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Intersectin goes nuclear: secret life of an endocytic protein

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Abstract

Intersectin 1-short (ITSN1-s) is a 1220 amino acids ubiquitously-expressed scaffold protein presenting a multi-domain structure that allows to spatiotemporally regulate the functional interaction of a plethora of proteins. Beside its well-established role in endocytosis, ITSN1-s is involved in regulation of cell signaling and is implicated in tumorigenesis processes, although the signaling pathways involved are still poorly understood. Here we identify ITSN1-s as a nucleocytoplasmic trafficking protein. We show that, by binding to importin (IMP) α , a small fraction of ITSN1-s localizes in the cell nucleus at the steady state, where it preferentially associates with the nuclear envelope (NE) and interacts with lamin A/C. However, upon pharmacological ablation of Chromosome region maintenance 1 (CRM-1) dependent nuclear export pathway, the protein accumulates into the nucleus, thus revealing its moonlighting nature. Analysis of deletion mutants revealed that the coiled coil (CC) and Src homology (SH3) regions play the major role in its nucleocytoplasmic shuttling. While no evident nuclear localization signal (NLS) was detected in the CC region, a functional bipartite NLS was identified within the SH3D region of ITSN1-s (**RKKNPGGWWEGELQARGKKRQIGW**-1127), capable of conferring energy-dependent nuclear accumulation to reporter proteins and whose mutational ablation affects nuclear import of the whole SH3 region. Thus, ITSN1-s is an endocytic protein, which shuttles between the nucleus and the cytoplasm in a CRM-1 and IMP α dependent fashion.

Introduction

Scaffold proteins (scaffolds), are important components of several cellular processes and signaling systems. Usually, scaffolds are soluble proteins devoid of enzymatic activity, containing several modular protein-interaction domains within their structure, together with sites for inducible posttranslational modifications [1]. Scaffolds control the assembly of multiprotein complexes, thus contributing to localize signaling molecules to specific cell compartments or/and to regulate the efficiency of signaling pathways [2]. For example, Grb2-Associated Binding Protein 2 (GAB2) is involved in the assembly of signaling systems downstream of receptor tyrosine kinases and non-receptor tyrosine kinases [3], while Shc proteins have roles in signaling via many different types of receptors, such as growth factor receptors, antigen receptors, cytokine receptors, G-protein coupled receptors, hormone receptors and integrins [4].

Accumulating evidence suggests that several cytoplasmic adaptor proteins involved in endocytosis, such as clathrin, adaptor protein containing a pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif 1 (APPL1) and Beta arrestin1, are capable of shuttling between the nucleus and the cytoplasm, being involved in nuclear signaling and transcriptional events in response to extracellular signals [5-7]. Therefore, a detailed understanding of the cellular compartmentalization dynamics of adaptor proteins is crucial to gain insights regarding their function.

In eukaryotes, nucleocytoplasmic transport of cargoes larger than 50-60 kDa is a signal- and energy-dependent process, which takes place across aqueous channels, delimited by nuclear envelope (NE)-embedded nuclear pore complexes (NPCs). Members of the karyopherin superfamily, recognize specific nuclear targeting signals (NTSs) responsible for targeting cargoes either into or out of the nucleus, nuclear localization or nuclear export signals - NLSs and NESs - respectively [8]. Usually nuclear import is mediated by importins (IMPs), such as IMP β 1 or one of its homologues, after recognition of cargo bearing NLSs, either directly or through the adaptor molecule IMP α . IMP α recognizes short basic NLSs - also named "classical" NLS (cNLS). cNLSs can be classified as monopartite - matching the consensus K-(K/R)-X-(K/R), or bipartite - matching the consensus: [(K/R)(K/R)-X₁₀₋₁₂-(K/R)_{3/5}], where X is any amino acid, and (K/R)_{3/5} represents 3 lysine or arginine residues out of 5 consecutive amino acids. Subsequently, complexes are translocated through the NPCs into the nucleus, whereby binding of RanGTP to IMP β promotes their dissociation and cargo release [9].

On the other hand, proteins are exported from the nucleus by exportins such as Chromosome region maintenance 1 (CRM-1), the so far best characterized exportin. In the nucleus, RanGTP-complexed CRM-1 recognizes cargoes bearing short NESs containing four non-consecutive

hydrophobic residues [10] and it translocates them to the cytoplasm, where RanGTP-CRM1-cargo complexes are dissociated and RanGTP is hydrolyzed to RanGDP and this allows CRM1 to be recycled back to the nucleus [11].

Intersectin 1 (ITSN1) is a ubiquitously expressed scaffold protein present in a long and short isoform of 190 and 145 kDa respectively. ITSN1-s contains two N-terminal Eps15 homology (EH) domains, a coiled coil (CC) region, and five SRC homology 3 (SH3A-E) domains. Due to its multimodular architecture, it interacts with several proteins involved in clathrin- and caveolin-mediated endocytosis, rearrangements of the actin cytoskeleton, cell signaling and survival [12, 13]. Indeed EH domains recognize the Asn-Pro-Phe (NPF) motif of many endocytic machinery proteins, including Epsin [14, 15] while the coiled coil (CC) region mediates protein dimerization and interacts with Eps15 and Eps15R [16], as well as with SNAP23, SNAP25 and HIP [17]. The SH3 domains, typical of cytoskeleton proteins and proteins involved in signal transduction, recognize proline rich motifs of endocytic proteins such as Dynamin, Synapsin and Synaptojanin [18], as well as the SH3 domain of Endophilin [19].

Analysis of HeLa nuclei phosphoproteome revealed the presence of peptides derived from ITSN1-s, suggesting that ITSN1-s could have access to the nucleus and play a role therein, similarly to other scaffold adaptor proteins [20].

In the present study, we report that a small, but significant fraction of ITSN1-s is present at the steady state in the nucleus of HeLa and HEK 293 cells, where it accumulates on the NE. We show here for the first time that ITSN1-s is able to bind to IMP α and shuttle between the cytoplasm and the nucleus in a CRM-1 dependent fashion. We also identify a bipartite NLS located at residues 1104-1127, capable of conferring energy and Ran dependent nuclear import abilities to a reporter protein.

117 **Experimental**

118

119 **Cell culture and transfections.** HeLa, HEK 293-A and HEK 293-T cells were maintained in
120 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum
121 (FBS), 50 U/ml penicillin, 50 U/ml streptomycin and 2 mM L-glutamine as described previously
122 [21-23]. For Confocal laser scanning microscopy (CLSM) experiments, 3×10^4 HEK 293-A or 2.5
123 $\times 10^4$ HeLa cells were seeded onto polyisinated 12 mm glass coverslips in 24-well plates 1 day
124 before transfection using Lipofectamine 2000 (Thermofisher) according to the manufacturer's
125 recommendations [24]. For live cell imaging experiments, 6×10^5 HeLa cells were seeded in a
126 glass bottom 6-well plate (P06G-0-10F, MatTec) 1 day before transfection using Lipofectamine
127 3000 (Thermofisher) according to the manufacturer's recommendations. For cytosol/nucleus
128 separation of endogenous and FLAG-ITSN1-s, and for immunoprecipitation between GFP-
129 IMP α Δ IBB and FLAG tagged fusion proteins 5×10^5 HeLa cells and HEK 293-T cells were
130 seeded in 60 mm diameter dish the day before transfection using Lipofectamine 2000. All
131 experiments were carried out 48 h post transfection.

132

133 **Plasmid construction.** Mammalian expression plasmids were generated using the GatewayTM
134 technology (Invitrogen). ITSN1-s regions of interest were amplified with appropriate primer pairs
135 containing attB sites using plasmid FLAG-ITSN1-s [25] (a kind gift of Peter S. McPherson,
136 McGill University, Montreal, Quebec, Canada) and cloned into plasmid vector pDNR207
137 (Invitrogen) via BP recombination, to generate entry clones, as previously described [26]. Entry
138 clones were then used to generate C-terminal YFP fusion Mammalian expression vectors
139 following LR recombination reactions with the pDESTnYFP [27] Gateway compatible vector, as
140 described previously [28]. All vectors were confirmed by sequencing. Point mutant derivatives
141 carrying amino acid substitutions of ITSN-NLS (**RKKNPGGWEGELQARGKKRQIGW**-1127,
142 to **aatNPGGWEGELQARGatsQIGW**-1127) were generated using appropriate oligo pairs and
143 the Quikchange mutagenesis kit (Agilent technologies) as described previously [29]. As positive
144 and negative controls for response to LMB treatment, plasmids GFP-Rev (2-116), encoding the
145 Rev protein from HIV-1, which shuttles between nucleus and cytoplasm in a CRM-1 dependent
146 manner, and GFP-UL44 Δ NLS driving the expression of an exclusively cytosolic version of human
147 cytomegalovirus DNA polymerase processivity factor UL44, were used in addition to the pEGFP-
148 C1 (Clontech) expression vector [30, 31]. Plasmid pEGFP-C1-mIMP α Δ IBB, encoding a GFP-
149 tagged deletion mutant of mouse IMP α 6 lacking the autoinhibitory IMP β binding (IBB) domain,
150 therefore binding to cNLSs with high affinity [32], was described elsewhere [33]. pDESTnFLAG-

151 UL44, a plasmid mediating expression of a FLAG-tagged version of UL44 which is known to be
152 transported into the nucleus by the IMP α / β heterodimer, was used as a positive control in
153 immunoprecipitation experiments with GFP-mIMP α Δ IBB [28]. Plasmid pDESTnYFP-NLS[R],
154 encoding a fusion protein between YFP and a minimal cNLS derived from Simian Virus 40 Large
155 Tumor antigen, mediating nuclear targeting via IMP α / β was described elsewhere (Smith et al.,
156 unpublished observations).

157

158 **Confocal laser scanning microscopy (CLSM).** HeLa cells were treated as described in [34].
159 Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 10 min at room
160 temperature (RT), permeabilized with 0.2% TritonX-100, 2mg/ml BSA, 1mM NaN₃ in PBS on ice
161 three times for 10 min. Cells were subsequently incubated with blocking solution (0.02% TritonX-
162 100, 3% BSA, 1mM NaN₃ in PBS). Primary antibodies α -FLAG (SIGMA), and α -lamin A/C
163 (Thermo fisher) were incubated for 30 min in blocking solution and washed three times for 10 min
164 with wash buffer (PBS 0.02% TritonX-100, 1.5% BSA, 1mM NaN₃). Secondary antibodies (goat
165 α -mouse Cy3, goat α -rabbit Cy2, Jackson ImmunoResearch) were incubated for 45 min and
166 washed as described above. Coverslips were mounted using an antifade mounting medium
167 (ProLong Gold-Invitrogen) on a glass slide. CLSM was performed on a ZEISS LSM 510 META
168 confocal laser scanning microscope using the 63X or the 100X Plan-NEOFLUAR oil immersion
169 objective. To analyze the subcellular distribution of spontaneously fluorescent fusion proteins
170 expressed in HEK 293-A cells, cells were transfected and, after 48 h, washed twice in PHEM
171 buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, and 4mM MgSO₄) to preserve the cellular
172 cytoskeleton [35], before being fixed with 3% PFA in PHEM buffer for 15 min at RT and mounted
173 onto glass coverslips with FluoromountG (Southern Biotech). Samples were processed by CLSM
174 using a Leica TCT-SP2 system, equipped with a Planapo fluor 63x oil immersion objective (Leica).
175 At least 4 randomly chosen fields were acquired, and a total of at least 30 cells, expressing the
176 fusion proteins of interest to similar levels, were analyzed for each repetition. The Fn/c values were
177 determined using the NIH ImageJ 1.62 public domain software, from single cell measurements for
178 each of the nuclear (Fn) and cytoplasmic (Fc) fluorescence, after the subtraction of fluorescence due
179 to autofluorescence/background [36]. Data was plotted and statistically analyzed using Prism 6
180 (GraphPad) software.

181

182 **Live Cell Imaging.** HeLa cells were transfected to express the spontaneously fluorescent fusion
183 protein of interest. 48h later, the medium was substituted with complete phenol red free DMEM
184 (A18967, Life Technology). Cells were imaged with a DMI8 inverted microscope (Leica),

185 equipped with a 40x NA 0.6 objective, a motorized stage and a heated/humidified chamber, at
186 37°C and 5% CO₂. Several positions have been recorded for each sample. Phase contrast image
187 served as reference for cytoplasm vs. nuclei masking. When required, the Fn/c values relative to
188 each fusion protein were calculated as described above.

189

190 **Cell treatments.** In indicated cases, Leptomycin B (LMB; Sigma L2913; 2.9 ng/ml) was added to
191 cells 8 h before processing samples for imaging, as previously described [37]. Intracellular ATP
192 was depleted by incubating cells for 2h at 37°C in DMEM lacking phenol red and glucose
193 (Thermofisher), supplemented with 10 mmol/L sodium azide and 6 mmol/L 2-deoxy-D-glucose
194 (Sigma), as described previously [38].

195

196 **Identification of putative NTSs on ITSN1-s.** The primary sequences of ITSN1-s (NCBI
197 Accession Number: NP_001001132.1) was scanned for putative NLS and NES using software
198 programs cNLSmapper [38] and NES finder, respectively [39].

199

200 **Western blot.** Samples were heated in reducing SDS sample buffer (80 mM Tris, pH 6.8, 2% SDS,
201 7.5% glycerol, 0.01% Bromophenol blue) supplemented with 2% 2-mercaptoethanol for 5 min at 95
202 °C and separated by SDS–PAGE on acrylamide/bisacrylamide gels and analyzed by Western blot
203 (WB) as previously described [21]. Briefly, samples were transferred for 1h onto PVDF membrane,
204 blocked for 1h at 37°C with 5% fat free milk, 0.05% Tween-20 in PBS. PVDF membranes were
205 incubated with specific antibodies: α-ITSN (Abcam), α-FLAG (SIGMA), α-lamin A/C (Thermo
206 fisher), α-ERK 1/2 (Santa Cruz), anti-histone H3 (Santa Cruz), α-tubulin (Santa Cruz), or α-
207 GFP/GST (a generous gift from Prof. Höning, University of Cologne) overnight in PBS Tween
208 0.05% + 1% fat free milk. The membrane was washed 3 times for 10 min with PBS Tween 0.05%
209 and incubated 1h with one of the following secondary antibodies horseradish peroxidase-
210 conjugated: goat α-mouse and goat α-rabbit (Bethyl), rabbit α-goat (Pierce). Blots were detected
211 using Immobilon Western Classico or Forte (Millipore). Images were acquired using a G:Box
212 Chemi XT Imaging system (Syngene)[40].

213

214 **Co-immunoprecipitation of protein complexes.** HeLa or HEK 293-T cells were washed with PBS
215 1X and harvested 48 h post transfection as described in [41]. Cells were centrifuged at 800 g for 10
216 min and pellet was resuspended in ice cold lysis buffer, 50 mM Hepes, 100 mM NaCl, 1% NP-40 +
217 Protease inhibitors cocktail [(PI), 1:1000 (Roche)] for 10 min on ice. Cells were then sonicated at

15% of instrument power (Sonopuls, Bandelin) for 10 sec. After clarification, supernatants were incubated with 4 μ g of the α -ITSN, α -Lamin, α -FLAG antibodies or without antibody as negative control as indicated, overnight at 4 °C, with gentle rocking. The following day, 50 μ L of protein A/G beads (Santa Cruz Biotechnology) were added and the mixtures incubated for 4 h. After 3 washes with PBS 1X, beads were resuspended in SDS sample buffer for 5 min at 95 °C and centrifuged at 800 g for 3 min before the supernatant were collected. For immunoprecipitation of GFP-mIMPdIBB in the presence of FLAG-ITSN1-s or not, the protocol has been adapted from [42]. HEK 293-T cells were washed with PBS 1X and harvested 48 h post transfection. Cells were centrifuged at 800 g for 10 min and pellet was resuspended in ice cold lysis buffer, 50 mM Hepes, 100 mM NaCl, 1mM MgCl₂, 1% NP-40 + Protease inhibitors cocktail [(PI), 1:1000 (Roche)] for 10 min on ice. Cells were then sonicated as described above. After clarification, supernatants were incubated with 4 μ g of the α -FLAG as indicated, overnight at 4 °C, with gentle rocking. The following day, 50 μ L of protein A/G beads were added and the mixtures incubated for 4 h. After 3 washes with 50 mM Hepes, 100 mM NaCl, 1mM MgCl₂, 1% NP-40, beads were resuspended in SDS sample buffer and treated as described above. Samples were subjected to SDS-PAGE/WB analysis.

Subcellular fractionation. In order to separate nuclei from cytoplasm a protocol from Nabbi et al. has been adapted [43]. Briefly, HeLa cells grown to 90% confluency in 10 cm dishes were washed with ice cold PBS and scraped. Cells were centrifuged at 10000 g for 10 sec. Cell pellets were resuspended in PBS with 0.1% NP-40 and triturated with a P1000 micropipette. The lysed cells suspension was centrifuged at 10000 g for 10 sec. The supernatant representing the cytoplasmic fraction was isolated, the nuclei were gently resuspended in PBS with 0.1% NP-40 and centrifuged again. Nuclear pellet was resuspended in SDS sample buffer and sonicated twice at 15% of instrument power (Sonopuls, Bandelin) for 6 sec on ice. Fractions were analyzed by SDS-PAGE/WB as described above.

Nucleoplasm/Nuclear envelope separation. To separate nucleoplasm and nuclear envelope, nuclei obtained as described above were resuspended in NP-40 buffer (PBS 0.5X, 10 mM MgCl₂, 50 mM MOPS pH 7.4, 0.5% NP-40, DNAase 5 U/mL, PI 1:1000, and incubated on ice 5 min. Samples were centrifuged at 20000 g for 10 min and supernatants were collected and considered as nucleosol. Pellet (nuclear envelope) were resuspended in RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 8, 0.1% SDS, 0.5% Sodium desossicolate, PI) and tip-sonicated 12 sec at 15%

251 of instrument power (Sonopuls, Bandelin). Fractions were analyzed by SDS-PAGE/WB as
252 described above.

253

254 **Chemical crosslinking to detect DNA-binding proteins.** In order to visualize if ITSN1-s is a
255 DNA binding protein a protocol from Qiu et al [44] has been adapted. HeLa cells (10 cm dish, 90%
256 confluency) were trypsinized and collected by centrifugation at 300 g for 5 min at 4 °C. Cells were
257 washed with ice-cold PBS to remove culture medium and FBS. *In vivo* cross-linking was achieved
258 by adding PFA to 1 mL of cell suspension in PBS to obtain a final concentration of 1% (w/v). After
259 incubating at RT, PFA was quenched by glycine to a final concentration of 125 mM and incubated
260 at RT for 5 min. The cross-linked cells were collected by centrifugation (300 g at 4 °C for 5 min),
261 and the cell pellet was washed twice with cold PBS. Nuclei isolation was carried out using a
262 protocol adapted from that reported by Henrich et al. [45, 46]. The cross-linked HeLa cell pellet (2x
263 10 cm dishes, 70% confluent) was resuspended in 10 volumes of ice-cold hypotonic lysis buffer A
264 containing 10 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, and PI 1:1000. After
265 incubation on ice for 30 min, 0.5% (v/v) NP-40 was added to the lysis buffer. Cells were then
266 gently lysed with a Dounce homogenizer and the nuclear fraction was collected by centrifugation.
267 The nuclear fraction resuspended in buffer B (250 mM sucrose, 10 mM MgCl₂, 20 mM Tris-HCl
268 (pH 7.4) and 1 mM DTT) was layered over a two-step sucrose gradient cushion [1.3 M sucrose,
269 6.25 mM MgCl₂, 20 mM Tris-HCl (pH 7.4), 0.5 mM DTT above 2.3 M sucrose in 2.5 mM MgCl₂
270 and 20 mM Tris-HCl (pH 7.4)] and centrifuged subsequently at 19000g at 4 °C for 45 min. The
271 isolated nuclei were washed with buffer A and collected by centrifugation at 1000g.
272 Isolation of DNA-Protein Complexes, cross-Linking Reversal and DNA Removal were performed
273 as described previously [44]. The purified DNA-binding proteins were separated using a NuPage 4-
274 12% Bis-Tris protein gel (Invitrogen).

275

276 **Statistical analysis.** Statistically significant differences between datasets were determined with
277 Student's *t*-Test (Graphpad software, Inc.). P values of less than 0.05 were considered statistically
278 significant with **p* ≤ 0.05, ** *p* ≤ 0.01, *** *p* ≤ 0.001. Values shown are the mean ± standard error
279 of the mean (SEM) relative to at least three independent experiments [47, 48].

280

281 **Results**

283 **A fraction of ITSN1-s localizes to the nucleus**

284 In order to investigate ITSN1-s subcellular localization, HeLa cells were transfected with a FLAG-
285 ITSN1-s expression plasmid and subjected to biochemical separation of nuclear and cytosolic
286 fractions. Mock-transfected cells were used as a negative control. Then, ITSN1-s distribution was
287 analyzed in the two fractions by SDS-PAGE/WB. ERK1/2 and lamin A/C were used as reference
288 proteins for cytosolic and nuclear compartments, respectively. Both FLAG-tagged and
289 endogenous ITSN1-s were enriched in the cytosol, but detectable in both fractions, while reference
290 proteins were localized exclusively in the specific fraction, proof of a clean compartment
291 separation (Fig. 1A). We also confirmed our results by immunofluorescence (IF), limiting our
292 analysis to FLAG-ITSN1-s due to the lack of suitable commercial antibodies. The protein
293 presented mainly a cytoplasmic localization, although it was also visible in the cell nucleus (Fig.
294 1B). Thus, our data demonstrate the existence of a nuclear pool of ITSN1-s.

296 **ITSN1-s is enriched in the nuclear envelope**

297 The relative amount of nuclear FLAG-ITSN1-s detectable in the nucleus after subcellular
298 fractionation appears higher than that observable by IF (Fig. 1), raising the possibility that the
299 majority of nuclear FLAG-ITSN1-s is associated to the nuclear envelope (NE) rather than in the
300 nucleoplasm. To verify this hypothesis, nucleoplasmic and NE fractions from purified nuclei of
301 HeLa cells transfected to express FLAG-ITSN1-s were analyzed by SDS-PAGE/WB to detect the
302 cytosolic marker ERK1/2, the NE marker lamin A/C, as well as FLAG-ITSN1-s. As expected,
303 while ERK1/2 was not detectable in the nuclear fraction, lamin A/C localized exclusively in the
304 NE fraction (Fig. 2A). FLAG-ITSN1-s was both detectable in the nucleoplasmic and the NE
305 fractions, but highly enriched in the latter, consistent with the idea that ITSN1-s could associate
306 with the NE. IF analysis revealed that FLAG-ITSN1-s partially colocalizes with lamin A/C on the
307 NE (Fig. 2B). Subsequently, we investigated the interaction between ITSN1-s and lamin A/C by
308 co-immunoprecipitation (co-IP) analysis. HeLa cells were lysed, and ITSN1-s complexes pulled
309 down using either an α -ITSN or an α -lamin A/C antibody. SDS-PAGE/WB analysis revealed the
310 presence of two bands corresponding to lamin A and C in the ITSN1-s pull down lane and of a
311 ITSN1-s band in the lamin A/C pull down (Fig. 2C).

312 Finally, since some endocytic proteins were found to be part of transcription complexes [49], we
313 tested the possibility that ITSN1-s could bind DNA. To this end, we adapted a protocol which
314 includes chemical cross-linking of cells followed by nuclei isolation and purification of covalently

bound DNA-protein complexes [44]. DNA protein complexes were isolated and analyzed by a SDS-PAGE/WB using α -ITSN1, in addition to α -tubulin and α -Histone H3 antibodies as negative and positive controls, respectively [50]. As expected, Histone H3 was detectable in the DNA protein complexes lane (DPC) as well as in the cell homogenate (H), whereas tubulin, which is incapable of binding to DNA, was detectable only in the cell homogenate (Fig. 2D). Importantly FLAG-ITSN1-s could not be detected in the DPC, suggesting that ITSN1-s does not interact with cellular DNA. Taken together our results indicate that a small, but detectable amount of ITSN1-s localizes within the cell nucleus at the steady state, where it preferentially associates with the NE, possibly interacting with lamin A/C.

324

325 **ITSN1-s interacts with IMP α**

326 The fact that a certain fraction of the 145 kDa protein ITSN1-s can gain access to the nucleus
327 implies the protein is actively translocated into the nucleus in a NLS-dependent fashion by IMPs.
328 A bioinformatics analysis using the NLS prediction software “cNLS MAPPER” revealed the
329 presence of a putative bipartite cNLS (**RKKNPGGWEGELQARGKKRQIGW-1127**), located
330 in the C-terminal, SH3 portion of ITSN1-s (Fig. 3A,B). Such sequence strongly resembles the
331 prototype bipartite cNLS originally described on nucleoplasmin (**KRPAATKKAGQAKKKK-**
332 **170**) and perfectly matches the consensus for such signals. This finding raised the possibility that
333 ITSN1-s could bind to the IMP α / β heterodimer. To assess this hypothesis by co-IP experiments,
334 HEK 293-T cells were transfected to express GFP-IMP α Δ IBB either individually or in the
335 presence of FLAG-ITSN1-s. The former is a GFP-tagged derivative of IMP α that lacks the
336 autoinhibitory IMP β binding (IBB) domain, and that therefore binds to NLSs with an affinity
337 comparable to that of the IMP α / β heterodimer [32]. As a positive control, GFP-IMP α Δ IBB was
338 also expressed in the presence of FLAG-UL44, a protein known to be recognized by the IMP α / β
339 heterodimer [28]. Proteins were subsequently immunoprecipitated in the presence or in the
340 absence of the FLAG antibody. As expected, FLAG-UL44 could be co-immunoprecipitated with
341 GFP-IMP α Δ IBB, whereas no GFP-IMP α Δ IBB was obtained after incubation of cell lysates in the
342 absence of the α FLAG antibody, indicating that GFP-IMP α Δ IBB did not interact unspecifically
343 with protein A/G beads (Fig. 4A). Importantly, GFP-IMP α Δ IBB could be also co-
344 immunoprecipitated by the α -FLAG antibody from cells co-expressing FLAG-ITSN1-s, but not
345 from cells expressing GFP-IMP α Δ IBB alone, indicating that GFP-IMP α Δ IBB did not interact
346 unspecifically with the α -FLAG antibody (Fig. 4B). Taken together these results show that
347 ITSN1-s is able to interact with IMP α , thus being actively transported into the nucleus.

348

349 **ITSN1-s is a nucleocytoplasmic trafficking protein**

350 Despite its ability to bind to $\text{IMP}\alpha$, at the steady state ITSN1-s preferentially localizes in the
351 cytosol. It is therefore possible that, as described for other endocytic proteins [6], ITSN1-s shuttles
352 between the nucleus and the cytoplasm thanks to the simultaneous presence of NLS and NES. A
353 bioinformatics analysis using the NES prediction software “NES finder”, integrated by visual
354 inspection based on a systematic analysis of NES consensus performed in eukaryotic cells [10],
355 identified 6 putative hydrophobic rich NESs [NES A-F] distributed throughout the 3 regions of
356 ITSN1-s (EH-like homology; EHs, aas 1-380; coiled coil; CC, aas 381-680 and; SH3s, aas 681-
357 1220) (Fig. 3A,B). The presence of putative hydrophobic NESs within ITSN1-s indeed suggested
358 that the protein could undergo nucleocytoplasmic shuttling in a CRM-1 dependent manner.
359 In order to confirm this hypothesis, the subcellular localization of ITSN1-s was tested in HeLa
360 cells upon LMB-mediated inhibition of CRM1 activity. FLAG-ITSN1-s transiently transfected
361 cells were treated or not with LMB, before CLSM analysis to quantify their levels of nuclear
362 accumulation. In addition to FLAG-ITSN1-s, cells were also transfected with GFP, GFP-Rev, a
363 protein known to accumulate in the nucleus after LMB treatment, as well as with GFP-
364 UL44 Δ NLS, a $\approx$ 180 kDa dimeric cytosolic protein which is excluded from the nucleus due to the
365 lack of a functional NLS. Addition of LMB did not affect the subcellular localization of either
366 GFP and GFP-UL44 Δ NLS, implying the treatment did not affect cell viability and morphology as
367 well as NPC permeability, while it caused GFP-Rev to strongly accumulate in the cell nucleus
368 (Fig. S1 and Table S1). Importantly, after treatment with LMB, FLAG-ITSN1-s nuclear staining
369 became significantly more evident (Fn/c from 0.15 to 0.45), although the protein did not
370 accumulate in the cell nucleus to high levels (Fig. 5A,B and Table S2). Thus, ITSN1-s can shuttle
371 between the nucleus and the cytosol in a $\text{IMP}\alpha/\beta$ and CRM1-dependent fashion.

372

373 **ITSN1-s CC and SH3 regions can independently undergo nucleocytoplasmic shuttling.**

374 In an effort to identify ITSN1-s functional NTSs among the putative ones predicted, we generated
375 several ITSN1-s deletion mutants fused to the C-terminus of YFP. We initially verified that
376 addition of a YFP-tag did not interfere with ITSN1-s subcellular localization and
377 nucleocytoplasmic shuttling abilities. To this end, HeLa cells were transfected to express YFP-
378 ITSN1-s, whose subcellular localization was initially monitored by live cell imaging every min for
379 a period of 30 min. As expected, YFP-ITSN1-s localized mainly in the cytoplasm, with evident
380 dots reminiscent of endocytic vesicles, which appeared to be highly mobile (Fig. S2 and Mov. S1).
381 In a second series of experiments, HeLa cells were transfected to express either YFP-ITSN1-s or

382 GFP-UL44ΔNLS, treated with either LMB or with solvent, and the subcellular localization of the
 383 fusion protein of interest visualized every 15 min for 10 h. As expected, YFP-ITSN1-s gradually
 384 accumulated into the nucleus, reaching a maximum approximately 5 h after LMB addition,
 385 whereas the negative control GFP-UL44ΔNLS remained mainly cytosolic for the whole duration
 386 of the experiment (Fig. S3 and Mov. S2). Therefore, YFP-ITSN1-s could be used to study the
 387 nucleocytoplasmic properties of ITSN1-s. Beside full length ITSN1-s (1-1220), we generated
 388 plasmids mediating the expression of five additional deletion mutants: YFP-ITSN1-s-ΔEHs (316-
 389 1220), lacking the N-terminal epsin like domain; YFP-ITSN1-s-ΔSH3s (1-680), lacking the C-
 390 terminal SH3 domains; YFP-ITSN1-s-SH3s (681-1220); YFP-ITSN1-s-EHs (1-315) and YFP-
 391 ITSN1-s-CC (316-680) (Fig. 6A). As a first step towards the characterization of such variants,
 392 their molecular weights were verified by SDS-PAGE/WB using an α-GFP antibody (Fig. 6B).
 393 Fusion proteins migrated at the expected molecular weights (YFP-ITSN1-s: 160 kDa, YFP-
 394 ITSN1-s-ΔEHs: 125kDa, YFP- ITSN1-s-ΔSH3s: 101 kDa, YFP-ITSN1-s-SH3s: 85 kDa, YFP-
 395 ITSN1-s-EHs: 61 kDa, YFP-ITSN1-s-CC: 66 kDa). Multiple bands of higher molecular weight
 396 could be detected for the YFP-ITSN1-s-CC fusion, most likely due to protein
 397 multimerization/aggregation. Secondly, we decided to investigate their subcellular localization
 398 either in the absence or in the presence of LMB. HEK 293-A cells were transiently transfected
 399 with appropriate expressing vectors and the subcellular localization of YFP-ITSN1-s was
 400 compared by quantitative CLSM to that of the above described deletion mutants. Along with YFP-
 401 ITSN1-s fusions, the previously described controls (GFP, GFP-Rev and GFP-UL44ΔNLS) were
 402 used to verify the functionality of LMB treatment (Fig. 6C). As expected, GFP evenly distributed
 403 between nucleus and cytoplasm both in absence and presence of LMB (Fn/c 1.27 ± 0.14 vs $1.50 \pm$
 404 0.28). Similarly, GFP-UL44ΔNLS, having a molecular weight $a \approx 180$ kDa, was retained in the
 405 cytosol in both cases (Fn/c 0.10 ± 0.09 vs 0.17 ± 0.08). Importantly, LMB addition significantly
 406 enhanced nuclear accumulation of GFP-Rev (Fn/c 0.16 ± 0.03 vs 16.97 ± 9.87), which strongly
 407 accumulated in the nucleoli. Taken together, our data indicate that LMB treatment functionally
 408 inhibited CRM-1 mediated nuclear export without affecting NE permeability (see Fig. 6C,D, *left*
 409 panel and Table S3). As expected, in the absence of LMB, YFP-ITSN1-s mainly localized in the
 410 cytoplasm of untreated cells (Fig. 6C and Fig. 2D, right panels and Table S4; Fn/c 0.11 ± 0.08)
 411 with a punctuate pattern present in the cytoplasm and in close proximity to the plasma membrane,
 412 thus resembling that of endocytic vesicles. Deletion of EH domains (YFP-ITSN1-s-ΔEH) altered
 413 ITSN1-s localization pattern, in that the punctuate structures observed for FL-ITSN1-s were not
 414 detectable, and large protein aggregates were often present in strongly expressing cells. However,
 415 the protein was still mainly detectable in the cytoplasm (Fn/c 0.07 ± 0.04). Removal of both EH

416 and CC regions (YFP-ITSN1-s-SH3s), caused the protein to localize in the cytosol with a mainly
417 diffuse pattern, although a few vesicle-like dots were still detectable (Fn/c 0.10 ± 0.04). On the
418 contrary, removal of ITSN1-s SH3 region (YFP-ITSN1-s- Δ SH3s) did not change the punctate
419 pattern described for ITSN1-s but caused a significant increase of nuclear signal (Fn/c $0.42 \pm$
420 0.09). When both the CC and SH3 regions were simultaneously deleted (YFP-ITSN1-s-EHs), the
421 subcellular localization was completely altered: this protein equally distributed between the
422 nucleus and the cytoplasm with a diffuse pattern (Fn/c 1.13 ± 0.35). In addition, vesicle-like dots
423 or protein aggregates were not detectable. Finally, YFP-ITSN1-s-CC fusion, devoid of both EH
424 and SH3 regions, localized mainly in the cytoplasm, but entered the nucleus slightly more
425 efficiently with respect to YFP-ITSN1-s (Fn/c 0.24 ± 0.08). The protein distributed with a diffuse
426 pattern in the cytoplasm, with the presence of large protein aggregates in the cytosolic
427 compartment of highly expressing cells.

428 Importantly, addition of LMB increased nuclear levels of YFP-ITSN1-s (Fn/c 0.11 ± 0.08
429 vs 0.41 ± 0.11), as well as of all YFP-ITSN1-s deletion mutants, apart from YFP-ITSN1-s-EHs
430 (Fn/c 1.13 ± 0.35 vs 1.34 ± 0.32 , see Fig. 6C,D). Indeed, both ITSN1-s-CC and ITSN1-s-SH3
431 responded to LMB treatment (Fn/c of 0.24 vs. 0.72 and 0.10 vs 0.78, respectively) similarly to
432 ITSN1-s- Δ EH and ITSN1-s- Δ SH3 (Fn/c of 0.07 vs. 0.32 and 0.42 vs 1.32, respectively).
433 Therefore, both ITSN1-s CC and SH3 regions were sufficient to confer LMB-dependent
434 nucleocytoplasmic shuttling properties to YFP. Taken together, the subcellular localization of
435 ITSN1-s and its deletion mutants at the steady state suggest that the N-terminal EH domain (to a
436 larger extent) and the C-terminally located SH3 regions play a role in ITSN1-s localization to
437 endocytic vesicles-like dots, while the CC domain can cause protein aggregation, when expressed
438 outside the physiological context of FL-ITSN1-s. In addition, LMB experiments suggest that
439 ITSN1-s could contain multiple NTSs located in the CC and SH3 regions, whereas ITSN-EH
440 domain does not actively contribute to the protein's nucleocytoplasmic shuttling ability, and NESs
441 [A-C] are not functional in terms of mediating CRM-1 dependent nuclear export.

442

443 **ITSN1-s residues 1104-1127 represent a functional cNLS.** As a first attempt towards the
444 characterization of ITSN1-s nucleo-cytoplasmic shuttling process, we decided to identify its NLS.
445 Our bioinformatics analysis could not detect any cNLS in ITSN1-s-CC region, but revealed the
446 presence of a putative bipartite cNLS in the SH3 region, within ITSN1-s-SH3D domain
447 (**RKKNPGGWWEGELQARGKKRQIGW**-1127) (Fig. 3). To validate its functionality, we
448 generated an expression plasmid encoding such sequence C-terminally fused to YFP (YFP-
449 ITSN1-s-NLS) and analyzed the ability of the YFP-ITSN1-s-NLS fusion protein to accumulate in

450 the nucleus in an energy-dependent manner. We also analyzed the subcellular localization of YFP
451 alone or of the control YFP-NLS[R] fusion protein, known to localize to the cell nucleus through
452 interaction with the IMP α/β heterodimer (Smith et al., unpublished observations). As expected,
453 when cells were maintained in normal media, YFP-NLS[R] accumulated into the nucleus of
454 transfected cells (Fn/c 2.8) in significantly higher levels than YFP alone (Fn/c 1.1; Fig. 7 and
455 Table S5). Depletion of intracellular ATP significantly decreased the nuclear accumulation of
456 YFP-NLS[R], without affecting the subcellular localization of YFP alone (Fn/c of 1.5 and 1.1
457 respectively; Fig. 7). Importantly, YFP-ITSN1-s-NLS accumulated into the nucleus at
458 significantly higher levels as compared to YFP alone when cells were maintained in normal media
459 (Fn/c 1.8), and its nuclear accumulation was significantly impaired after incubation in the energy
460 depletion media (Fn/c 1.3). These data clearly show that ITSN1-s residues 1104-1127 represent a
461 basic NLS capable of conferring energy dependent nuclear localization to heterologous proteins.

462

463 **ITSN-NLS is essential for nuclear targeting of ITSN1-s C-terminal domain.** We decided to test
464 the impact of ITSN1-s-NLS on nuclear targeting of the protein. We compared the subcellular
465 localization of YFP-ITSN1-s to that of its derivative YFP-ITSN1-s Δ NLS, where basic residues of
466 its NLS (**RKKNPGGWWEGELQARGKKRQIGW**-1127) were substituted with hydrophobic ones
467 (Δ NLS; **aatNPGGWWEGELQARGatsQIGW**-1127; see Fig. 8A), either in the absence or in the
468 presence of LMB. Under both conditions, the NLS defective derivative localized with a very similar
469 pattern as compared to the wild-type protein (Fig. 8B). This was not surprising, since deletion of the
470 whole ITSN1-s-SH3 region, comprising ITSN1-s-NLS, did not impair the ability of the protein to
471 enter the nucleus upon LMB treatment, implying a contribution of ITSN1-s-CC region in
472 nucleocytoplasmic shuttling of ITSN1-s (Fig. 6). In order to study the contribution of ITSN1-s-NLS
473 to nuclear import of the protein independently of the contribution of ITSN1-s-CC region, we
474 analyzed the subcellular localization of a series of ITSN1-s C-terminal domain deletions, including
475 YFP-ITSN1-s-SH3s, encompassing residues 681-1220; YFP-ITSN1-s-SH3(A-D) comprising
476 residues 681-1173; and YFP-ITSN1-s-SH3(D-E) comprising ITSN1-s residues 1074-1220, either
477 carrying the wild-type or the mutated NLS (Fig. 8). In the absence of LMB both YFP-ITSN1-s-SH3
478 and YFP-ITSN1-s-SH3(A-D) localized mainly in the cytosol, and inactivation of ITSN-NLS did not
479 affect the proteins subcellular localization (Fn/c c. 0.2, see Fig. 8). Upon LMB treatment, both YFP-
480 ITSN1-s-SH3 and YFP-ITSN1-s-SH3(A-D) significantly accumulated into the nucleus (Fn/c of c.
481 0.8 and 1.1, respectively), while their NLS mutated counterparts were mainly retained in the cytosol
482 (Fn/c of c. 0.4), clearly showing the importance of ITSN1-s residues 1104-1127 for nuclear
483 targeting. The contribution of ITSN1-s-NLS to nuclear import was even more evident when

analyzing the subcellular localization of YFP-ITSN1-s-SH3(D-E). In absence of LMB, this protein was detectable in the nucleus, compatible with its ability to passively enter the nucleus by passive diffusion (Fn/c 0.9), while addition of LMB increased its nuclear accumulation (Fn/c 1.5). Importantly, in the absence of LMB, YFP-ITSN1-s-SH3(D-E) Δ NLS localized to the nucleus to lower levels (Fn/c of 0.5) as compared to its NLS bearing counterpart, further confirming the importance of ITSN1-s-NLS in nuclear import of the protein (Fig. 8B,C and Table S6). Overall our data also suggest that ITSN1-s-NESE -located within SH3D domain - is the functional NES within the SH3 region, while NESF - located within SH3E domain- does not contribute to CRM-1 nuclear export. Indeed, both YFP-ITSN1-s-SH(A-D) and YFP-ITSN1-s-SH3(D-E), accumulate to the nucleus in the presence of LMB. Furthermore, site specific mutagenesis for NESF did not abolish LMB responsiveness, nor increased steady state nuclear localization either in the SH3(A-E) or SH3(D-E) context (data not shown). However, attempts to mutate NESE hydrophobic residues resulted in increased nuclear accumulation in the presence of LMB (data not shown), probably by interfering with the nearby-located NLS, thus precluding formal proof that NESE is a functional NES.

499

ITSN1s-CC shuttles between the nucleus and the cytoplasm independently of the presence of putative NTSs. Our data indicate that ITSN1-s-CC is capable of nucleocytoplasmic shuttling. However, no evident NTSs are present within its sequence, with the exclusion of a stretch of aa containing four L residues, which could resemble the NES originally described on HIV-1 Rev (NESD: LELEKQLEKQREL-419, see Fig. 3). Since NESD is located at the N-terminal portion of the CC region, we decided to test its functionality by analyzing the subcellular localization of the N-terminal (residues 316-456, containing NESD) and of the C-terminal (residues 457-680) portions of ITSN1-s CC region, either in the absence or in the presence of LMB (Fig. 9A,B,C and Table S7). Strikingly, in the absence of LMB both YFP-ITSN1-s(316-456) and YFP-ITSN1-s(457-680) mainly localized in the cytosol of transfected cells, (Fn/c of 0.7 and 0.3, respectively) but accumulated in the nucleus upon LMB treatment (Fn/c of 1.4 and 1.2, respectively). Surprisingly, mutation of ITSN1-s-NESD hydrophobic residues (LELEKQLEKQREL-419 to qEaEKQqEKQREL-419), did not affect protein subcellular localization either in the absence or in the presence of LMB. Therefore, ITSN1-s-CC appears capable of shuttling between nucleus and cytosol in the absence of evident NTSs.

515

516

Discussion

518

519 In the present work we showed for the first time that the adaptor scaffold protein ITSN1-s, known
520 to be involved in several signaling and endocytic processes [1, 13, 51], and interacting with a
521 plethora of factors involved in endocytosis, cytoskeleton rearrangements, cell signaling and survival
522 [52-54], is a nucleocytoplasmic shuttling protein, which accumulates in the cell nucleus upon
523 pharmacological ablation of CRM1-dependent nuclear export. At the steady state, a fraction of
524 ITSN1-s localizes at the nucleus and is enriched at the nuclear envelope, where it interacts with
525 lamin A/C.

526 The identification of ITSN1-s nucleocytoplasmic shuttling sequences has been hampered by the
527 evidence that both ITSN1-s CC and SH3 regions can shuttle between nucleus and cytosol
528 independently of each other (Fig. 6). Moreover, the ITSN-1s CC region is apparently devoid of
529 evident NTSs, bearing no putative cNLSs and only one putative leucine-rich NES (NESD:
530 LELEKQLEKQREL-419), whose mutation to qEaEKQqEKQREL-419 did not affect LMB
531 responsiveness (Fig. 9). Therefore, it is not known how ITSN-1s CC could undergo cytoplasmic
532 shuttling. One possibility is that the α -helix rich CC domain can interact with the FG repeats of the
533 NPC to allow passage in and out of the nucleus, in analogous fashion as it has been demonstrated
534 for HEAT repeats containing proteins such as IMP β [55]. Another possibility is that one of the
535 several cellular proteins capable of interacting with ITSN1-s-CC region, is responsible for transport
536 across the NPCs through a “piggy-back” mechanism. Such proteins include the nucleocytoplasmic
537 shuttling Eps15 and HIP1 proteins, as well as endogenous ITSN1-s, ITSN1-L and ITSN2-s [16, 17,
538 56-58]. Despite the confounding effect due to the presence of the CC-region, we were able to
539 clearly demonstrate the existence of a functional bipartite cNLS in the SH3 region of ITSN1-s
540 (ITSN1-s-NLS: **RKKNPGGWEGELQARGKKRQIGW**-1127). ITSN1-s-NLS is capable of
541 conferring ATP dependent nuclear targeting to YFP (Fig.7), and mutation of its basic residues
542 impairs nuclear targeting of several YFP-ITSN1-s SH3 deletion mutants in the presence of LMB
543 (Fig. 8). However, mutation of ITSN1-s-NLS in the context of the full length protein does not
544 abolish its ability to enter the nucleus, most likely due to the fact that the CC region can mediate
545 nuclear import and that deletion of the SH3 region of ITSN1-s similarly does not abolish the
546 protein's nucleocytoplasmic shuttling properties (see Fig. 6). The bipartite nature of the ITSN1-s-
547 NLS was confirmed by CLSM quantitative analysis showing that deletion of upstream basic cluster
548 alone only partially affected nuclear accumulation as compared to mutation of both clusters, in the
549 context of the YFP-ITSN1-s-SH3(D-E) fusion protein (not shown), implying that both basic
550 stretches of aa are required for optimal NLS activity [59].

551 Similarly to ITSN1-s, several endocytic adaptor proteins undergo nucleocytoplasmic
552 trafficking, mainly to perform additional, specialized tasks within the nucleus, thus being dubbed

553 "moonlighting" proteins [56, 60-62]. A number of moonlighting proteins, such as Paxilin and EHD2
554 migrate to the nucleus to regulate cellular proliferation and transcription processes [63, 64]. Our
555 data, showing that ITSN1-s concentrates on the NE, where it interacts with lamin A/C (Fig. 2A-C),
556 and does not bind to cellular DNA (Fig. 2D), rather suggest the possibility that ITSN1-s might play
557 a role at the NE. It has been recently shown that a fourth endosomal route, besides recycling
558 endosomes, endolysosomes or Golgi apparatus, transports cell surface receptors to the nucleoplasm
559 through docking and membrane fusion of a population of endosomes with the nuclear envelope
560 [65]. Such Nuclear Envelope Associated Endosomes (NAE) route may be an alternative mechanism
561 by which external stimuli can influence cellular activity independently of the conventional signaling
562 cascades that operate in the cytosol, and ITSN1-s could therefore be a new player in such route,
563 helping to transport molecules from the plasma membrane to the nucleoplasm, as it has been
564 hypothesized for Epidermal Growth Factor Receptor. It is very likely that this process comprises a
565 series of tightly regulated events, hence experiments to unravel the mechanisms regulating ITSN1-s
566 shuttling to the nucleus are undergoing in our laboratories. Furthermore, we do not exclude that the
567 process of nuclear localization might be dependent on cell cycle phase, as it happens for other
568 proteins [6, 66-68].

569 Since modifications of the endocytic process have been recently linked to malignancy [69,
570 70], it is likely that rerouting of endocytic proteins to other pathways or compartments due to
571 moonlighting functions could be functionally linked to tumorigenesis. In this context, a very recent
572 study showed that APPL1 and APPL2, Rab5 effector proteins and multifunctional adaptors
573 containing different domains, implicated in several signaling pathways, and recently discovered as
574 nucleocytoplasmic shuttling proteins [49], are required for the nuclear translocation of type I
575 serine/threonine kinase receptors intracellular domain (T β RI-ICD), thereby promoting progression
576 of prostate cancer cells [71]. Our results might have important implications for the process of
577 carcinogenesis. ITSN1-s is highly expressed in pancreatic, lung, liposarcomas and Wilm's tumors,
578 as shown in ONCOMINE database. Furthermore, ITSN1-s is necessary for malignant glioma cell
579 proliferation and for *in vitro* and *in vivo* tumorigenic properties of primary human neuroblastoma
580 tumors [72, 73]. It is evident that ITSN1-s plays a critical role in this process due to its tertiary
581 structure, allowing its domains to make contacts with many specific targets. Furthermore, its role in
582 tumorigenesis has been linked to signaling regulation rather than endocytosis although the signaling
583 pathways involved have only been started to be unveiled.

584 In conclusion, our results suggest a new scenario that foresees the nucleocytoplasmic
585 shuttling of ITSN1-s as an important clue for understanding the physiological and disease-related
586 role of this scaffold protein.

587

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592

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596

597 **Conflict of interest**

598 The authors declare that they have no conflict of interest.

599

600 **Author contribution statement**

601 AR and DR conceived the project. AR and GA designed the experiments. AR, GA, LP, NM, AC,
602 AC, VdA, CZ, MT performed the experiments. AR, GA and LP wrote the paper. All authors
603 analyzed the data and proofread the paper prior to submission.

604

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792

793

794 **FIGURE LEGENDS**

795

796 **Fig. 1 A fraction of endogenous and overexpressed ITSN1-s localizes within the cell nucleus.**

797 (A) HeLa cells, untransfected or transfected to express FLAG-ITSN1-s, were processed for nucleus
 798 (N)/cytosol (C) separation and the obtained fractions subjected to SDS-PAGE/WB using the
 799 indicated antibodies. The image is representative of three independent experiments. (B) HeLa cells
 800 transfected to express FLAG-ITSN1-s were fixed with PFA and processed for CLSM as described
 801 in the Experimental section. The DAPI channel, depicting cell nuclei is shown on the *left panel*, the
 802 FITC channel, relative to FLAG-ITSN1-s is shown on the *middle panel*, whereas a merged image of
 803 both channels is shown on the *right panel*. Scale bars, 5 μ m. The inset represent a 1,5X
 804 magnification of the boxed area.

805

806 **Fig. 2 Nuclear ITSN1-s is associated with the nuclear envelope.**

807 (A) 48 h after being transfected
 808 to express FLAG-ITSN1-s, HeLa cells were lysed and processed as described in the Experimental
 809 section to obtain whole cell lysate (W), cytosol (C), nuclei (N), nucleoplasm (NP) and nuclear
 810 envelope (NE) fractions. The respective distribution of the indicated proteins was investigated by
 811 SDS-PAGE/WB using the indicated antibodies, as described in the Experimental section. The
 812 amounts loaded were W 10%, C 30%, N 5%, NP 25%, NE 25% of the total cell extracts,
 813 respectively. The image is representative of three independent experiments. (B) FLAG-ITSN1-s
 814 transfected HeLa cells were fixed with 4% PFA, and processed for IF as described in the
 815 Experimental section to allow detection of FLAG-ITSN1-s (*left panel*), lamin A/C (*middle panel*),
 816 cell nuclei (DAPI, *right panel*). A merged image of all channels is shown on the right. Scale bars,
 817 5 μ m. The inset represents a 1,5 X magnification of the boxed area. Shown is a representative
 818 image chosen among three independent experiments. (C) HeLa cells were harvested, and 500 μ g
 819 of the whole cell lysates were immunoprecipitated either in the presence of the α -ITSN1-s
 820 endogenous, α -lamin A/C or in the absence (k-) of antibodies, as described in the Experimental
 821 section. After SDS-PAGE/WB, purified proteins were detected using α -ITSN endogenous or α -
 822 lamin A/C antibodies. Whole cell lysates [50 μ g (I)] were also loaded as further reference. The
 image is representative of three independent experiments.

823 (D) HeLa cells were treated with PFA 1% and processed to obtain a homogenate fraction (H, 50
824 μ g) and DNA protein complexes (DPC, 10 cm dish content) as described in the Experimental
825 section. Fractions were analyzed by SDS-PAGE/WB using the α -ITSN endogenous, α -tubulin and
826 α -histone H3 (H3) antibodies. The image is representative of three independent experiments.

827

828 **Fig. 3 ITSN1-s contains several putative NTSs.** The primary sequences of ITSN1-s (NCBI
829 Accession Number: NP_001001132.1) was scanned for putative NLS and NES using different
830 bioinformatics tools, as described in the Experimental section. (A) A graphic representation of
831 ITSN1s multi-domain structure is shown, along with the respective position of putative NLS
832 (NLS; *gray oval*), and NESs (A-F; *blue stars*). Epsin-like domains (EH; *orange ovals*). Coiled coil
833 domain (CC; *red square*). Src-Homology 3 domains (SH3; *green hexagons*). (B) The sequence of
834 each putative NLS and NES is shown, along with the method used for their identification and a
835 color code, indicating the position with respect to ITSN1-s domains identical to that used in panel
836 a. The single letter amino acid code is used. Basic residues forming the putative NLS are in *bold*
837 *face*. Hydrophobic residues forming putative NESs are *underlined*.

838

839 **Fig. 4 ITSN1-s interacts with IMP α .**(A) HEK 293-T cells were transfected to express GFP-
840 IMP α Δ IBB in the presence of FLAG-UL44. Cells were harvested 48 h after transfection and 500
841 μ g of whole cell lysates were immunoprecipitated either in the presence (FLAG) or in the absence
842 (k-) of the α -FLAG mAb, as described in the Experimental section. Whole cell lysates [50 μ g (I)],
843 and immunoprecipitated proteins (IP) were separated by SDS-PAGE/WB and the presence of the
844 indicated proteins revealed using either α -FLAG or α -GFP mAbs. (B) HEK 293-T cells were
845 transfected to express GFP-IMP α Δ IBB in the presence of (+), or in the absence (-) of FLAG-
846 ITSN1-s. Cells were harvested 48 h after transfection and 1 mg of transfected cell lysates was
847 immunoprecipitated in the presence of the α -FLAG mAb, as described in the Experimental
848 section. Whole cell lysates [50 μ g (I)] and immunoprecipitated proteins (IP-FLAG) were separated
849 by SDS-PAGE/WB, and purified proteins were detected using either α -FLAG or α -GFP mAbs
850 The image is representative of three independent experiments.

851

852 **Fig. 5 ITSN1-s shuttles between nucleus and cytoplasm in a CRM-1 dependent fashion.** HeLa
853 cells were transfected to transiently express FLAG-ITSN1-s. Eight hours before processing samples
854 for IF and CLSM analysis, cells were treated either with LMB (2.9 ng/ μ l 0.01% Methanol) or with
855 solvent (0.01% Methanol). (A) Representative microscopic images of cells treated with solvent (-
856 LMB; *top panels*), or LMB (+ LMB; *bottom panels*) are shown relative to ITSN1s (*first panels*),

857 lamin A/C (*second panels*) and nuclei (*DAPI, third panels*). A merged image is shown (*right*
858 *panels*). Scale bars, 5 μ m. (B) Digital images such as those shown in (A) were quantitatively
859 analyzed using software ImageJ to calculate the Fn/c ratio relative to ITSN1-s, as described in the
860 Experimental section. The mean $\pm$ SEM relative to at least three independent experiments ($n \geq 3$) is
861 shown, along with the p value relative to the Student t -test between LMB treated and untreated
862 cells. ** = $p \leq 0.01$

863

864 **Fig. 6 ITSN1-s CC and SH3 regions can independently undergo nucleocytoplasmic shuttling.**

865 (A) HEK 293 cells were transfected to transiently express the indicated YFP-ITSN1-s fusion
866 proteins. The presence of the putative NLS (NLS; *gray oval*), and NESs (A-F; *blue stars*) is
867 indicated. (B) 48 h post transfections cells were lysed and processed as described in the
868 Experimental section for SDS-PAGE/WB analysis, to allow detection of the indicated fusion
869 protein using a polyclonal α -GFP antibody. The presence of the YFP-ITSN1-s fusion of interest at
870 the expected molecular weight is indicated by a pink arrowhead, whereas the presence of YFP-
871 ITSN-CC aggregates of higher molecular weight is highlighted by black arrowheads. The image is
872 representative of three independent experiments. (C) Eight hours before being processed for
873 imaging by CLSM, cells were treated with either LMB (2.9 ng/ μ l 0.01% Methanol) or solvent
874 (0.01% Methanol). Representative microscopic images of cells treated with solvent (- LMB; *top*
875 *panels*), or LMB (+ LMB; *bottom panels*) are shown. Scale bars, 10 μ m (D) Digital images such
876 as shown in (C) were quantitatively analyzed using software ImageJ to calculate the Fn/c ratio
877 relative to the indicated proteins, as described in the Experimental section. The mean $\pm$ SEM
878 relative to at least three independent experiments is shown, along with the p value relative to the
879 Student t test between LMB treated and untreated cells. * = $p \leq 0.05$

880

881 **Fig. 7 ITSN1-s residues 1104-1127 represent a bipartite cNLS able to confer energy**
882 **dependent nuclear targeting properties to heterologous proteins.** (A) HEK 293-A cells were

883 transfected to transiently express the indicated fusion proteins. Thirty min before processing the
884 cells for CLSM analysis, media was changed either with fresh DMEM (+ ATP, *left panels*) or with
885 an energy depletion media (- ATP, *right panels*; see Experimental section). Scale bars, 10 μ m (B)
886 Digital images such as those shown in (A) were quantitatively analyzed using software ImageJ to
887 calculate the Fn/c ratio relative to each fusion protein, as described in the Experimental section.
888 The mean $\pm$ SEM relative to pooled data from two independent experiments ($n > 40$) is shown,
889 along with the p value relative to the Student t test between cells expressing YFP-NLS fusions and

890 YFP alone in the presence of ATP, or relative to cells expressing the individual YFP fusions, in
891 the presence or in the absence of ATP . * = $p \leq 0.05$

892

893 **Fig. 8 ISTN-NLS is important for ITSN-SH3 region nuclear accumulation.** (A) HEK 293-A
894 cells were transfected to transiently express the YFP-ITSN1s fusions shown. The presence of the
895 putative NLS (NLS; *gray oval*), and NESs (A-F; *blue stars*) is indicated. Mutated NLS are shown
896 as *black ovals*. (B) Six hours before being processed for CLSM analysis, cells were treated with
897 either LMB (2.9 ng/μl in 0.01% Methanol) or solvent (0.01% Methanol). Representative images
898 relative to the indicated YFP-ITSN1s point mutant are shown either in the absence (- LMB, *upper*
899 *panels*) or in the presence (+ LMB, *bottom panels*) of LMB. Scale bars, 10 μm (C) Digital images
900 such as those shown in (B) were quantitatively analyzed using software ImageJ to calculate the
901 Fn/c ratio relative to each fusion protein, as described in the Experimental section. The mean ±
902 SEM relative to at least three independent experiments is shown, along with the p value relative to
903 the Student t test between the indicated conditions. * = $p \leq 0.05$

904

905

906 **Fig. 9 ITSN1s-CC possesses an intrinsic capability to undergo nucleocytoplasmic shuttling.**
907 (A) HEK 293-A cells were transfected to transiently express the YFP-ITSN1-s fusions shown. The
908 presence of the putative NLS (NLS; *gray oval*), and NESs (A-F; *blue stars*) is indicated. Mutated
909 NLS are shown as *black ovals*. (B) Six hours before being processed for CLSM analysis, cells were
910 treated with either LMB (2.9 ng/μl 0.01% Methanol) or solvent (0.01% Methanol). Representative
911 images relative to the indicated YFP-ITSN1s point mutant are shown either in the absence (- LMB;
912 *upper panels*) or in the presence (+ LMB; *bottom panels*) of LMB. Scale bars, 10 μm (C) Digital
913 images such as those shown in (B) were quantitatively analyzed using software ImageJ to calculate
914 the Fn/c ratio relative to each fusion protein, as described in the Experimental section. The mean ±
915 SEM is shown ($n \geq 3$), along with the p value relative to the Student t test between LMB treated and
916 untreated cells. * = $p \leq 0.05$

917

918

919 **Fig. S1 GFP-fusion proteins were used as controls for LMB treatment.** (A) HeLa cells were
920 transfected to transiently express GFP, GFP-REV or GFP-UL44. Eight hours before processing
921 samples for IF and CLSM analysis, cells were treated either with LMB (2.9 ng/μl 0.01% Methanol)
922 or with solvent (0.01% Methanol). Representative microscopic images of cells treated with solvent
923 (- LMB; *top panels*), or LMB (+ LMB; *bottom panels*) are shown relative to different constructs. A

924 merged image, including DAPI staining to facilitate visualization of cell nuclei is shown (*right*
925 *panels*). (B) Digital images such as shown in (A) were quantitatively analyzed using software
926 ImageJ to calculate the Fn/c ratio relative to GFP and GFP-fusions, as described in the
927 Experimental section. The mean $\pm$ SEM relative to at least three independent experiments ($n \geq 3$) is
928 shown. The p value relative to the Student t -test between LMB treated and untreated cells is shown.
929 *** = $p \leq 0.001$

930

931 **Fig S2. YFP-ITSN1-s allows direct visualization of ITSN1-s in living cells.** HeLa cells were
932 transfected to transiently express YFP-ITSN1-s and imaged live for 30 min using a Leica DMI8
933 inverted epifluorescent microscope. Images relative to Bright field and nuclei (not shown) as well
934 as of the YFP channel (gray) were acquired every minute. Scale bars, 20 μ M. Positions of a large
935 (red arrow) and small (green arrow) ITSN1-s vesicles is shown.

936

937 **Fig. S3. Time dependent nuclear accumulation of YFP-ITSN1-s in the nucleus of living cells**
938 **upon addition of LMB.** HeLa cells were transfected to transiently express YFP-ITSN1-s. 15 min
939 after addition of LMB or vehicle, the subcellular localization of spontaneously fluorescent fusion
940 proteins was monitored for 10h with a frequency of 15 min using a Leica DMI8 inverted
941 epifluorescent microscope. (A) The subcellular localization of a cell expressing YFP-ITSN1-s is
942 shown, along with the time after addition of LMB. (B) Quantification of the levels of nuclear
943 accumulation of YFP-ITSN1-s at the indicated time points after the addition of LMB (*red circles*)
944 or vehicle (*blue circles*). Data are the mean $\pm$ standard error of the mean relative to > 5 cells. (C)
945 Quantification of the levels of nuclear accumulation YFP-ITSN1-s (red circles) of the control fusion
946 protein GFP-UL44DNLS (*blue circles*) at the indicated time points after addition of LMB. Data are
947 the mean $\pm$ SEM relative to > 5 cells.

948

949 **Movie S1. YFP-ITSN1-s allows direct visualization of ITSN1-s in living cells.** HeLa cells were
950 transfected to transiently express YFP-ITSN1-s and imaged live for 30 min using a Leica DMI8
951 inverted microscope. Images relative to Bright field and nuclei (not shown) as well as of the YFP
952 channel (gray) were acquired every minute. Scale bars, 20 μ M. Positions of a large (red arrow) and
953 small (green arrow) ITSN1-s vesicles is shown.

954

955 **Movie S2. Time dependent nuclear accumulation of YFP-ITSN1-s in the nucleus of living cells**
956 **upon addition of LMB.** HeLa cells were transfected to transiently express YFP-ITSN1-s. 15 min
957 after addition of LMB or vehicle, the subcellular localization of spontaneously fluorescent fusion

958 proteins was monitored for 10h with a frequency of 15 min using a Leica DMI8 inverted
959 epifluorescent microscope. The subcellular localization (top panels) and relative Fn/c quantification
960 (*bottom panels*) relative to cells expressing YFP-ITSN1-s either in the absence (*left panels*) or
961 presence (*midldle panels*) of LMB, as well as to cells expressing GFP-UL44DNLS after addition of
962 LMB (*right panels*) is shown. Data are the mean $\pm$ SEM relative to > 5 cells.

Figure 1

A

Figure 2

Figure 3

A

B

SIGNAL NAME	SEQUENCE	METHOD
NESA	<u>M</u> DQVEFS <u>I</u> AMKLI <u>K</u> L <u>K</u> L 89	NES FINDER
NESB	<u>I</u> LMQSSLPQAQLAS <u>I</u> WN <u>L</u> 265	VISUAL
NESC	<u>F</u> ILAMH <u>L</u> IDVAM 290	NES FINDER
NESD	<u>L</u> E <u>L</u> E <u>K</u> Q <u>L</u> E <u>K</u> QREL 419	VISUAL
NESE	<u>L</u> T <u>L</u> APGQ <u>L</u> IL <u>I</u> 1103	VISUAL
NESF	<u>L</u> AAVCQV <u>I</u> GM 1163	NES FINDER
NLS	RKKNPGGWWE <u>G</u> ELQARGKKRQIGW 1127	cNLS MAPPER

Figure 4

A

B

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Supplementary Table S1, data to Figure S1

Plasmid	- LMB			+ LMB		
	mean	S.E.M.	n	mean	S.E.M.	n
Rev-GFP	0.43	0.1	3	15.47	7.22	3
GFP UL44 Δ NLS	0.24	0.13	4	0.3	0.07	4
GFP	1.23	0.1	3	1.53	0.3	3

Supplementary Table S1. Data to Figure S1. Digital images such as shown in Figure S1, were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. For each experiment, an average of 30 cells was analyzed. The mean, and standard error of the mean (S.E.M.) relative to n experiments are shown.

Supplementary Table S2, data to Figure 5

		- LMB		+ LMB		
Plasmid	mean	S.E.M.	n	mean	S.E.M.	n
FLAG-ITSN1-s	0.17	0.02	3	0.43	0.02	3

Supplementary Table S2. Data to Figure 5. Digital images such as shown in Figure 5 were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. For each experiment, an average of 30 cells was analyzed. The mean, and standard error of the mean (S.E.M.) relative to n experiments are shown.

Supplementary Table S3, data to Figure 6d, *left panels*

Plasmid	- LMB			+ LMB		
	mean	S.E.M.	n	mean	S.E.M.	n
Rev-GFP	0.16	0.06	8	19.31	4.00	7
GFP/YFP	1.27	0.05	7	1.50	0.11	6
GFP UL44 Δ NLS	0.10	0.04	4	0.17	0.05	3

Supplementary Table S3. Data to Figure 6d, *left panels*. Digital images such as shown in Figure 6c, *left panel*, were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. For each experiment, an average of 30 cells was analyzed. The mean, and standard error of the mean (S.E.M.) relative to n experiments are shown.

Supplementary Table S4, data to Figure 6d, *right panels*

YFP-ITSN1-s	- LMB			+ LMB		
	mean	S.E.M.	n	mean	S.E.M.	n
[1-1220]	0.11	0.03	7	0.41	0.04	6
[316-1220]	0.07	0.02	4	0.32	0.06	3
[1-680]	0.42	0.05	4	1.32	0.09	3
[1-315]	1.13	0.16	5	1.34	0.18	3
[316-680]	0.24	0.04	5	0.72	0.07	4
[681-1220]	0.10	0.01	9	0.78	0.09	8

Supplementary Table S4. Data to Figure 6d, *right panels*. Digital images such as shown in Figure 6c, *right panel*, were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. For each experiment, an average of 30 cells was analyzed. The mean, and standard error of the mean (S.E.M.) relative to n experiments are shown.

Supplementary Table S5, data to Figure 7b

	YFP		YFP-ITSN-1s-NLS		YFP-NLS[R]	
	+ ATP	- ATP	+ ATP	- ATP	+ ATP	- ATP
Number of cells	215	105	104	52	83	43
Mean	1.110	1.179	1.830	1.351	2.810	1.469
S.E.M.	0.02	0.03	0.07	0.05	0.18	0.09

Supplementary Table S5. Data to Figure 7b. Digital images such as shown in Figure 7a, were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. Data from two independent experiments were pooled. The mean and standard error of the mean (S.E.M.) relative the indicated number of cells are shown.

Supplementary Table S6, data to Figure 8c

- LMB				+ LMB		
YFP-ITSN1-s	mean	S.E.M.	n	mean	S.E.M.	n
[1-1220]	0.11	0.03	7	0.41	0.04	6
[1-1220]-ΔNLS	0.18	0.03	3	0.39	0.03	3
[681-1220]	0.10	0.01	9	0.78	0.09	8
[681-1220]-Δ NLS	0.14	0.02	4	0.38	0.06	4
[681-1137]	0.16	0.02	3	1.14	0.09	3
[681-1137]-Δ NLS	0.18	0.09	3	0.50	0.08	3
[1074-1220]	0.91	0.06	9	1.85	0.04	8
[1074-1220]-Δ NLS	0.50	0.04	4	1.48	0.21	4

Supplementary Table S6. Data to Figure 8c. Digital images such as shown in Figure 8b, were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. For each experiment, an average of 30 cells was analyzed. The mean, and standard error of the mean (S.E.M.) relative to n experiments are shown.

Supplementary Table S7, data to Figure 9c

YFP-ITSN1-s	- LMB			+ LMB		
	mean	S.E.M	n	mean	S.E.M.	n
[316-680]	0.24	0.04	5	0.72	0.07	4
[316-456]	0.65	0.02	5	1.37	0.10	4
[316-456]-mNESD	0.57	0.03	5	1.37	0.06	4
[461-680]	0.29	0.04	4	1.07	0.14	3

Supplementary Table S7. Data to Figure 9c. Digital images such as shown in Figure 9b, were quantitatively analysed using software ImageJ to calculate the Fn/C ratio relative to the indicated proteins, as described in Material and Method section. For each experiment, an average of 30 cells was analyzed. The mean, and standard error of the mean (S.E.M.) relative to n experiments are shown.

Figure S1

Figure S2

Figure S3

A YFP-ITSN1-s + LMB

B

YFP-ITSN1s

C

LMB treatment