. **Irie F., Yamaguchi Y.** EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. *Nat Neurosci.* 2002, Vol. 5, 11, pp. 1117-8.

122. **Malacombe M., Ceridono M., Calco V., Chasserot-Golaz S., McPherson P.S., Bader M.F., Gasman S.** Intersectin-1L nucleotide exchange factor regulates secretory granule exocytosis by activating Cdc42. *EMBO J.* 2006, Vol. 25, 15, pp. 3494-503.

123. **Momboisse F., Ory S., Calco V., Malacombe M., Bader M.F., Gasman S.** Calcium-regulated exocytosis in neuroendocrine cells: intersectin-1L stimulates actin polymerization and exocytosis by activating Cdc42. *Ann N Y Acad Sci.* 2009, Vol. 1152, pp. 209-14.

124. **Keating D.J., Chen C., Pritchard M.A.** Alzheimer's disease and endocytic dysfunction: clues from the Down syndrome-related proteins, DSCR1 and ITSN1. *Ageing Res Rev.* 2006, Vol. 5, 4, pp. 388-401.

125. **Ma Y., Wang B., Li W., Ying G., Fu L., Niu R., Gu F.** Reduction of intersectin1-s induced apoptosis of human glioblastoma cells. *Brain Res.* 2010, Vol. 1351, pp. 222-8.

126. **Ma Y., Wang B., Li W., Liu X., Wang J., Ding T., Zhang J., Ying G., Fu L., Gu F.** Intersectin1-s is involved in migration and invasion of human glioma cells. *J Neurosci Res.* 2011, Vol. 89, 7, pp. 1079-90.

127. **Russo A., O'Bryan J.P.** Intersectin 1 is required for neuroblastoma tumorigenesis. *Oncogene.* 2012, Vol. 31, 46, pp. 4828-34.

128. **Dergai O., Dergai M., Skrypkina I., Matskova L., Tsyba L., Gudkova D., Rynditch A.** The LMP2A protein of Epstein-Barr virus regulates phosphorylation of ITSN1 and Shb adaptors by tyrosine kinases. *Cell Signal.* 2013, Vol. 25, 1, pp. 33-40.

129. **Saras J., Wollberg P., Aspenström P.** Wrch1 is a GTPase-deficient Cdc42-like protein with unusual binding characteristics and cellular effects. *Exp Cell Res.* 2004, Vol. 299, 2, pp. 356-69.

130. **Tao W., Pennica D., Xu L., Kalejta R.F., Levine A.J.** Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. *Genes Dev.* 2001, Vol. 15, 14, pp. 1796-807.

131. **Berzat A.C., Buss J.E., Chenette E.J., Weinbaum C.A., Shutes A., Der C.J., Minden A., Cox A.D.** Transforming activity of the Rho family GTPase, Wrch-1, a Wnt-regulated Cdc42 homolog, is dependent on a novel carboxyl-terminal palmitoylation motif. *J Biol Chem.* 2005, Vol. 280, 38, pp. 33055-65.

132. **Brazier H., Stephens S., Ory S., Fort P., Morrison N., Blangy A.** Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. *J Bone Miner Res.* 2006, Vol. 21, 9, pp. 1387-98.

133. **Schiavone D., Dewilde S., Vallania F., Turkson J., Di Cunto F., Poli V.** The RhoU/Wrch1 Rho GTPase gene is a common transcriptional target of both the gp130/STAT3 and Wnt-1 pathways. *Biochem J.* 2009, Vol. 421, 2, pp. 283-92.

134. **Bhavsar P.J., Infante E., Khwaja A., Ridley A.J.** Analysis of Rho GTPase expression in T-ALL identifies RhoU as a target for Notch involved in T-ALL cell migration. *Oncogene.* 2012.

135. **Chuang Y.Y., Valster A., Coniglio S.J., Backer J.M., Symons M.** The atypical Rho family GTPase Wrch-1 regulates focal adhesion formation and cell migration. *J Cell Sci.* 2007, Vol. 120, pp. 1927-34.

136. **Artaza J.N., Sirad F., Ferrini M.G., Norris K.C.** 1,25(OH)2vitamin D3 inhibits cell proliferation by promoting cell cycle arrest without inducing apoptosis and modifies cell morphology of mesenchymal multipotent cells. *J Steroid Biochem Mol Biol.* 2010, Vol. 119, 1-2, pp. 73-83.

137. **Kirikoshi H., Katoh M.** Expression of WRCH1 in human cancer and down-regulation of WRCH1 by beta-estradiol in MCF-7 cells. *Int J Oncol.* 2002, Vol. 20, 4, pp. 777-83.

138. **Alan J.K., Berzat A.C., Dewar B.J., Graves L.M., Cox A.D.** Regulation of the Rho family small GTPase Wrch-1/RhoU by C-terminal tyrosine phosphorylation requires Src. *Mol Cell Biol.* 2010, Vol. 30, 17, pp. 4324-38.

139. **Tu S., Wu W.J., Wang J., Cerione R.A.** Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase. *J Biol Chem.* 2003, Vol. 278, 49, pp. 49293-300.

140. **Chang F., Lemmon C., Lietha D., Eck M., Romer L.** Tyrosine phosphorylation of Rac1: a role in regulation of cell spreading. *PLoS One.* 2011, Vol. 6, 12, p. e28587.

141. **Ory S., Brazier H., Blangy A.** Identification of a bipartite focal adhesion localization signal in RhoU/Wrch-1, a Rho family GTPase that regulates cell adhesion and migration. *Biol Cell.* 2007, Vol. 99, 12, pp. 701-16.

142. **Brady D.C., Alan J.K., Madigan J.P., Fanning A.S., Cox A.D.** The transforming Rho family GTPase Wrch-1 disrupts epithelial cell tight junctions and epithelial morphogenesis. *Mol Cell Biol.* 2009, Vol. 29, 4, pp. 1035-49.

143. **Shutes A., Berzat A.C., Cox A.D., Der C.J.** Atypical mechanism of regulation of the Wrch-1 Rho family small GTPase. *Curr Biol.* 2004, Vol. 14, 22, pp. 2052-6.

144. **Ruusala A., Aspenström P.** The atypical Rho GTPase Wrch1 collaborates with the nonreceptor tyrosine kinases Pyk2 and Src in regulating cytoskeletal dynamics. *Mol Cell Biol.* 2008, Vol. 28, 5, pp. 1802-14.

145. **Brazier H., Pawlak G., Vives V., Blangy A.** The Rho GTPase Wrch1 regulates osteoclast precursor adhesion and migration. *Int J Biochem Cell Biol.* 2009, Vol. 41, 6, pp. 1391-401.

146. **Zhang J.S., Koenig A., Young C., Billadeau D.D.** GRB2 couples RhoU to epidermal growth factor receptor signaling and cell migration. *Mol Biol Cell.* 2011, Vol. 22, 12, pp. 2119-30.

147. **Fort P., Guémar L., Vignal E., Morin N., Notarnicola C., de Santa Barbara P., Faure S.** Activity of the RhoU/Wrch1 GTPase is critical for cranial neural crest cell migration. *Dev Biol.* 2011, Vol. 320, 2, pp. 451-63.

148. **Loebel D.A., Studdert J.B., Power M., Radziewic T., Jones V., Coultas L., Jackson Y., Rao R.S., Steiner K., Fossat N., Robb L., Tam P.P.** Rhou maintains the epithelial architecture and facilitates differentiation of the foregut endoderm. *Development.* 2011, Vol. 128, 20, pp. 4511-22.

149. **Herbst R.S.** Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys.* 2004, Vol. 59, 2 Suppl, pp. 21-6.

150. **Naji L., Pacholsky D., Aspenström P.** ARHGAP30 is a Wrch-1-interacting protein involved in actin dynamics and cell adhesion. *Biochem Biophys Res Commun.* 2011, Vol. 409, 1, pp. 96-102.

151. **Notarnicola C., Le Guen L., Fort P., Faure S., de Santa Barbara P.** Dynamic expression patterns of RhoV/Chp and RhoU/Wrch during chicken embryonic development. *Dev Dyn.* 2008, Vol. 237, 4, pp. 1165-71.

152. **Faure S., Fort P.** Atypical RhoV and RhoU GTPases control development of the neural crest. *Small GTPases.* 2011, Vol. 2, 6, pp. 310-313.

153. **Gubar O.S., Kropyvko S.V., Tsyba L.O., Gasman S., Rynditch A.V.** Alternatively spliced short and long isoforms of adaptor protein intersectin 1 form complexes in mammalian cells. *Biopolymers and Cell.* 2012, Vol. 28, 6, pp. 429-433.

154. **O'Bryan J.P.** Intersecting pathways in cell biology. *Sci Signal.* 2010, Vol. 3, 152, p. re10.

155. **Willingham M.C., Hanover J.A., Dickson R.B., Pastan I.** Morphologic characterization of the pathway of transferrin endocytosis and recycling in human KB cells. *Proc Natl Acad Sci U S A.* 1984, Vol. 81, 1, pp. 175-9.

156. **Ullrich O., Reinsch S., Urbé S., Zerial M., Parton R.G.** Rab11 regulates recycling through the pericentriolar recycling endosome. *J Cell Biol.* 1996, Vol. 135, 4, pp. 913-24.

157. **Mart-Renom M.A., Stuart A.C., Fiser A., Snchez R., Melo F., Sali A.** Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct.* 2000, 29, pp. 291-325.

158. **Rossman K.L., Worthylake D.K., Snyder J.T., Siderovski D.P., Campbell S.L., Sondek J.** A crystallographic view of interactions between Dbs and Cdc42: PH domain-assisted guanine nucleotide exchange. *EMBO J.* 2002, Vol. 21, 6, pp. 1315-26.

159. **Glaven J.A., Whitehead I.P., Nomanbhoy T., Kay R., Cerione R.A.** Lfc and Lsc oncoproteins represent two new guanine nucleotide exchange factors for the Rho GTP-binding protein. *J Biol Chem.* 1996, Vol. 271, 44, pp. 27374-81.

160. **Wang J.B., Wu W.J., Cerione R.A.** Cdc42 and Ras cooperate to mediate cellular transformation by intersectin-L. *J Biol Chem.* 2005, Vol. 280, 24, pp. 22883-91.

161. **Billuart P., Bienvenu T., Ronce N., des Portes V., Vinet M.C., Zemni R., Roest Crollius H., Carrié A., Fauchereau F., Cherry M., Briault S., Hamel B., Fryns J.P., Beldjord C., Kahn A., Moraine C., Chelly J.** Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature.* 1998, Vol. 392, 6679, pp. 923-6.

162. **Fauchereau F., Herbrand U., Chafey P., Eberth A., Koulakoff A., Vinet M.C., Ahmadian M.R., Chelly J., Billuart P.** The RhoGAP activity of OPHN1, a new F-actin-binding protein, is negatively controlled by its amino-terminal domain. *Mol Cell Neurosci.* 2003, Vol. 23, 4, pp. 574-86.

163. **Govek E.E., Newey S.E., Akerman C.J., Cross J.R., Van der Veken L., Van Aelst L.** The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis. *Nat Neurosci.* 2004, Vol. 7, 4, pp. 364-72.

164. **Nadif Kasri N., Nakano-Kobayashi A., Malinow R., Li B., Van Aelst L.** The Rho-linked mental retardation protein oligophrenin-1 controls synapse maturation and plasticity by stabilizing AMPA receptors. *Genes Dev.* 2009, Vol. 23, 11, pp. 1289-302.

165. **Khelfaoui M., Pavlowsky A., Powell A.D., Valnegri P., Cheong K.W., Blandin Y., Passafaro M., Jefferys J.G., Chelly J., Billuart P.** Inhibition of RhoA pathway rescues the endocytosis defects in Oligophrenin1 mouse model of mental retardation. *Hum Mol Genet.* 2009, Vol. 18, 14, pp. 2575-83.

166. **Nakano-Kobayashi A., Kasri N.N., Newey S.E., Van Aelst L.** The Rho-linked mental retardation protein OPHN1 controls synaptic vesicle endocytosis via endophilin A1. *Curr Biol.* 2009, Vol. 19, 13, pp. 1133-9.

167. **Vaudry D., Stork P.J., Lazarovici P., Eiden L.E.** Signaling pathways for PC12 cell differentiation: making the right connections. *Science.* 2002, Vol. 296, 5573, pp. 1648-9.

168. **Westerink R.H., Ewing A.G.** The PC12 cell as model for neurosecretion. *Acta Physiol (Oxf).* 2008, Vol. 192, 2, pp. 273-85.

169. **Maignan S., Guilloteau J.P., Fromage N., Arnoux B., Becquart J., Ducruix A.** Crystal structure of the mammalian Grb2 adaptor. *Science.* 1995, Vol. 268, 5208, pp. 291-3.

170. **Elion E.A.** The Ste5p scaffold. *J Cell Sci.* 2001, Vol. 114(Pt 22), pp. 3967-78.

171. **Dergai M. V., Dergai O. V., Tsyba L. O., Novokhatska O. V., Skrypkina I. Ya., Rynditch A. V.** Novel isoform of adaptor protein ITSN1 forms homodimers via its C-terminus. *Biopolym. Cell.* 2011, Vol. 27, 4, pp. 306-309.

172. **Feng Q., Baird D., Cerione R.A.** Novel regulatory mechanisms for the Dbl family guanine nucleotide exchange factor Cool-2/alpha-Pix. *EMBO J.* 2004, Vol. 23, 17, pp. 3492-504.

173. **Arthur W.T., Ellerbroek S.M., Der C.J., Burridge K., Wennerberg K.** XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. *J Biol Chem.* 2002, Vol. 277, 45, pp. 42964-72.

174. **Guo X., Stafford L.J., Bryan B., Xia C., Ma W., Wu X., Liu D., Songyang Z., Liu M.** A Rac/Cdc42-specific exchange factor, GEFT, induces cell proliferation, transformation, and migration. *J Biol Chem.* 2003, Vol. 278, 15, pp. 13207-15.

175. **Zhang B., Zhang Y., Wang Z., Zheng Y.** The role of Mg2+ cofactor in the guanine nucleotide exchange and GTP hydrolysis reactions of Rho family GTP-binding proteins. *J Biol Chem.* 2000, Vol. 275, 33, pp. 25299-307.

176. GTPase Protocols: The Ras Superfamily. [ed.] E. Manser and T. Leung. *Methods in Molecular Biology.* 2002, Vol. 189.

177. **Ferrel J.E.** What Do Scaffold Proteins Really Do? *Sci STKE.* 2000, Vol. 52.

178. **Mani M., Lee S.Y., Lucast L., Cremona O., Di Paolo G., De Camilli P., Ryan T.A.** The dual phosphatase activity of synaptojanin1 is required for both efficient synaptic vesicle endocytosis and reavailability at nerve terminals. *Neuron.* 2007, Vol. 56, 6, pp. 1004-18.

179. **Zanazzi G., Matthews G.** A doubleheader in endocytosis. *Neuron.* 2007, Vol. 56, 6, pp. 939-42.

180. **Yamazaki T., Zaal K., Hailey D., Presley J., Lippincott-Schwartz J., Samelson L.E.** Role of Grb2 in EGF-stimulated EGFR internalization. *J Cell Sci.* 2002, Vol. 115, pp. 1791-802.

181. **Jiang X., Huang F., Marusyk A., Sorkin A.** Grb2 regulates internalization of EGF receptors through clathrin-coated pits. *Mol Biol Cell.* 2003, Vol. 14, 3, pp. 858-70.

182. **Huang .F, Kirkpatrick D., Jiang X., Gygi S., Sorkin A.** Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol Cell.* 2006, Vol. 21, 6, pp. 737-48.

183. **Sorkina T., Bild A., Tebar F., Sorkin A.** Clathrin, adaptors and eps15 in endosomes containing activated epidermal growth factor receptors. *J Cell Sci.* 1999, Vol. 112, pp. 317-27.

184. **Myromslien F.D., Grøvdal L.M., Raiborg C., Stenmark H., Madshus I.H., Stang E.** Both clathrin-positive and -negative coats are involved in endosomal sorting of the EGF receptor. *Exp Cell Res.* 2006, Vol. 312, 16, pp. 3036-48.

185. **Sigismund S., Argenzio E., Tosoni D., Cavallaro E., Polo S., Di Fiore P.P.** Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell.* 2008, Vol. 15, 2, pp. 209-19.

186. **Wu W.J., Tu S., Cerione R.A.** Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. *Cell.* 2003, Vol. 114, 6, pp. 715-25.

187. **Nicholson R.I., Gee J.M., Harper M.E.** EGFR and cancer prognosis. *Eur J Cancer.* 2001, Vol. 37, Suppl.4, pp. S9-15.

188. **Fisher R., Pusztai L., Swanton C.** Cancer heterogeneity: implications for targeted therapeutics. *Br J Cancer.* 2013, p. doi: 10.1038/bjc.2012.581.

189. **Brosnan J.A., Iacobuzio-Donahue C.A.** A new branch on the tree: next-generation sequencing in the study of cancer evolution. *Semin Cell Dev Biol.* 2012, Vol. 23, 2, pp. 237-42.

190. **Heng H.H., Stevens J.B., Bremer S.W., Ye K.J., Liu G., Ye C.J.** The evolutionary mechanism of cancer. *J Cell Biochem.* 2010, Vol. 109, 6, pp. 1072-84.

191. **Kim J.E., Stones C., Joseph W.R., Leung E., Finlay G.J., Shelling A.N., Phillips W.A., Shepherd P.R., Baguley B.C.** Comparison of growth factor signalling pathway utilisation in cultured normal melanocytes and melanoma cell lines. *BMC Cancer.* 2012, Vol. 12, 141.

192. **Duesberg P., Li R., Sachs R., Fabarius A., Upender M.B., Hehlmann R.** Cancer drug resistance: the central role of the karyotype. *Drug Resist Updat.* 2007, Vol. 10, 1-2, pp. 51-8.

193. **Marusyk A., Polyak K.** Tumor heterogeneity: causes and consequences. *Biochim Biophys Acta.* 2010, Vol. 1805, 1, pp. 105-17.

194. **Qin Y., Meisen W.H., Hao Y., Macara I.G.** Tuba, a Cdc42 GEF, is required for polarized spindle orientation during epithelial cyst formation. *J Cell Biol.* 2010, Vol. 189, 4, pp. 661-9.

195. **Chabu C., Doe C.Q.** Dap160/intersectin binds and activates aPKC to regulate cell polarity and cell cycle progression. *Development.* 2008, Vol. 135, 16, pp. 2739-46.

196. **Rodriguez-Fraticelli A.E., Vergarajauregui S., Eastburn D.J., Datta A., Alonso M.A., Mostov K., Martín-Belmonte F.** The Cdc42 GEF Intersectin 2 controls mitotic spindle orientation to form the lumen during epithelial morphogenesis. *J Cell Biol.* 2010, Vol. 189, 4, pp. 725-38.

197. **Pechstein A., Shupliakov O., Haucke V.** Intersectin 1: a versatile actor in the synaptic vesicle cycle. *Biochem Soc Trans.* 2010, Vol. 38, pp. 181-6.

198. **Gasman S., Chasserot-Golaz S., Hubert P., Aunis D., Bader M.F.** Identification of a potential effector pathway for the trimeric Go protein associated with secretory granules. Go stimulates a granule-bound phosphatidylinositol 4-kinase by activating RhoA in chromaffin cells. *J Biol Chem.* 1998, Vol. 273, 27, pp. 16913-20.

199. **Vaughan E.M., Miller A.L., Yu H.Y., Bement W.M.** Control of local Rho GTPase crosstalk by Abr. *Curr Biol.* 2011, Vol. 21, 4, pp. 270-7.

200. **Somers W.G., Saint R.** A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis. *Dev Cell.* 2003, Vol. 4, 1, pp. 29-39.

201. **Peter B.J., Kent H.M., Mills I.G., Vallis Y., Butler P.J., Evans P.R., McMahon H.T.** BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science.* 2004, Vol. 303, 5657, pp. 495-9.

202. **Gubar O.S., Kropyvko S.V., Tsyba L.O., Gasman S. , Rynditch A.V.** Alternatively spliced short and long isoforms of adaptor protein intersectin 1 form complexes in mammalian cells. *Biopolymers and Cell.* 2012, Vol. 28, 6, pp. 429-433.

203. **Cupers P., ter Haar E., Boll W., Kirchhausen T.** Parallel dimers and anti-parallel tetramers formed by epidermal growth factor receptor pathway substrate clone 15. *J. Biol. Chem.* . 1997, Vol. 272, 52, pp. 33430-33434.

204. **Wong K.A., Wilson J., Russo A., Wang L., Okur M.N., Wang X., Martin N.P., Scappini E., Carnegie G.K., O'Bryan J.P. .** Intersectin (ITSN) family of scaffolds function as molecular Hubs in protein interaction networks. *PLoS One.* 2012, Vol. 7, 4.

205. **Kristensen O., Guenat S., Dar I., Allaman-Pillet N., Abderrahmani A., Ferdaoussi M., Roduit R., Maurer F., Beckmann J.S., Kastrup J.S., Gajhede M., Bonny C.** A unique set of SH3-SH3 interactions controls IB1 homodimerization. *EMBO J.* 2006, Vol. 25, 4, pp. 785-97.

206. **Levinson N.M., Visperas P.R., Kuriyan J.** The tyrosine kinase Csk dimerizes through Its SH3 domain. *PLoS One.* 2009, Vol. 4, 11, p. e7683.

207. **Mattila P.K., Lappalainen P.** Filopodia: molecular architecture and cellular functions. *Nat Rev Mol Cell Biol.* 2008, Vol. 9, 6, pp. 446-54.

208. **Tashiro A., Yuste R.** Role of Rho GTPases in the morphogenesis and motility of dendritic spines. *Methods Enzymol.* 2008, Vol. 439, pp. 285-302.

209. **Chen Y., Kramár E.A., Chen L.Y., Babayan A.H., Andres A.L., Gall C.M., Lynch G., Baram T.Z.** Impairment of synaptic plasticity by the stress mediator CRH involves selective destruction of thin dendritic spines via RhoA signaling. *Mol Psychiatry.* 2012, p. doi: 10.1038/mp.2012.17. [Epub ahead of print].

210. **Brooks B.R., Bruccoleri R.E., Olafson B.D., States D.J., Swaminathan S., Karplus M.** CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. *J Comp Chem.* 1983, Vol. 4, 2, pp. 187–217.

211. **Shen M.-Y., Sali A.** Statistical potential for assessment and prediction of protein structures. *Protein Sci.* 2006, 15, pp. 2507-2524.

212. **Davis I.W., Leaver-Fay A., Chen V.B., Block J.N., Kapral G.J., Wang X., et al.** MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucl. Acids Res.* 2007, pp. W375-W383.

213. **DeLano W.L.** The PyMOL User's Manual. *DeLano Scientific.* Palo Alto, CA, USA : s.n., 2001.

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ГТФазо-активуючий білок олігофренін 1 – новий партнер багатофункціонального адапторного білка інтерсектину 1

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Інтерсектин 1 (ITSN1) – багатофункціональний адапторний білок, що бере участь в ендоцитозі, екзоцитозі та передачі клітинних сигналів, а також асоційований з такими патологіями, як синдром Дауна і хвороба Альцгеймера. ***Мета****. Виявлення нових білкових партнерів ITSN1, залучених до процесів мембранного транспорту.* ***Методи****. Аналізом in silico за допомогою програми Scansite виявлено ГТФазо-активуючий білок олігофренін 1 (OPHN1) як потенційний партнер SH3A-домену ITSN1. Для підтвердження формування комплексу між ITSN1 і OPHN1 застосовано тест на взаємодію in vitro з використанням GST-злитих білків та імунопреципітацію. Локалізацію білків у клітині визначали методами імунофлуоресценції та конфокальної мікроскопії.* ***Результати****. Показано, що мозкоспецифічна ізоформа SH3A-домену ITSN1 і SH3A-домен, який експресується в усіх тканинах, взаємодіють з OPHN1. ITSN1 і OPHN1 формують комплекси у клітинах лінії PC12 як у стані спокою, так і при стимулюванні екзоцитозу.* ***Висновки****. ГТФазо-активуючий білок OPHN1 та адапторний білок ITSN1 взаємодіють у клітинах лінії PC12 незалежно від стимулювання екзоцитозу.*

Ключові слова: інтерсектин 1, олігофренін 1, PC12, екзоцитоз.

Вступ. Інтерсектин 1 (ITSN1) – мультидоменний і мультифункціональний адапторний білок, що бере участь у клатрин- і кавеолін-опосередкованому ендоцитозі [1, 2] та в передачі клітинних сигналів [3, 4]. ITSN1 залучений до рециркуляції синаптичних пухирців [5], росту, розвитку та виживання нейронів [6]. Нещодавно показано, що ITSN1 причетний до прогресії гліоми та нейробластоми [7, 8].

Родина інтерсектинів є еволюційно консервативною. Личинки *Drosophila* з мутацією, яка спричиняє втрату функції гена *Dap160* (гомолог ITSN1), не доживають до пізніх стадій розвитку [9], а у мишей, нокаутних за геном *Itsn1*, порушено ендоцитоз і везикулярний трафік у нейронах [10]. Ген *ITSN1* людини локалізований на хромосомі 21, і рівень його експресії при синдромі Дауна підвищується [11]. За останніми даними, ITSN1 може бути одним із білків ендоцитозу, пов'язаних з розвитком спонтанної хвороби Альцгеймера [12].

ITSN1 має дві основні ізоформи, що утворюються в результаті альтернативного сплайсингу: коротку (ITSN1-S) і довгу (ITSN1-L) [13]. Коротка ізо-

форма складається з двох N-кінцевих EH (Eps15 homology)-доменів, центральної α-спіральної (або KLERQ) ділянки і п'яти послідовних С-кінцевих SH3 (src homology 3)-доменів. Цю ізоформу виявлено в усіх досліджених тканинах людини, у тому числі в ембріональних. Довга ізоформа переважно експресується в мозку і містить три додаткових С-кінцевих домени: DH (dbl homology), PH (pleckstrin homology) і C2 – два перших (тандем DH-PH-доменів) обумовлюють приналежність ITSN1-L до Dbl-родини гуаніннуклеотидобмінних факторів (GEF) і беруть участь в активації малої ГТФази Cdc42 (cell division cycle protein 42), ініціюючи заміну зв'язаного ГДФ на ГТФ [14].

На сьогодні однією з найдослідженіших функцій ITSN1 є участь у клатрин-опосередкованому ендоцитозі [1], у тому числі в компенсаторному ендоцитозі синаптичних пухирців [5, 15]. Нещодавно встановлено також залучення ITSN1-L до процесів екзоцитозу в хромафінних клітинах ссавців, зокрема, в клітинах наднирників щура лінії PC12 [16, 17]. Таким чином, ITSN1 може бути одним із білків, що об'єднують ці процеси. Як відомо, особливо тісно екзо- та ендоцитоз пов'язані в синапсах, але механізми цих подій ще вивчено недостатньо. Тому особливої актуальності набуває дослідження зв'язків між згаданими процесами і молекул, що ці зв'язки опосередковують.

У результаті аналізу *in silico* (за допомогою інтернет-ресурсу Scansite (http://scansite.mit.edu/) виявлено білок олігофренін 1 (OPHN1) як можливий партнер SH3A-домену ITSN1. OPHN1 є ГТФазо-активуючим білком для Rho-родини малих ГТФ-аз і бере участь в ендоцитозі [18] та екзоцитозі (Уї та ін., неопубліковане). Він складається з N-кінцевого BAR (Bin-Amphiphysin-Rvs)-домену, PH-домену, ГТФазо-активуючого домену та трьох С-кінцевих пролінзбагачених ділянок. OPHN1 на високому рівні експресується у мозку, зокрема присутній у пре- та постсинаптичних сайтах, а втрата його функції призводить до розвитку Х-зчепленої розумової відсталості [19, 20]. Враховуючи вищевикладене, нам видалося доцільним дослідити його можливу взаємодію з ITSN1.

Матеріали і методи. *Антитіла*. Використовували мишачі моноклональні антитіла Omni D8 (sc-7270) і поліклональні антитіла кози OPHN1 C-19 (sc-8374) («Santa-Cruz Biotechnology», США); кролячі поліклональні антитіла до GST («Invitrogen», США); кролячі поліклональні антитіла до GFP («Clinisciences», Франція); вторинні антитіла, мічені пероксидазою хрону, проти імуноглобулінів миші, кроля і кози («Invitrogen»). Вторинні антитіла для імунофлуоресценції з вислюка проти імуноглобулінів кози, кон'юговані з Alexa Fluor 488, та проти імуноглобулінів миші, кон'юговані з Cy3, придбано в «Invitrogen» і «Millipore» (США) відповідно. Фалоїдин, кон'югований з Atto-665, отримано із «Sigma» (США).

Плазмідні ДНК-конструкції. ДНК-конструкції SH3-доменів ITSN1 у векторі *pGEX-4T-3*, що експресують GST-злиті білки, описано в [21, 22]. кДНК повної кодуючої послідовності ITSN1-L одержано методом ЗТ-ПЛР з використанням тотальної РНК ембріонального мозку людини та клоновано у векторі *pcDNA4/HisMax* («Invitrogen»), що містить Omni-таг. ДНК конструкції OPHN1, злиті з HA- або GFP-тагами, люб'язно надано П. Білюартом [23].

Культура клітин і трансфекція. Лінію PC12 хромафінних клітин наднирників щура культивували за температури 37 °С у зволоженій атмосфері 5 % CO_2. Клітини вирощували в модифікованому Дюльбеко середовищі Ігла (DMEM) з 4,6 г/л глюкози і додаванням 5 % ембріональної сироватки теляти (FBS) та 10 % сироватки коня (HS) («Sigma»), 50 мкг/мл пеніциліну і 100 мкг/мл стрептоміцину. Трансфекцію проводили реагентом Lipofectamine 2000 («Invitrogen») згідно з протоколом виробника.

Аналіз взаємодії білків, злитих з GST, і Вестерн-блот. GST-злиті SH3-домени ITSN1 експресували в клітинах *Escherichia coli* штаму BL21, після чого імобілізували на глутатіон-сефарозі 4B («GE Healthcare», США) згідно з протоколом виробника. Клітини PC12 з чашок діаметром 10 см, що надекспресували один із варіантів OPHN1, через 48 год після трансфекції лізували в 1 мл розчину 50 мМ трису, pH 7,5, 150 мМ NaCl, 10 %-го гліцерину, 0,1 %-го NP40, 1 × PIC (суміш інгібіторів протеїназ, «Sigma»), центрифугували протягом 15 хв при 16000 *g*. До 2 мг тотального білка додавали зв'язані з сефарозою білки та інкубували за температури 4 °С з активним перемішуванням. Преципітат осаджували

центрифугуванням при 500 *g* протягом 1 хв, промивали тричі 50 мМ трисом, pH 7,5, 150 мМ NaCl, 0,1 %-м NP40 та інкубували з відповідною кількістю 2 × буфера Леммлі за температури 95 °С. Білки розділяли у 4–15 %-му ПААГ і переносили на нітроцелюлозну мембрану. Фрагмент гелю, що містив GST-злиті білки, фарбували Кумасі. Блоти блокували 5 %-м розчином молока у 1 × TBS з 0,1 %-м твіном-20, інкубували упродовж 1 год з первинними антитілами, відмивали та обробляли відповідними вторинними антитілами, кон'югованими з пероксидазою хрону. Детекцію проводили, використовуючи реагент SuperSignal West Dura Extended Duration Substrate («Thermo Scientific», США) і апарат ChemiCapt («Fischer Biobloc Scientfic», США).

Імунопреципітація. Клітини PC12 з чашок діаметром 10 см через 48 год після трансфекції стимулювали до екзоцитозу, як описано нижче, відразу лізували в 1 мл розчину IP (20 мМ трис, pH 7,5, 150 мМ NaCl, 10 %-й гліцерин, 0,5 %-й NP40), 1 × PIC та центрифугували протягом 15 хв при 16000 *g*. Імунопреципітацію здійснювали впродовж 4 год за методом [24].

Стимулювання екзоцитозу та імунофлуоресценція. Клітини висівали на покривні скельця, після 48-год трансфекції промивали тричі по 5 хв теплим буфером LOCKE (140 мМ NaCl, 4,7 мМ KCl, 2,5 мМ $CaCl_2$, 1,2 мМ KH_2PO_4, 1,2 мМ $MgSO_4$, 10 мкМ EDTA, 15 мМ HEPES, 11 мМ глюкоза) за температури 37 °С. Після цього екзоцитоз стимулювали впродовж 5 хв (37 °С) буфером LOCKE з 300 мкМ АТФ. Контрольні клітини інкубували за тих же умов в LOCKE без додавання АТФ. Надалі клітини фіксували у 4 %-му розчині параформальдегіду в PBS протягом 15 хв на льоду. Залишки формальдегіду відмивали, мембрани пермеабілізували 8-хв інкубацією в 0,1 %-му тритоні X-100 в PBS. Клітини блокували 3 %-м розчином БСА та 10 %-м розчином сироватки віслюка в PBS протягом 30 хв. Далі інкубували з первинними антитілами (1 год), промивали тричі PBS та інкубували з відповідними вторинними антитілами (1 год) і знову промивали, як описано раніше. Слайди робили, застосовуючи середовище Mowiol («Sigma») для приготування препаратів. Одержані препарати аналізували за допомогою конфокального лазерного скануючого мікроскопа Leica SP5.

Результати і обговорення. Для перевірки взаємодії *in vitro* SH3A-домену ITSN1 з OPHN1 рекомбінантні GST-злиті ізоформи SH3A-домену (рис. 1, *а*) іммобілізували на глутатіон-сефарозі та інкубували з лізатом хромафінних клітин лінії PC12 надниркових щура, які надекспресували один із варіантів GFP-злитого OPHN1 (рис. 1, *б*). Оскільки відомо, що N-кінцева частина олігофреніну блокує його ГТФазну активність, вона, ймовірно, може блокувати і потенційний сайт зв'язування з ITSN1 [23]. Тому використано два варіанти олігофреніну: повнорозмірний і С-кінцеву частину, яка містить лише ГТФазо-активуючий домен і пролін-збагачені ділянки. Також до експерименту залучено дві ізоформи SH3A-домену ITSN1 (нейронспецифічну і форму, присутню в усіх тканинах), які утворюються в результаті альтернативного сплайсингу і різняться за специфічністю взаємодії з пролін-багатими ділянками білків-партнерів [21]. Кількість GST-злитих білків контролювали фарбуванням за Понсо, преципітовані GFP-злиті OPHN1 або OPHN1 C-ter детектували Вестерн-блот-аналізом з антитілами проти GFP.

Контролем слугували іммобілізований на глутатіон-сефарозі GST і лізат клітин лінії PC12, в яких надекспресували GFP. Виявилося, що обидва варіанти OPHN1 зв'язуються з двома SH3A-доменами. Отже, інтерсектин може взаємодіяти з олігофренін і в аутоінгібованій формі.

Щоб довести специфічність взаємодії OPHN1 саме з SH3A-доменом ITSN1, ми провели аналогічний експеримент з перевірки взаємодії *in vitro* з кожним окремим SH3-доменом (A, B, C, D, E) (рис. 1, *в*). GST-злиті SH3-домени іммобілізували на глутатіон-сефарозі та інкубували з лізатом клітин лінії PC12, у яких надекспресували гемаглютинін(HA)-злитий OPHN1. Кількість GST-злитих білків контролювали фарбуванням Кумасі, преципітований OPHN1-HA детектували Вестерн-блот-аналізом з антитілами проти OPHN1. Як контроль використано іммобілізований на глутатіон-сефарозі GST і лізат клітин лінії PC12, в яких надекспресували порожній вектор.

Згідно з передбаченням програми Scansite, лише SH3A-домени (обидва варіанти) преципітували OPHN1.

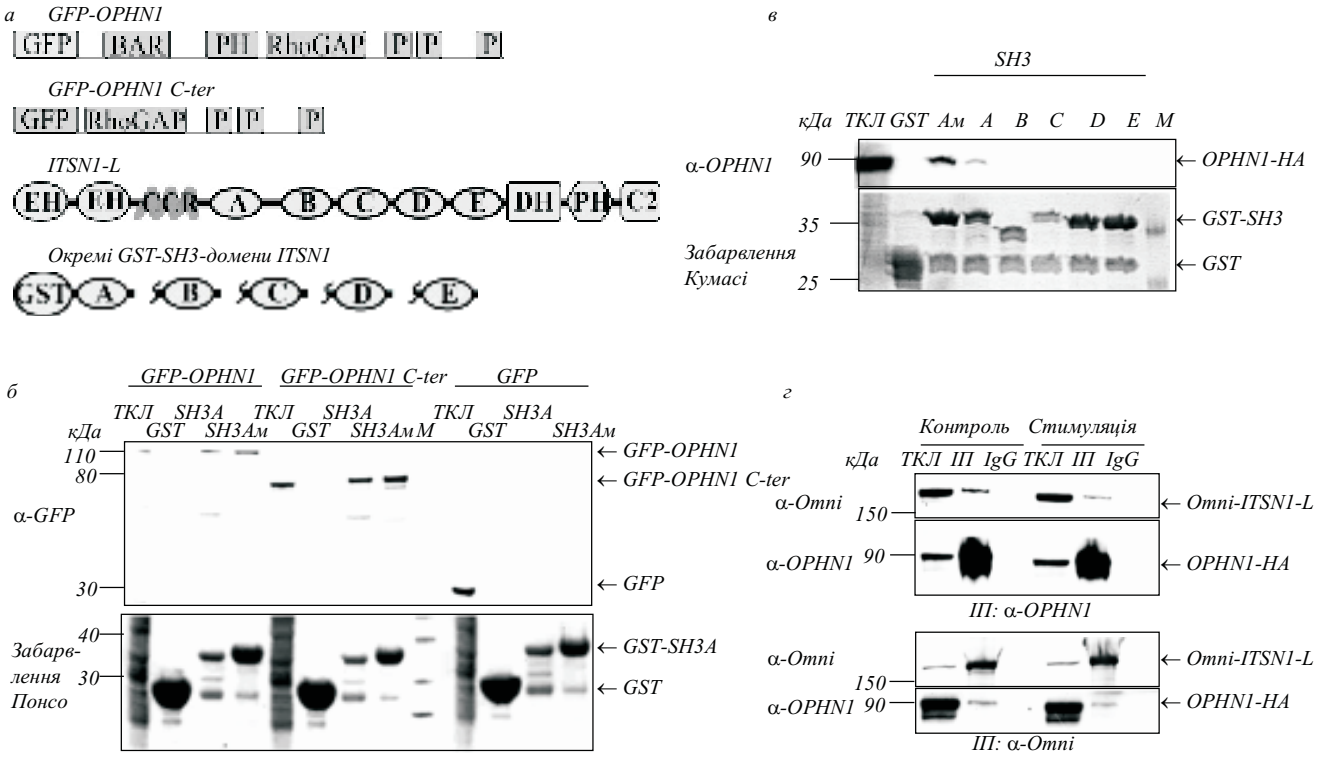

Рис. 1. ITSN1-L і OPHN1 взаємодіють *in vitro* та копреципітуються з клітинних лізатів: *а* – доменна організація повнорозмірних і делетованих форм ITSN1 і OPHN1, використаних у роботі; *б* – взаємодія SH3A-домену ITSN1 з повнорозмірним OPHN1 і його С-кінцевою частиною (OPHN1 C-ter) *in vitro* (кількість GST-злитих білків контролювали фарбуванням Понсо (нижня панель); SH3Ам – мозкоспецифічна ізоформа SH3A-домену ITSN1); *в* – SH3A-домен ITSN1 відповідає за зв'язування з OPHN1 *in vitro* (кількість GST-злитих білків контролювали фарбуванням Кумасі (нижня панель)); *г* – стимулювання екзоцитозу не впливає на формування комплексів між ITSN1-L і OPHN1 у клітинах лінії PC12 (*ІП* – імунопреципітація; *IgG* – імунопреципітація з контрольними імуноглобулінами; *ТКЛ* – тотальний клітинний лізат; *М* – маркер)

Наступним етапом стало дослідження наявності комплексів ITSN1 з OPHN1 у клітинах ссавців. Для цього застосовано метод імунопреципітації у клітинах лінії PC12 (рис. 1, *г*). Цю лінію хромафінних клітин наднирників щура обрано як робочу модель, оскільки її визнано класичною моделлю нейронального розвитку, а також нейросекреції [25]. До того ж вона експресує довгу і коротку ізоформи ITSN1.

Omni-ITSN1-L і OPHN1-HA коекспресували у клітинах лінії PC12. Для експерименту ITSN1-L обрано через те, що він експресується переважно в нейронах. Через 48 год після трансфекції клітини стимулювали до екзоцитозу, як описано в «Матеріалах і методах». Після цього їх лізували і отриманий лізат використовували для імунопреципітації з антитілами проти Omni-тагу або OPHN1. Преципітовані Omni-ITSN1-L або OPHN1-HA детектували Вестерн-блот- аналізом з відповідними антитілами. Контроль імунопреципітації здійснювали за допомогою IgG відповідного походження. Білки копреципітувалися в обох напрямках, що дійсно підтверджує формування комплексу ITSN1 з OPHN1 у клітинах. Кількість комплексів у контрольних і стимульованих клітинах практично не відрізняється, що свідчить про незалежність взаємодії ITSN1 з OPHN1 від екзоцитозу.

Одержані дані збігаються з результатами експерименту з колокалізації в клітинах лінії PC12 (рис. 2, див. уклейку). Клітини трансфікували Omni-ITSN1-L та OPHN1-HA. Через 48 год після трансфекції їх стимулювали до екзоцитозу, як описано у «Матеріалах і методах», і фіксували. Відповідні білки детектували методом імунофлуоресценції. Для кожної клітини робили два конфокальних зрізи у різних площинах: через цитоплазму та через ядро. При стимулюванні екзоцитозу OPHN1 частково релокалізується до периферії клітини (рис. 2, *б*), тоді як

Fig. 2 to article by Gubar O. S. et al.

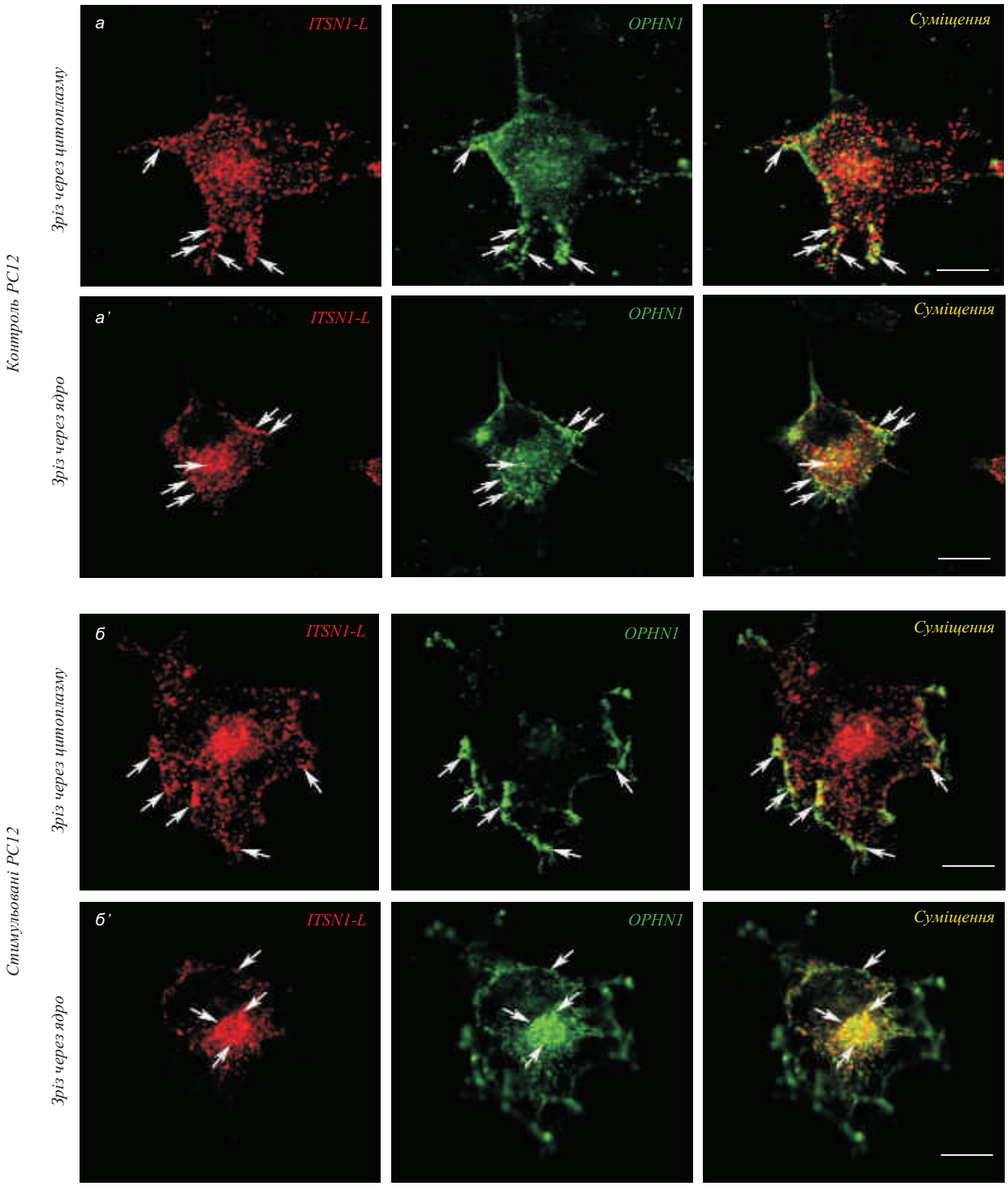

Рис. 2. ITSN1 і OPHN1 колокалізуються в клітинах наднирників щура лінії PC12. Клітини лінії PC12 трансфікували Omni-ITSN1-L і OPHN1-HA з наступним стимулюванням екзоцитозу. Білки детектували методом імунофлуоресценції (див. «Матеріали і методи»): Omni-ITSN1-L забарвлено червоним кольором, OPHN1-HA – зеленим. Для кожної клітини робили два конфокальних зрізи у різних площинах: через цитоплазму (верхній рядок, позначено літерами) і через ядро (нижній рядок, позначено тими самими літерами, але із штрихами: *а*,*а'* – контрольні клітини; *б*,*б'* – клітини, стимульовані до екзоцитозу). Масштаб відповідає 5 мкм

розташування ITSN1-L практично не змінюється. Коекспресія цих білків не впливає на їхню локалізацію у клітині. ITSN1-L і OPHN1 частково колокалізуються на периферії клітини (рис. 2, *а*, *б*), а також у приядерній частині цитоплазми (рис. 2, *а*', *б*'). Причому їхня колокалізація також не залежить від стимулювання. Структури на периферії клітини можуть бути сайтами ендоцитозу, хоча це, звичайно, потребує подальшого дослідження. Відомо, що у нейронах і нейросекреторних клітинах екзоцитоз тісно пов'язаний з компенсаторним ендоцитозом, який відбувається відразу ж після секреції (а при тривалому стимулюванні – і одночасно з нею) [26, 27]. Оскільки є дані, що і ITSN1, і OPHN1 також залучені до ендоцитозу [5, 15, 18], вищезгадані білки можуть разом брати участь у компенсаторному ендоцитозі в нейросекреторних клітинах і, можливо, нервових закінченнях. Перевірка останнього і становитиме предмет наших подальших досліджень.

Висновки. Таким чином, нами виявлено новий партнер багатофункціонального адапторного білка ITSN1 – ГТФазо-активуючий білок OPHN1, що специфічно зв'язується з SH3A-доменом ITSN1. Також уперше показано, що обидва білки формують комплекси і частково колокалізуються у секреторних клітинах лінії PC12 як у стані спокою, так і під час регульованого екзоцитозу.

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GTPase-activating protein oligophrenin 1 is a new partner of multifunctional adapter protein intersectin 1

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Summary

Aim. *Intersectin 1 (ITSN1) is a multifunctional adaptor protein which is involved in endocytosis, exocytosis and cellular signaling and it is also associated with such pathologies as Down syndrome and Alzheimer's disease. The aim of this study was to identify new ITSN1 protein partners which are implicated in membrane trafficking.* ***Methods***. *In silico analysis by Scansite online resource had identified a GTPase activating protein oligophrenin 1 (OPHN1) as a potential partner of ITSN1 SH3A domain. GST pull-down and immunoprecipitation were used to prove complex formation between ITSN1 and OPHN1. Subcellular protein localization was determined by immunofluorescence and confocal microscopy.* ***Results***. *We have shown that brain-specific and ubiquitously expressed SH3A domain isoforms of ITSN1 interact with OPHN1. ITSN1 and OPHN1 form complexes in both resting and stimulated to exocytosis PC12 cell line.* ***Conclusions***. *GTPase activating protein OPHN1 and adaptor protein ITSN1 interact in PC12 cell line independently of exocytosis stimulation.*

Keywords: intersectin 1, oligophrenin 1, PC12, exocytosis.

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ГТФазо-активирующий белок олигофренин 1 – новый партнер многофункционального адапторного белка интерсектина 1

Резюме

Интерсектин 1 (ITSN1) – многофункциональный адапторный белок, участвующий в эндоцитозе, экзоцитозе и передаче клеточных сигналов, а также ассоциированный с такими патологиями, как синдром Дауна и болезнь Альцгеймера. ***Цель***. *Выявление новых белковых партнеров ITSN1, вовлеченных в процессы мембранного транспорта.* ***Методы***. *Анализом in silico с помощью программы Scansite выявлен ГТФазо-активирующий белок олигофренин 1 (OPHN1) как потенциальный партнер SH3A-домена ITSN1. Для подтверждения формирования комплекса между ITSN1 и OPHN1 применен тест на взаимодействие in vitro с использованием GST-слитых белков и иммунопреципитация. Локализацию белков в клетке определяли методами иммунофлуоресценции и конфокальной микроскопии.* ***Результаты***. *Показано, что мозгоспецифическая изоформа SH3A-домена ITSN1 и SH3A-домен, экспрессирующийся во всех тканях, взаимодействуют с OPHN1. ITSN1 и OPHN1 формируют комплексы в клетках линии PC12 как в состоянии покоя, так и при стимулировании экзоцитоза.* ***Выводы***. *ГТФазо-активирующий белок OPHN1 и адапторный белок ITSN1 взаимодействуют в клетках линии PC12 независимо от стимулирования экзоцитоза.*

Ключевые слова: интерсектин 1, олигофренин 1, PC12, экзоцитоз.

REFERENCES

1. *Hussain N. K., Yamabhai M., Ramjaun A. R., Guyi A. M., Baranesi D., O'Bryan J. P., Der C. J., Kay B. K., McPherson P. S.* Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells // J. Biol. Chem.–1999.–**274**, N 22.–P. 15671–15677.
2. *Predescu S. A., Predescu D. N., Timblin B. K., Stan R. V., Malik A. B.* Intersectin regulates fission and internalization of caveolae in endothelial cells // Mol. Biol. Cell.–2003.–**14**, N 12.–P. 4997–5010.
3. *Tong X. K., Hussain N. K., Adams A. G., O'Bryan J. P., McPherson P. S.* Intersectin can regulate the Ras/MAP kinase pathway independent of its role in endocytosis // J. Biol. Chem.–2000.–**275**, N 38.–P. 29894–29899.
4. *Adams A., Thorn J. M., Yamabhai M., Kay B. K., O'Bryan J. P.* Intersectin, an adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways // J. Biol. Chem.–2000.–**275**, N 35.–P. 27414–27420.
5. *Pechstein A., Bacetic J., Vahedi-Faridi A., Gromova K., Sundborger A., Tomlin N., Krainer G., Vorontsova O., Schafer J. G., Owe S. G., Cousin M. A., Saenger W., Shupliakov O., Haucke V.* Regulation of synaptic vesicle recycling by complex formation between intersectin 1 and the clathrin adaptor complex AP2 // Proc. Natl Acad. Sci. USA.–2010.–**107**, N 9.–P. 4206–4211.

6. *Das M., Scappini E., Martin N. P., Wong K. A., Dunn S., Chen Y. J., Miller S. L., Domin J., O'Bryan J. P.* Regulation of neuron survival through an intersectin-phosphoinositide 3-Kinase C2β-AKT pathway // Moll. Cell. Biol.–2007.–**27**, N 22.–P. 7906–7917.
7. *Ma Y., Wang B., Li W., Liu X., Wang J., Ding T., Zhang J., Ying G., Fu L., Gu F.* Intersectin1-s is involved in migration and invasion of human glioma cells // J. Neurosci. Res.–2011.–**89**, N 7.–P. 1079–1090.
8. *Russo A., O'Bryan J. P.* Intersectin 1 is required for neuroblastoma tumorigenesis // Oncogene.–2012.–doi: 10.1038/onc.2011.643.
9. *Marie B., Sweeney S. T., Poskanzer K. E., Roos J., Kelly R. B., Davis G. W.* Dap160/Intersectin scaffolds the periactive zone to achieve high-fidelity endocytosis and normal synaptic growth // Neuron.–2004.–**43**, N 2.–P. 207–219.
10. *Yu Y., Chu P. Y., Bowser D. N., Keating D. J., Dubach D., Harper I., Tkalcevic J., Finkelstein D. I., Pritchard M. A.* Mice deficient for the chromosome 21 ortholog Itsn1 exhibit vesicle-trafficking abnormalities // Hum. Mol. Genet.– 2008.–**17**, N 21.–P. 3281–3290.
11. *Hunter M. P., Nelson M., Kurzer M., Wang X., Kryscio R. J., Head E., Pinna G., O'Bryan J. P.* Intersectin 1 contributes to phenotypes *in vivo*: implications for Down's syndrome // Neuroreport.–2011.–**22**, N 15.–P. 767–772.
12. *Keating D. J., Chen C., Pritchard M. A.* Alzheimer's disease and endocytic dysfunction: clues from the Down syndrome-related proteins, DSCR1 and ITSN1 // Ageing Res. Rev.–2006.–**5**, N 4.–P. 388–401.
13. *Guipponi M., Scott H. S., Chen H., Schebesta A., Rossier C., Antonarakis S. E.* Two isoforms of human intersectin (ITSN) protein are produced by brain-specific alternative splicing in a stop codon // Genomics.–1998.–**53**, N 3.–P. 369–376.
14. *Hussain N. K., Jenna S., Glogauer M., Quinn C. C., Wasiak S., Guipponi M., Antonarakis S. E., Kay B. K., Stossel T. P., Lamarche-Vane N., McPherson P. S.* Endocytic protein intersectin-1 regulates actin assembly via Cdc42 and N-WASP // Nat. Cell. Biol.–2001.–**3**, N 10.–P. 927–932.
15. *Koh T. W., Verstreken P., Bellen H. J.* Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis // Neuron.–2004.–**43**, N 2.–P. 193–205.
16. *Malacombe M., Ceridono M., Calco V., Chasserot-Golaz S., McPherson P. S., Bader M. F., Gasman S.* Intersectin-1L nucleotide exchange factor regulates secretory granule exocytosis by activating Cdc42 // EMBO J.–2006.–**25**, N 15.–P. 3494–3503.
17. *Momboisse F., Ory S., Calco V., Malacombe M., Bader M. F., Gasman S.* Calcium-regulated exocytosis in neuroendocrine cells: intersectin-1L stimulates actin polymerization and exocytosis by activating Cdc42 // Ann. NY Acad. Sci.–2009.–**1152**.–P. 209–214.
18. *Nakano-Kobayashi A., Kasri N. N., Newey S. E., Van Aelst L.* The Rho-linked mental retardation protein OPHN1 controls synaptic vesicle endocytosis via endophilin A1 // Curr. Biol.–2009.–**19**, N 13.–P. 1133–1139.
19. *Billuart P., Bienvenu T., Ronce N., des Portes V., Vinet M. C., Zemni R., Roest Crollius H., Carrie A., Fauchereau F., Cherry M., Briault S., Hamel B., Fryns J. P., Beldjord C., Kahn A., Moraine C., Chelly J.* Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation // Nature.–1998.–**392**, N 6679.–P. 923–926.
20. *Govek E. E., Newey S. E., Akerman C. J., Cross J. R., Van der Veken L., Van Aelst L.* The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis // Nat. Neurosci.–2004.–**7**, N 4.–P. 364–372.
21. *Tsyba L., Gryaznova T., Dergai O., Dergai M., Skrypkina I., Kropyvko S., Boldyryev O., Nikolaienko O., Novokhatska O., Rynditch A.* Alternative splicing affecting the SH3A domain controls the binding properties of intersectin 1 in neurons // Biochem. Biophys. Res. Commun.–2008.–**372**, N 4.–P. 929–934.
22. *Nikolaienko O., Skrypkina I., Tsyba L., Fedyshyn Y., Morderer D., Buchman V., de la Luna S., Drobot L., Rynditch A.* Intersectin 1 forms a complex with adaptor protein Ruk/CIN85 *in vivo* independently of epidermal growth factor stimulation // Cell. Signal.–2009.–**21**, N 5.–P. 753–759.
23. *Fauchereau F., Herbrand U., Chafey P., Eberth A., Koulakoff A., Vinet M. C., Ahmadian M. R., Chelly J., Billuart P.* The Rho GAP activity of OPHN1, a new F-actin-binding protein, is negatively controlled by its amino-terminal domain // Mol. Cell. Neurosci.–2003.–**23**, N 4.–P. 574–586.
24. *Dergai O. V., Dergai M. V., Skrypkina I. Ya., Tsyba L. O., Yaruchik A. M., Rynditch A. V.* Amphiphysin 1 and 2 interact with membrane protein 2A of Epstein-Barr virus and regulate its exosomal secretion // Biopolym. Cell.–2012.–**28**, N 3.–P. 234–238.
25. *Westerink R. H., Ewing A. G.* The PC12 cell as model for neurosecretion // Acta Physiol. (Oxf).–2008.–**192**, N 2.–P. 273–285.
26. *Gundelfinger E. D., Kessels M. M., Qualmann B.* Temporal and spatial coordination of exocytosis and endocytosis // Nat. Rev. Mol. Cell. Biol.–2003.–**4**, N 2.–P. 127–139.
27. *Jarousse N., Kelly R. B.* Endocytotic mechanisms in synapses // Curr. Opin. Cell. Biol.–2001.–**13**, N 4.–P. 461–469.

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Annex-2: a short communication in Biopolymers and Cell

"Alternatively spliced short and long isoforms of adaptor protein intersectin 1 form complexes in mammalian cells"

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Alternatively spliced short and long isoforms of adaptor protein intersectin 1 form complexes in mammalian cells

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Intersectin 1 (ITSN1) is an adaptor protein involved in membrane trafficking and cell signaling. Long and short isoforms of ITSN1 (ITSN1-L and ITSN1-S) are produced by alternative splicing. The ***aim*** *of our study was to investigate whether ITSN1-L and ITSN1-S could interact in mammalian cells.* ***Methods****. During this study we employed immunoprecipitation and confocal microscopy.* ***Results****. We have shown that endogenous ITSN1-S co-precipitates with overexpressed ITSN1-L in PC12, 293 and 293T cells. Long and short isoforms of ITSN1 also co-localize in 293T cells.* ***Conclusions****. ITSN1-L and ITSN1-S form complexes in mammalian cells.*

Keywords: ITSN1, alternatively spliced isoforms, adaptor/scaffold proteins.

Introduction. ITSN1 is a multidomain and multifunctional adaptor protein which is involved in clathrin- and caveolin-dependent endocytosis [1, 2], Ca^{2+}-regulated exocytosis [3] and synaptic vesicles retrieval [4]. It is also implicated in cellular signaling [5–7] and neuron survival [8]. Abnormalities of ITSN1 expression are associated with the endocytic anomalies reported in Down syndrome brains and early stages of Alzheimer's disease as well as with neurodegeneration in Huntington's disease [9, 10]. ITSN1 is also associated with glioma and neuroblastoma tumorigenesis [11, 12].

Two major ITSN1 isoforms are produced by alternative splicing [13]. Ubiquitously expressed ITSN1-S consists of two N-terminal EH (Eps15 homology) domains, coiled-coil region (CCR) and five SH3 (Src homology) domains. EH domains interact with NPF motifs and are highly involved in clathrin coated pits assembling [14], and SH3 domains interact with proline-rich PXXP motifs and provide protein-protein interactions in many cellular processes, including membrane trafficking and signaling [15]. The long isoform of ITSN1 (ITSN1-L) is expressed predominantly in neurons and has three additional C-terminal domains: DH (dbl homology), PH (pleckstrin homology) and C2. The tandem of DH-PH domains is a GEF (guanine nucleotide exchange factor) for the Rho-family small GTPase Cdc42 [16].

ITSN1 domain composition implies that this is an adaptor/scaffold protein. These proteins possess multiple modular interaction domains and play a crucial role in spatial and temporal organization of cellular processes. Scaffolds regulate selectivity in signaling pathways using tethering mechanism and physically assembling chosen components of signaling pathway or network [17]. It is known that some scaffold proteins dimerize or even oligomerize to perform their function [18, 19]. As we have recently shown that ITSN1-S forms a heterodimer with its minor isoform 22a [20, 21], we presumed that probably other isoforms of ITSN1 are able to interact with each other thereby assembling protein supercomplexes.

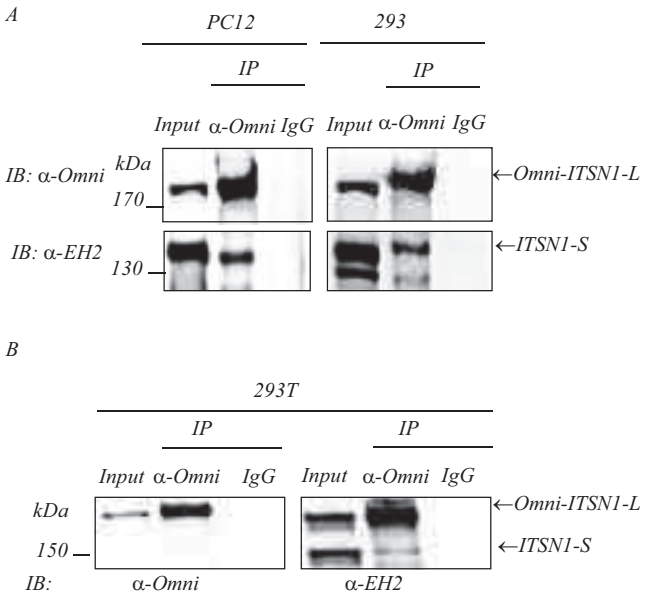

Fig. 1. ITSN1-L and ITSN1-S co-precipitate in mammalian cells. PC12 and 293 cells (*A*) or 293T cells (*B*) were transfected with Omni-ITSN1-L and subjected to immunoprecipitation (*IP*) with anti-Omni antibody or control IgG followed by immunoblotting (*IB*) with anti-EH2 (anti-ITSN1) antibody

Materials and methods. *Antibodies*. A monoclonal anti-Omni (D-8) antibody was from «Santa Cruz Biotechnology» (USA). Rabbit polyclonal antibodies against the EH2 domain of ITSN1 were described previously [22]. HRP-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from «Invitrogen» (USA).

DNA plasmid constructs. The coding sequence of ITSN1-L was amplified from human embryonic brain cDNA and cloned into the *pcDNA4His/Max C* vector («Invitrogen»).

Plasmids encoding GFP-ITSN1-L and mCherry-ITSN1-S were described previously [23, 24].

Cell culture and transfection. 293 and 293T cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum («Sigma», USA), 50 U/ml penicillin and 100 mg/ml streptomycin. The cells were transiently transfected using JetPEI transfection reagent (Polyplus Transfection) according to manufacturer recommendations and further processed 24 h after transfection.

PC12 cells were maintained in DMEM supplemented with 5 % fetal calf serum («Sigma»), 10 % horse serum («Sigma»), 50 U/ml penicillin and 100 mg/ml streptomycin. The cells were transiently transfected using Lipofectamine 2000 transfection reagent («Invitrogen») according to manufacturer recommendations and further processed 48 h after transfection.

Immunoprecipitation and Western blot analysis. The immunoprecipitation and Western blot were performed as described previously [25]. In brief, the cells were lysed in IP buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 10 % glycerol, 0.5 % NP40, protease inhibitors cocktail («Sigma») and centrifuged for 15 min at 16,000 g. Supernatant (2 mg of proteins) was incubated with 2 μg an- ti-Omni antibodies and 20 μl Protein A/G Plus Ultralink Resin («Thermo Scientific», USA) for 4 h at 4 °C. Then beads were washed four times with IP buffer without inhibitors. Immunoprecipitated complexes were eluted with Laemmli buffer, resolved by SDS-PAGE and transferred to nitrocellulose membranes («Bio-Rad», USA). The membranes were blocked with 5 % non-fat milk in TBS-T (1 × TBS («Euromedex», France), 0,1 % Tween 20) for 1 h, incubated with anti-Omni or anti-EH2 antibodies and washed. Detection was performed by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies.

Confocal microscopy. 293T cells were transfected with fluorescent protein constructs, fixed in 4 % paraformaldehyde in PBS 24 h after transfection, washed two times with PBS and mounted in Mowiol medium («Sigma»). The slides were analyzed using Leica SP5 confocal microscope.

Results and discussion. In order to prove the existence of ITSN1 macromolecular complexes in cells, we have performed a co-immunoprecipitation assay in different mammalian cell lines (Fig. 1).

Omni-tagged ITSN1-L was overexpressed in rat pheochromocytoma PC12 cell line and in human 293 or 293T cell lines. The immunoprecipitation was carried out with anti-Omni antibodies and further immunoblotting was performed with anti-EH2 antibodies to detect precipitated ITSN1-S. As a control of non-specific binding appropriate amount of mouse IgG was used. In all tested cell lines endogenous ITSN1-S readily co-precipitated with recombinant ITSN1-L. 293T cell line was of particular interest as it expressed endogenous ITSN1-L at relatively high level in contrast to 293 cell line (data not shown). And in addition to full-size ITSN1-L protein in 293T cells a minor band with slightly lower molecular weight is present which is presumab-

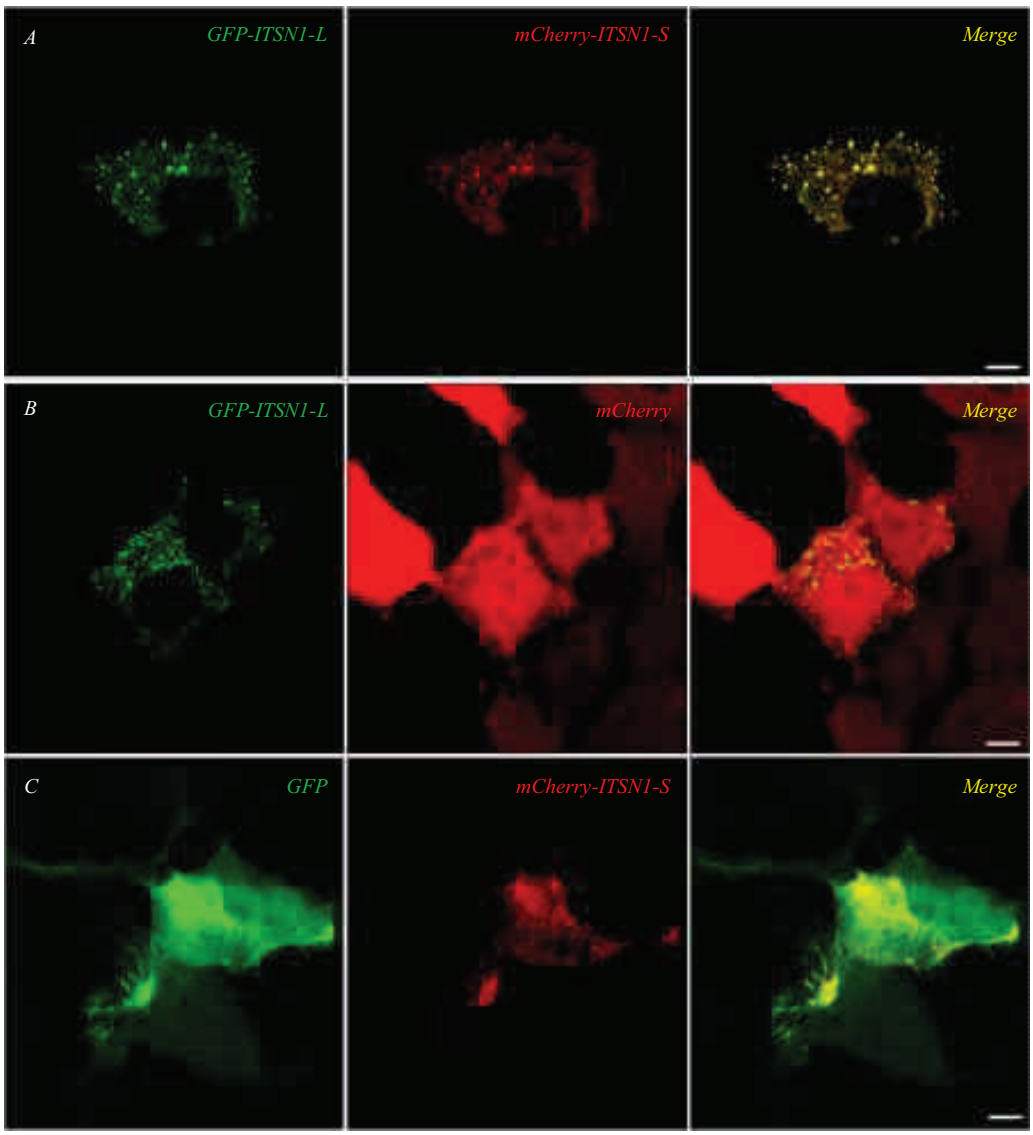

Fig. 2. Overexpressed GFP-ITSN1-L and mCherry-ITSN1-S colocalize in 293T cells. 293T cells were transfected with both GFP- ITSN1-L and mCherry-ITSN1-S (*A*) or with GFP-ITSN1-L alone and empty pmCherry vector (*B*) or with mCherry-ITSN1-S alone and empty pGFP-C1 vector (*C*). Scale bar represents 5 μm

ly one of the possible alternatively spliced isoforms of ITSN1-L. The bands below the ITSN1-S can also refer to one of its minor isoforms, e. g. ITSN1-22a [20, 21]. And these minor isoforms also co-precipitate with overexpressed ITSN1-L suggesting that ITSN1 can form heterodimers of different isoform composition (Fig. 1, *B*). To explore whether ITSN1-S and ITSN1-L have similar subcellular localization, we overexpressed GFP-tagged ITSN1-L and mCherry-tagged ITSN1-S in 293T cells (Fig. 2, *A*).

As a control we co-expressed each of the proteins with empty vector (Fig. 2, *B*, *C*). Obtained data clearly indicate that both isoforms perfectly colocalize and have similar subcellular distribution.

So we have demonstrated that ITSN1 isoforms interact in mammalian cells, thereby forming large complexes with varying protein composition that can function in membrane trafficking as well as in cell signaling. However the molecular mechanism controlling this clusterization remains unclear.

Such ITSN1-ITSN1 interaction possibly could be mediated by CCR as it is the case for Eps15, another CCR and EH domain containing protein [26]. This supposition is also supported by the work of Wong et al. [27]. They have used a high throughput yeast two hybrid screening to define the possible partners of the ITSN scaffolds and have identified ITSN1 as a target of a prey containing its EH2 domain and a half of CCR

(204–522 aa). They have further shown the existence of ITSN1 homo- and heteromeric complexes by bimolecular fluorescence complementation.

But we assume that ITSN1 SH3 domains may also contribute to this interaction as it has already been shown that some other proteins dimerize via their SH3 domains [28, 29]. These assumptions will be tested in our further research.

Conclusions. We have shown that ITSN1 long and short isoforms form complexes in different mammalian cell lines.

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Альтернативно сплайсовані коротка і довга ізоформи адапторного білка інтерсектину 1 формують комплекси у клітинах ссавців

Резюме

Інтерсектин 1 (ITSN1) – адапторний білок, залучений до мембранного транспорту та передачі клітинних сигналів. Довга і коротка ізоформи ITSN1 (ITSN1-L і ITSN1-S) утворюються в результаті альтернативного сплайсингу. ***Метою*** *роботи було встановити, чи можуть ITSN1-L і ITSN1-S взаємодіяти в клітинах ссавців.* ***Методи****. Використано методи імунопреципітації та конфокальної мікроскопії.* ***Результати****. Показано, що ендогенний ITSN1-S копреципітується з надекспресованим ITSN1-L у клітинах PC12, 293 і 293T. Довга і коротка ізоформи ITSN1 також колокалізуються у клітинах 293T.* ***Висновки****. ITSN1-L і ITSN1-S формують комплекси в клітинах ссавців.*

Ключові слова: ITSN1, альтернативно сплайсовані ізоформи, адапторні білки.

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Альтернативно сплайсированные короткая и длинная изоформы адапторного белка интерсектина 1 формируют комплексы в клетках млекопитающих

Резюме

Интерсектин 1 (ITSN1) – адапторный белок, участвующий в мембранном транспорте и передаче клеточных сигналов. Длинная и короткая изоформы ITSN1 (ITSN1-L и ITSN1-S) образуются вследствие альтернативного сплайсинга. ***Целью*** *исследования было выяснить, могут ли ITSN1-L и ITSN1-S взаимодействовать в клетках млекопитающих.* ***Методы****. Использованы методы иммунопреципитации и конфокальной микроскопии.* ***Результаты****. Показано, что эндогенный ITSN1-S копреципитируется со сверхэкпрессированным ITSN1-L в клетках PC12, 293 и 293T. Длинная и короткая изоформы ITSN1 также колокализуются в клетках 293T.* ***Выводы****. ITSN1-L и ITSN1-S формируют комплексы в клетках млекопитающих.*

Ключевые слова: ITSN1, альтернативно сплайсированные изоформы, адапторные белки.

REFERENCES

1. *Hussain N. K., Yamabhai M., Ramjaun A. R., Guy A. M., Baranes D., O'Bryan J. P., Der C. J., Kay B. K., McPherson P. S.* Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells // J. Biol. Chem.–1999.–**274**, N 22.–P. 15671–15677.
2. *Predescu S. A., Predescu D. N., Timblin B. K., Stan R. V., Malik A. B.* Intersectin regulates fission and internalization of caveolae in endothelial cells // Mol. Biol. Cell.–2003.–**14**, N 12.– P. 4997–5010.
3. *Momboisse F., Ory S., Calco V., Malacombe M., Bader M. F., Gasman S.* Calcium-regulated exocytosis in neuroendocrine cells: intersectin-1L stimulates actin polymerization and exocytosis by activating Cdc42 // Ann. NY Acad. Sci.–2009.–**1152**.–P. 209–214.
4. *Pechstein A., Bacetica J., Vahedi-Faridi A., Gromovaa K., Sundborgerb A., Tomlinb N., Krainerc G., Vorontsova O., Schafera J. G., Owed S. G., Cousine M. A., Saengera W., Shupliakov O., Haucke V.* Regulation of synaptic vesicle recycling by complex formation between intersectin 1 and the clathrin adaptor complex AP2 // Proc. Natl Acad. Sci. USA.–2010.–**107**, N 9.–P. 4206–4211.
5. *Tong X., Hussain N. K., Adams A. G., O'Bryan J. P., McPherson P. S.* Intersectin can regulate the Ras/MAP kinase pathway independent of its role in endocytosis // J. Biol. Chem.–2000.–**275**, N 38.–P. 29894–29899.
6. *Adams A., Thorn J. M., Yamabhai M., Kay B. K., O'Bryan J. P.* Intersectin, an adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways // J. Biol. Chem.–2000.–**275**, N 35.–P. 27414–27420.
7. *Novokhatska O. V., Skrypkina I. Ya., Dergai M. V., Tsyba L. O., Rynditch A. V*. RTK signaling regulator SPRY2 associates with endocytic adaptor ITSN1 *in vivo* // Biopolym. Cell.–2012.–**28**, N 4.–P. 314–316.
8. *Das M., Scappini E., Martin N. P., Wong K. A., Dunn S., Chen Y. J., Miller S. L., Domin J., O'Bryan J. P.* Regulation of neuron survival through an intersectin-phosphoinositide 3'-kinase C2 beta-AKT pathway // Moll. Cell. Biol.–2007.–**27**, N 22.–P. 7906–7917.
9. *Scappini E., Koh T. W., Martin N. P., O'Bryan J. P.* Intersectin enhances huntingtin aggregation and neurodegeneration through activation of c-Jun-NH2-terminal kinase // Hum. Mol. Genet.–2007.–**16**, N 15.–P. 1862–1871.
10. *Keating D. J., Chen C., Pritchard M. A.* Alzheimer's disease and endocytic dysfunction: clues from the Down syndrome-related proteins, DSCR1 and ITSN1 // Ageing Res. Rev.–2006.–**5**, N 4.–P. 388–401.
11. *Ma Y., Wang B., Li W., Liu X., Wang J., Ding T., Zhang J., Ying G., Fu L., Gu F.* Intersectin1-s is involved in migration and invasion of human glioma cells // J. Neurosci. Res.–2011.–**89**, N 7.–P. 1079–1090.
12. *Russo A., O'Bryan J. P.* Intersectin 1 is required for neuroblastoma tumorigenesis // Oncogene.–2012.–doi: 10.1038/onc.2011.643.
13. *Guipponi M., Scott H. S., Chen H., Schebesta A., Rossier C., Antonarakis S. E.* Two isoforms of a human intersectin (ITSN) protein are produced by brain-specific alternative splicing in a stop codon // Genomics.–1998.–**53**, N 3.–P. 369–376.
14. *Morgan J. R., Prasad K., Jin S., Augustine G. J., Lafer E. M.* Eps15 homology domain-NPF motif interactions regulate clathrin coat assembly during synaptic vesicle recycling // J. Biol. Chem.–2003.–**278**, N 35.–P. 33583–33592.
15. *Li S. S.* Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction // Biochem. J.–2005.–**390**, pt 3.–P. 641–653.
16. *Hussain N. K., Jenna S., Glogauer M., Quinn C. C., Wasiak S., Guipponi M., Antonarakis S. E., Kay B. K., Stossel T. P., Lamar-*

che-Vane N., McPherson P. S. Endocytic protein intersectin-1 regulates actin assembly via Cdc42 and N-WASP // Nat. Cell. Biol.–2001.–**3**, N 10.–P. 927–932.
17. *Good M. C., Zalatan J. G., Lim W. A.* Scaffold proteins: hubs for controlling the flow of cellular information // Science.–2011.–**332**, N 6030.–P. 680–686.
18. *Maignan S., Guilloteau J. P., Fromage N., Arnoux B., Becquart J., Ducruix A.* Crystal structure of the mammalian Grb2 adaptor // Science.–1995.–**268**, N 5208.–P. 291–293.
19. *Elion E. A.* The Ste5p scaffold // J. Cell. Sci.–2001.–**114**, N 22.–P. 3967–3978.
20. *Dergai M., Skrypkina I., Dergai O., Tsyba L., Novokhatska O., Filonenko V., Drobot L., Rynditch A.* Identification and characterization of a novel mammalian isoform of the endocytic adaptor ITSN1 // Gene.–2011.–**485**, N 2.–P. 120–129.
21. *Dergai M. V., Dergai O. V., Tsyba L. O., Novokhatska O. V., Skrypkina I. Ya., Rynditch A. V.* Novel isoform of adaptor protein ITSN1 forms homodimers via its C-terminus // Biopolym. Cell.–2011.–**27**, N. 4.–P. 306–309.
22. *Nikolaienko O., Skrypkina I., Tsyba L., Fedyshyn Y., Morderer D., Buchman V., de la Luna S., Drobot L., Rynditch A.* Intersectin 1 forms a complex with adaptor protein Ruk/CIN85 *in vivo* independently of epidermal growth factor stimulation // Cell. Signal.–2009.–**21**, N 5.–P. 753–759.
23. *Morderer D., Nikolaienko O., Skrypkina I., Cherkas V., Tsyba L., Belan P., Rynditch A.* Endocytic adaptor protein intersectin 1 forms a complex with microtubule stabilizer STOP in neurons // Gene.–2012.–**505**, N 2.–P. 360–364.
24. *Dergai O., Novokhatska O., Dergai M., Skrypkina I., Tsyba L., Moreau J., Rynditch A.* Intersectin 1 forms complexes with SGIP1 and Reps1 in clathrin-coated pits // Biochem. Biophys. Res. Commun.–2010.–**402**, N 2.–P. 408–413.
25. *Gubar O. S., Houy S. , Billuart P. , Kropyvko S. V., Tsyba L. O., Gasman S., Rynditch A. V.* GTPase-activating protein oligophrenin 1 is a new partner of multifunctional adapter protein intersectin 1 // Biopolym. Cell.–2012.–**28**, N 5.–P. 357–362.
26. *Cupers P., ter Haar E., Boll W., Kirchhausen T.* Parallel dimers and anti-parallel tetramers formed by epidermal growth factor receptor pathway substrate clone 15 // J. Biol. Chem.–1997.–**272**, N 52.–P. 33430–33434.
27. *Wong K. A., Wilson J., Russo A., Wang L., Okur M. N., Wang X., Martin N. P., Scappini E., Carnegie G. K., O'Bryan J. P.* Intersectin (ITSN) family of scaffolds function as molecular Hubs in protein interaction networks // PLoS One.–2012.–**7**, N 4.–e36023.
28. *Kristensen O., Guenat S., Dar I., Allaman-Pillet N., Abderrahmani A., Ferdaoussi M., Roduit R., Maurer F., Beckmann J. S., Kastrup J. S., Gajhede M., Bonny C.* A unique set of SH3-SH3 interactions controls IB1 homodimerization // EMBO J.–2006.–**25**, N 4.–P. 785–797.
29. *Levinson N. M., Visperas P. R., Kuriyan J.* The tyrosine kinase Csk dimerizes through Its SH3 domain // PLoS One.–2009.–**4**, N 11.–e7683.

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Rôle de l'intersectin-1 au cours du trafic membranaire:

Identification de nouveaux partenaires moléculaires.

Résumé

L'homéostasie cellulaire est intimement liée au trafic membranaire, processus dynamique qui permet les échanges de lipides et de protéines entre les compartiments cellulaires mais aussi entre la cellule et le milieu extracellulaire. L'intersectin-1 (ITSN1) est une protéine d'échafaudage multifonctionnelle, impliquée dans les processus d'endocytose, d'exocytose, diverses voies de signalisation ainsi que dans la survie cellulaire.

L'ensemble de mes travaux de doctorat a permis d'identifier deux nouveaux partenaires de l'ITSN1, RhoU et l'OPHN1, et de montrer leur implication dans le trafic membranaire. De plus je démontre que les variants d'épissage de l'ITSN1 pourraient avoir une spécificité d'interaction différente vis-à-vis de ses partenaires. Nous montrons aussi que l'ITSN1 est capable de former des complexes entre ses différentes isoformes. Ainsi, l'ensemble de ces données apportent de nouvelles connaissances sur l'interactôme d'ITSN1.

Résumé en anglais

The cellular homeostasis is tightly linked to the membrane trafficking, a dynamic process which allows lipid and protein exchange between the cellular compartments as well as the cell and the environment. Intersectin1 (ITSN1) is a multifunctional scaffold protein implicated in the processes of endocytosis and exocytosis, different signaling pathways and cell survival.

In present study I have identified two new partners of ITSN1, RhoU and OPHN1, and demonstrated their implication in membrane trafficking. Surprisingly, I have also found that the alternative splicing of ITSN1-L can lead to the change of the specificity of its interaction with binding partners. In addition, I have shown that different ITSN1 isoforms are capable to form complexes with each other. All together these data add new knowledge to ITSN1 interactome.