Intersectin 1 enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction

Mustafa Nazir Okur[1,2], Jolene Ooi Yu Zhu[3], Chee Wai Fong[3], Natalia Martinez[4], Carlota Garcia-Dominguez[4], Jose M. Rojas[4], Graeme Guy[3], John P. O'Bryan[1,5]

[1]Departments of Pharmacology, [2]Biochemistry and Molecular Genetics, University of Illinois at Chicago; [3]Institute of Molecular and Cell Biology, Singapore; [4]Unidad de Biologia Celular, Área de Biología Celular y del Desarrollo, Centro Nacional de Microbiología, Instituto de Salud Carlos III (ISCIII), 28220 Majadahonda, Madrid, Spain.

Running Title: ITSN1 regulation of receptor ubiquitylation

[5]Address correspondence to: John P. O'Bryan, Department of Pharmacology, University of Illinois College of Medicine, 835 S. Wolcott, E403 M/C 868, Chicago, IL 60612: Tel: 312-996-6221; Fax: 312-996-1225; Email: obryanj@uic.edu

Keywords: receptor tyrosine kinase, epidermal growth factor receptor, ubiquitylation, endocytosis, trafficking, signal transduction, ITSN

Word Count: Materials and Methods- 746; Introduction, Results, and Discussion-2845

Character number (without spaces, excluding Materials and Methods): 23,160

Abstract

Ubiquitylation of receptor tyrosine kinases plays a critical role in regulating the trafficking and lysosomal degradation of these important signaling molecules. We identified the multi-domain scaffolding protein intersectin 1 (ITSN1) as an important regulator of this process. ITSN1 stimulates ubiquitylation of the epidermal growth factor receptor (EGFR) through enhancing the activity of the Cbl E3 ubiquitin ligase. However, the precise mechanism through which ITSN1 enhanced Cbl activity was unclear. In this study, we find that ITSN1 enhances Cbl activity through disrupting the interaction of Cbl with the Sprouty2 (Spry2) inhibitory protein. We demonstrate that ITSN1 binds Pro-rich regions in both Cbl and Spry2 and that interaction of ITSN1 with Spry2 disrupts Spry2-Cbl interaction resulting in enhanced ubiquitylation of the EGFR. Disruption of ITSN1 binding to Spry2 through point mutation of the Pro-rich, ITSN1 binding site in Spry2 results in enhanced Cbl-Spry2 interaction and inhibition of receptor ubiquitylation. This study demonstrates that ITSN1 enhances Cbl activity by modulating the interaction of Cbl with Spry2. In addition, our results reveal a new level of complexity in the regulation of Cbl through the interaction with ITSN1 and Spry2.

Introduction

Receptor tyrosine kinases (RTKs) play critical roles in the regulation of multiple aspects of metazoan life. Binding of ligand stimulates the intrinsic kinase activity of the receptor leading to the recruitment and activation of numerous intracellular signaling pathways. However, a number of mechanisms exist to regulate the extent and duration of RTK

signaling. One such mechanism involves the covalent attachment of ubiquitin to activated receptors. This post-translational modification targets the activated receptors for lysosomal degradation (19). Thus, regulation of RTK ubiquitylation represents a critical step in cellular signaling.

Cbl is a RING (really interesting new gene) domain E3 ubiquitin ligase that specifically regulates RTK ubiquitylation (28). Although binding of Cbl to activated RTKs represents an important step in regulation of RTK ubiquitylation, Cbl activity is modulated by both post-translational modifications as well as interactions with numerous proteins (28). One such protein is the intersectin1 (ITSN1) scaffold protein. Although initially identified as a regulator of clathrin-dependent endocytosis, ITSN1 regulates a number of additional biochemical pathways (25). Recently, we demonstrated that ITSN1 enhances Cbl-dependent ubiquitylation of the EGFR leading to enhanced degradation of the activated receptor (20). However the mechanism underlying the increase in Cbl activity was unclear. We postulated that ITSN1 either promoted Cbl binding to an activator or prevented Cbl interaction with a negative regulator. In this study, we have defined a novel role for ITSN1 in attenuating Cbl inhibition by Spry2, a negative regulator of Cbl (9, 15). Our results demonstrate that ITSN1 binds both Cbl and Spry2 and that ITSN1 releases Cbl from Spry2 inhibition leading to enhanced EGFR ubiquitylation.

Materials and Methods:

Cell lines and reagents

HEK293T human kidney epithelial cells and COS-1 monkey kidney cells were maintained in DMEM with 10 fetal bovine serum. Human IMR-5 neuroblastoma cells were grown in RPMI media supplemented with 10% fetal bovine serum. All cells were grown at 37 ^{o}C in a humidified chamber with 5% CO_2/95% air. Epidermal Growth Factor was purchased from Millipore. The antibodies used in this study were: N-Spry2 and ubiquitin P4D1 antibodies from Santa Cruz; EGFR AB12 and EGFR AB13 antibody from Thermo Scientific; monoclonal anti-hemagglutinin (HA) antibody was purchased from Covance.

DNA constructs and transfection

An amino-terminal HA epitope-tagged full-length ITSN1 (mouse) in pCGN construct was previously described (24). HA-tagged wild-type (WT) human c-Cbl was a gift from Drs. Yosef Yarden (Weizmann Institute of Science, Rehovot, Israel) and has been described previously (18). The pHM6-HA-Spry2 and its empty vector, pHM6-HA, were kindly provided by Dr. Tarun Patel (Loyola University, Chicago, IL) and described previously (38). COS-1 cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) according to the protocol provided by manufacturer. GST-tagged SH3 domains of ITSN were created by subcloning the individual SH3 domains into the mammalian expression vector pEFG (26). The single amino acid mutants of Spry2 (Y55F, P59A, P65A, P69A, P71A, P73A, P304A, P308A) were generated from the plasmid pCEFL-KZ-AU5-Spry2 WT (4, 22) by site-directed PCR mutagenesis using specific primers. The sequences of

all PCR-generated constructs were verified by direct sequencing and those of the oligonucleotides used are available upon request. Spry2 WT, Y55F, P59A, and P308A fragments were subcloned into pHA-VC155 kindly provided by Dr. Chang-Deng Hu (Purdue University, West Lafayette, IN)

COOH-terminal truncated constructs of Spry2 from amino acid 301 (T301) in pXJ40-FLAG have been described (17). Spry2N and Spry2C were also previously described (2). Various truncation mutants of the short isoform of ITSN1 were generated using reverse primer 5'CGGGGTACCCCGAGATGCAGGTCTGAGCACC3' and forward primers as follows: ΔEH1- 5'ATAAGAATGCGGCCGCTGTCATGA AACAGGCAACCAGTG3' ΔEH1 + EH2- 5'ATAAGAATGCGGCCGCTCAGCCACTGC CGCCCGTC3' and ΔEH1 + EH2 + CC- 5'ATAAGAATGCGGCCGCTCATCAGGAGCCA GCTAAGCTG3'. The N-terminal truncation mutants were cloned into pXJ40-Myc using *Not*I and *Kpn*I sites.

Immunoprecipitation and immunoblotting

Whole-cell extracts were prepared as described previously (26). For the analysis of endogenous levels of ubiquitin in COS-1 cells, lysis buffer was supplemented with 5 mM N-ethylmaleimide. EGFR immunoprecipitation and ubiquitylation levels were determined as previously described (20). For detection of Spry2, EGFR and HA-tagged proteins, standard protocols suggested by the manufacturers were used.

GST pull down assays

Samples were lysed in a Tris-based buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM sodium orthovanadate and a cocktail of protease inhibitors) and centrifuged at 13,200 rpm for 15 minutes at 4^{o}C. Fifteen microliters of glutathione Sepharose4B beads (Amersham Biosciences, Buckinghamshire, HP) was added to the supernatant to precipitate the GST epitope. The resulting immunoprecipitates were separated on SDS-PAGE.

Yeast two hybrid screening

Analysis was performed through a contract with Myriad Genetics essentially as described previously (3, 33) except using the various individual domains of mouse or human ITSN1 as bait. Multiple mouse and human Spry2 clones were identified as binding to the first SH3 domain of ITSN1.

Peptide screening of SH3 domain blots

SH3 domain blots were obtained from Panomics, Inc. (Redwood City, CA). Biotinylated peptides (1µg/ml) coding for the Pro-rich region of Spry2, TVCCKVPTVPPRNFEKPT, or a control peptide, TVCCKVATVPANFEKPT, were incubated with membranes overnight at 4°C. The membranes were washed with PBS containing 0.01% Tween-20 (PBST) for 3 x 15 minutes and then incubated with streptavidin-conjugated horseradish peroxidase (1:100,000 in PBST). After 3x15 minute washes with PBST, enhanced chemiluminescent system (Amersham Pharmacia Biotech, Buckinghamshire, HP) was used to detect bound peptides.

Bimolecular Fluorescence Complementation (BiFC)

BiFC was performed essentially as described (36). Briefly, COS-1 cells were seeded on glass bottom plate and in a 6-well dish and transfected with 0.5 ug of plasmids encoding proteins fused to pHA-VC155N and pFLAG-VN173N. Twenty four hours post-transfection, the glass bottom dishes were fixed on ice in 3.7% formaldehyde for 20 min, rinsed 2x with PBS, and stored with PBS at 4°C in the dark. Zeiss LSM 510 META confocal microscope was used to image samples. CFP positive cells were selected and imaged for BiFC signal in the YFP channel. BiFC was quantified and expressed as average fluorescence intensity per pixel using ImageJ available from the NIH as described (36). In parallel with imaging, cells in 6-well dishes were lysed and expression levels of transfected proteins were determined by Western blot analysis.

Results:

Spry2 is an ITSN binding partner. Spry2 was identified in a high throughput yeast two-hybrid (Y2H) screen designed to identify ITSN-binding partners (Wong, et. al., unpublished observations). The SH3A domain of ITSN1 (amino acid 730-816) isolated both human and mouse Spry2 clones as targets. The COOH-terminus of Spry2 contains a consensus Pro-rich sequence (PTVPPRN) resembling the ligand for ITSN1's first SH3 domain, SH3A (3, 20). Using a biotinylated peptide derived from the Spry2 sequence encompassing this site (TVCCKVPTVPPRNFEKPT), we identified the SH3 domains of both ITSN1 and ITSN2 as potential binding partners for Spry2. Thus, both Y2H and peptide screening experiments suggest that ITSN1 and Spry2 may represent binding partners *in vivo*.

ITSN1 and Spry2 interact in cells. Immunocytochemical staining of cells reveals that a portion of endogenous ITSN1 and Spry2 co-localize in cells (Fig. 1A). Although we were unable to co-precipitate endogenous ITSN1 and Spry2 from cells possibly due to the fact that the antibodies target epitopes in the regions of interaction between the two proteins, we analyzed the interaction of epitope-tagged versions of the two proteins (Fig. 1B). Using HA-epitope tagged versions of the major ITSN1 isoforms (25), we demonstrated that both ITSN1-S and ITSN1-L interact with Spry2 suggesting that the presence of the guanine nucleotide exchange factor (GEF) domain on ITSN1-L does not interfere with Spry2 interaction (Fig. 1B). Spry2 is a member of the Spry family of proteins consisting of Spry1-4 (15). To determine the specificity of ITSN1 for specific Spry members, we co-expressed ITSN1 with different Spry isoforms (Fig. 1C&D). ITSN1 specifically interacted with full-length Spry2 and this binding was abolished by deletion of the COOH-terminal Pro-rich tail in Spry2 T301 truncation mutant. Spry4, which lacks a comparable Pro-rich sequence, did not interact with ITSN1 (Figs. 1C&D).

Using truncation mutants of ITSN1, we observed that ITSN1's SH3 domains mediated Spry2 binding (see Fig 4). Given the presence of five SH3 (A-E) domains in ITSN1, we examined the specificity of Spry2 for each of these SH3 domains. The five SH3 domains were individually cloned into the mammalian expression vector pEFG (26) as described in the *Materials and Methods*. These SH3 constructs were co-transfected into HEK293T cells along with FLAG-Spry2. Following immunoprecipitation with anti-FLAG antibody, we observed that the SH3A domain of ITSN1 but not any of the other SH3

180 domains specifically interacted with full-length Spry2 (Fig. 2A). Although Spry2 contains
181 two Pro-rich stretches (aa 59-PTVVPRP-65; and aa 304-PTVPPRN-310), only mutation
182 of Pro304 to Ala (P304A) in the COOH-terminal Pro-rich sequence disrupted binding of
183 ITSN1 (Fig. 2B).
184
185 To examine the interaction of Spry2 and ITSN1 in whole cells, we utilized bimolecular
186 fluorescence complementation (BiFC) (Fig. 3). As seen with the individual SH3A domain
187 of ITSN1, Spry2 interaction with full-length ITSN1 was disrupted by the P304A mutation
188 but not by the Y55A mutation (Fig. 3). Mutation of P59A resulted in slight but significant
189 reduction in ITSN1 interaction. These differences in BiFC signal were not due to
190 differences in expression of the various Spry2 mutants and thus likely reflect true
191 differences in the affinity of ITSN1 for these mutants (Fig. 3C). These findings
192 demonstrate that ITSN1 specifically interacts with the COOH-terminal Pro-rich
193 sequence in Spry2.
194
195 **SH3 binding to targets is negatively regulated by ITSN1's EH and CC domains.**
196 During the course of our investigations, we observed that Spry2 interacted better with
197 the isolated SH3A domain than with full-length ITSN1 (data not shown). One possible
198 explanation for these results is that the regions NH_2-terminal to the SH3 domains, i.e.,
199 the EH and CC domains, may sterically hinder SH3 binding to targets such as Spry2. To
200 test this possibility, we created a series of NH_2-terminal ITSN1 truncations which were
201 tested for interaction with Spry2 (Fig. 4A). Myc-tagged ITSN1 full-length or truncation
202 mutants were co-expressed with Spry2 in HEK293T cells. Immunoprecipitation of Spry2

revealed increased binding to ITSN1 with progressive truncation of the NH_2-terminus (Fig. 4B). Deletion of the EH1 domain enhanced Spry2 binding to ITSN1 compared to full-length ITSN1. Although not visible on the gel in Fig. 4B, full-length ITSN1-S and ITSN1-L do indeed interact with Spry2 by co-immunoprecipitation (Fig. 1B). Removal of both EH domains of ITSN1 did not appear to further enhance binding to Spry2. However, deletion of the EH and CC domains further enhanced Spry2-ITSN1 interaction. Similar results were observed in the binding of another ITSN1 target, N-WASP, which also interacts with ITSN1's SH3 domains (data not shown). Although Spry2 bound exclusively to SH3A (Fig. 2A), N-WASP interacted with multiple SH3 domains (SH3A>SH3C>SH3E>SH3D). However, SH3B did not interact with N-WASP. These findings are consistent with previous reports demonstrating ITSN1 binding to N-WASP proteins (13, 40). To further confirm that the SH3 domains of ITSN1 are sterically hindered in the full-length protein and to circumvent the possibility that the Pro-rich motif of Spry2 or N-WASP may not be properly presented for binding, a biotinylated Pro-rich Spry2 peptide was used in a pull-down assay. Biotinylated peptides were incubated with cell lysates from HEK293T cells transfected with the various NH_2-terminal truncation mutant of the ITSN1 short isoform (ITSN1-S). The biotinylated peptides were pre-incubated with streptavidin-conjugated Sepharose beads and then mixed with cell lysates. Consistent with the results in Fig. 4B, we observed increased ITSN1 binding to the Spry2 peptide upon progressive NH_2-terminal truncations in ITSN1, with the isolated SH3 region binding most avidly to the biotinylated Spry2 peptide (Fig. 4C).

ITSN1 disrupts Spry2 interaction with Cbl to enhance EGFR ubiquitylation. We previously demonstrated that ITSN1 regulates EGFR degradation through enhancing Cbl ubiquitylation of the activated EGFR (20). Since ITSN1 did not affect Cbl binding to EGFR, Cbl phosphorylation, or Cbl stability (20), we speculated that ITSN1 might activate Cbl by disrupting the interaction with Cbl inhibitory proteins. Thus, the identification of Spry2 (a Cbl inhibitor) as an ITSN1 binding partner suggests that ITSN1 might activate Cbl by disrupting the Spry2-Cbl interaction leading to enhanced ubiquitylation of the EGFR. To test this possibility, we examined the effect of ITSN1 overexpression on Spry2-Cbl interaction and EGFR ubiquitylation. Using BiFC to quantify Spry2-Cbl binding, we observed that ITSN1 decreased Spry2-Cbl binding in a dose-dependent manner (Fig. 5A&B). The loss of Spry2-Cbl BiFC signal was not due to changes in the expression of VN-Spry2 or VC-Cbl (Fig. 5C). Using epitope-tagged versions of these proteins instead of BiFC, we also demonstrate that ITSN1 dose-dependently decreased the co-immunoprecipitation of Spry2 with Cbl thus corroborating the BiFC data (Fig. 5D).

Given the ability of ITSN1 to disrupt Spry2-Cbl interaction, we next tested the possibility that increasing ITSN1 levels might reverse Spry2 inhibition of Cbl. Transient overexpression of Cbl enhanced EGF-stimulated ubiquitylation of endogenous EGFR and co-expression of Spry2 with Cbl inhibited this effect (Fig. 6, compare lanes 2-4) (7, 27, 35). However, addition of ITSN1 reversed the inhibitory effect of Spry2 on Cbl leading to enhanced ubiquitylation of endogenous EGFR (Fig. 6, compare lanes 4 and

5). These results demonstrate that ITSN1 overexpression disrupts Spry2-Cbl binding resulting in enhanced Cbl activity toward the activated EGFR.

ITSN1 enhances the inhibitory effect of Spry2 P304A mutant. ITSN1's SH3 domains bind the Pro-rich tail of Cbl (20) as well as Spry2 (Figs. 1 & 4). Since Cbl and Spry2 interact with each other, we next examined the effect of mutating the ITSN1-binding site of Spry2 on the interaction between Spry2 and Cbl. Spry2 P304A interacts with Cbl as measured by BiFC (Fig. 7A, left panel). Surprisingly, increasing ITSN1 expression resulted in increased interaction between Cbl and Spry2 P304A in a dose-dependent manner (Fig. 7A&B). The increased in Spry2-Cbl BiFC signal was not due to changes in the expression of VN-Spry2 or VC-Cbl (Fig. 7C). To confirm these BiFC results, we again used epitope-tagged versions of these proteins and tested the effect of ITSN1 on co-precipitation of Spry2 P304A with Cbl. In agreement with the BiFC results, we observed that ITSN1 dose dependently increased association of Spry2 P304A with Cbl Fig. 7D.

Given this increased interaction between Spry2 P304A and Cbl in the presence of ITSN1, we next tested the consequence on EGFR ubiquitylation. Co-expression of Spry2 P304A with Cbl inhibited Cbl activity and thus decreased EGFR ubiquitylation (Fig. 8, compare lanes 3 and 4). These results are comparable to results with WT Spry2 (Fig. 6, compare lanes 3 and 4). However, in contrast to the results with wild-type Spry2, co-expression of ITSN1 with Spry2 P304A and Cbl further inhibited EGFR ubiquitylation compared to Spry P304A and Cbl consistent with the increased

interaction of Cbl and Spry2 P304A in the presence of ITSN1 (Fig. 8, compare lanes 4 and 5).

Discussion:

We have identified a novel molecular link between ITSN1 and Spry2 through two independent observations. First, a high throughput Y2H screen for ITSN1 binding proteins identified multiple Spry2 clones as SH3-interacting proteins. Second, a peptide screen of SH3 domains from various proteins revealed ITSN1 (and ITSN2) as a potential interacting partner of Spry2. Our results (Figs 1-3) demonstrate that Spyr2, but not other Spry isoforms, is a *bona fide* ITSN1 target. Furthermore, this association is mediated predominantly through ITSN1's SH3 domains binding Spry2's C-terminal Pro-rich site (aa 304-310). Indeed, this Pro-rich sequence conforms to previously identified ITSN1 binding sites (3, 20, 37).

Our previous work demonstrated a novel role for ITSN1 in regulating Cbl-dependent ubiquitylation of the EGFR resulting in increased degradation of the receptor following growth factor stimulation (20). However, the mechanism by which ITSN1 enhanced Cbl activity was unclear. The identification of Spry2 as an ITSN1 target provides a potential answer to this question. Cbl regulation is quite complex, involving post-translational modifications as well as association of Cbl with numerous activators and inhibitors (28). Although ITSN1's ability to activate Cbl did not stem from alterations in Cbl binding to the EGFR, changes in Cbl stability, or altered tyrosine phosphorylation of Cbl, we proposed that ITSN1 activation of Cbl may occur through enhancing Cbl binding to an

activator or inhibiting Cbl interaction with an inhibitor (20). Our current results demonstrate that ITSN1 regulates Cbl, in part, through disrupting the inhibitory effect of Spry2 on Cbl thereby enhancing EGFR ubiquitylation by Cbl. The importance of this regulation by ITSN1 is highlighted by the finding that EGFR ubiquitylation is not necessary for internalization of the receptor but rather necessary for the sorting of the receptor in the multivesicular endosomes/bodies for degradation in the lysosome (6, 12). Thus, enhancing ubiquitylation of the EGFR leads to enhanced EGFR turnover thereby altering EGFR signaling.

It should be noted that although the observed effects of ITSN1, Cbl, and Spry2 (Spry2 P304A) are rather modest, we are likely underestimating the effects of these proteins on EGFR ubiquitylation since we are measuring ubiquitylation of endogenous EGFR in the total population of cells yet are only able to transfect approximately 50% of cells. This approach allows us to measure the effects on endogenous receptor using endogenous ubiquitin and therefore avoids problems of uneven expression of epitope-tagged ubiquitin between samples (23). In addition, this approach also reduces the number of plasmids that are being transfected in any given sample which also results in more consistent expression of the given proteins between experiments.

Our findings reveal a complex network of interactions between ITSN1 and the Pro-rich regions of both Cbl and Spry2 resulting in either activation or inhibition of Cbl depending on how ITSN1 interacts with each of these components. Thus, modulating the interaction of ITSN1 with Spry2 and Cbl may lead to activation or repression of Cbl's

316 ubiquitin ligase activity to regulate EGFR ubiquitylation. Both Cbl and Spry2 possess
317 Pro-rich motifs that bind ITSN1 (Fig. 1D) (20). Surprisingly, disrupting the binding of
318 ITSN1 to Spry2 enhanced interaction between Cbl and Spry2 P304A leading to
319 decreased EGFR ubiquitylation (Fig. 6). This result suggests that ITSN binding to the
320 Pro-rich tail of Cbl may promote a conformational change that enhances the interaction
321 of Spry2 with Cbl. While Spry2 binds Cbl through phosphotyrosine-dependent and -
322 independent mechanisms [reviewed in (15)], ITSN overexpression does not alter the
323 tyrosine phosphorylation of Cbl following growth factor stimulation (20). Thus, we do not
324 believe that the enhanced interaction of Spry2 P304A with Cbl is due to altered
325 phosphorylation of Cbl. However, it is unclear whether ITSN1 overexpression alters the
326 phosphorylation of Spry2 to facilitate interaction with Cbl.
327
328 ITSN1 regulates numerous biological processes including endocytosis and cellular
329 signaling (25). The modular structure of ITSN1 allows for interaction with a variety of
330 targets. Furthermore, intra- and intermolecular interaction of these domains appears to
331 play an important role in ITSN function. For example, overexpression of ITSN1's SH3
332 domains inhibits the formation of clathrin-coated pits as well as ITSN-regulated
333 signaling pathways (29, 30, 32) indicating that SH3 domain availability must be strictly
334 regulated to maintain proper ITSN1 function. Our data suggest that the EH and CC
335 domains may negatively regulate SH3 domain availability as progressive NH_2-terminal
336 deletions in ITSN1 enhanced binding to Spry2 as well as N-WASP. This regulation of
337 SH3 binding may also have important implications for Cdc42 regulation by the long
338 isoform of ITSN1 (ITSN1-L). ITSN1-L GEF activity is autoinhibited through an

intramolecular interaction of the GEF domain with the linker region between the SH3E and the DH domain (16). Furthermore, interaction of ITSN1-L with N-WASP relieves this inhibition (13). Thus, EH binding to endocytic proteins such as epsin (29), stonin (21), SCAMP1 (8) FCHo proteins (11), AP180 (34) and Dab (34), may enhance interaction of the SH3 domains with their targets to relieve this autoinhibition thereby resulting in Cdc42 activation.

The 'activation' of ITSN1 likely requires a complex of contributing proteins (25). While binding to targets as noted above may regulate ITSN1 function, localization also likely plays an important role in ITSN1 function. Interaction of ITSN1 with endocytic proteins facilitates ITSN1's translocation to the plasma membrane were it participates in vesicle assembly (11). However, this recruitment may also allow for cross-talk with RTK-associated Cbl and regulation of receptor ubiquitylation. In addition, EH domain binding to components of the JNK MAPK pathway (1, 24) may also free the SH3 domains for interaction with various targets such as Cbl and Spry2.

The identification of this novel ITSN1-Spry2 connection raises new questions in the pathophysiology of several diseases. ITSN1 has been implicated in the pathology of Down Syndrome and Alzheimer's Disease due to an increased expression of ITSN1 in patients and its participation in neuronal survival and differentiation (3, 5, 14, 39). There is a high co-morbidity of the obstructive gastrointestinal disorder, Hirschsprung Disease, in Down Syndrome. Hirschsprung is caused by a failure of enteric nerve ganglia to migrate to the gut. Interestingly, Spry2 has been reported to regulate neurite outgrowth

in the sympathetic neuron-like PC12 cells (10). Moreover, Spry2 deficient mice develop enteric nerve hyperplasia (31). The development of esophageal achalasia and intestinal pseudo-obstruction in these mice is reminiscent of Hirschsprung Disease and together these data suggest that pathogenesis of the disease may lie in the interaction between ITSN1 and Spry2.

Acknowledgements:

The authors declare that there are no competing financial interests. We wish to thank Tarun Patel for providing the HA-Spry2 expression construct, Chang-Deng Hu for providing the BiFC vectors, and Pritin Soni for construction of the Spry2 BiFC constructs. We also thank members of the O'Bryan lab and the Carnegie lab for many helpful comments and discussions. This work was funded in part by grants to JMR from FIS (PI09/0562), RETIC (RD06/0020/0003), and AECC and grants to J.P.O. from the NIH (RO1 HL090651), Department of Defense (PR080428), the Foundation Jerome Lejune, and the St. Baldrick's Foundation.

References:

1. **Adams, A., J. M. Thorn, M. Yamabhai, B. K. Kay, and J. P. O'Bryan.** 2000. Intersectin, an adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways. J Biol Chem **275:**27414-20.
2. **Chow, S. Y., C. Y. Yu, and G. R. Guy.** 2009. Sprouty2 interacts with protein kinase C delta and disrupts phosphorylation of protein kinase D1. J Biol Chem **284:**19623-36.
3. **Das, M., E. Scappini, N. P. Martin, K. Wong, S. A. Dunn, Y.-J. Chen, S. L. H. Miller, J. Domin, and P. O'Bryan J.** 2007. Regulation of neuron survival through an intersectin (ITSN)-phosphoinositide 3'-kinase C2β-AKT pathway. Mol Cell Biol **27:**7906-17.
4. **de Alvaro, C., N. Martinez, J. M. Rojas, and M. Lorenzo.** 2005. Sprouty-2 overexpression in C2C12 cells confers myogenic differentiation properties in the presence of FGF2. Mol Biol Cell **16:**4454-61.
5. **Dierssen, M., E. Marti, C. Pucharcos, V. Fotaki, X. Altafaj, K. Casas, A. Solans, M. L. Arbones, C. Fillat, and X. Estivill.** 2001. Functional genomics of Down syndrome: a multidisciplinary approach. J Neural Transm Suppl**:**131-48.
6. **Eden, E. R., F. Huang, A. Sorkin, and C. E. Futter.** "Accepted Article". The role of EGF receptor ubiquitination in regulating its intracellular traffic. Traffic.
7. **Egan, J. E., A. B. Hall, B. A. Yatsula, and D. Bar-Sagi.** 2002. The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. Proc Natl Acad Sci U S A **99:**6041-6.

8. **Fernandez-Chacon, R., M. Achiriloaie, R. Janz, J. P. Albanesi, and T. C. Sudhof.** 2000. SCAMP1 function in endocytosis. J Biol Chem **275:**12752-6.

9. **Guy, G. R., E. S. Wong, P. Yusoff, S. Chandramouli, T. L. Lo, J. Lim, and C. W. Fong.** 2003. Sprouty: how does the branch manager work? J Cell Sci **116:**3061-8.

10. **Hanafusa, H., S. Torii, T. Yasunaga, and E. Nishida.** 2002. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. Nat Cell Biol **4:**850-8.

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

12. **Huang, F., L. K. Goh, and A. Sorkin.** 2007. EGF receptor ubiquitination is not necessary for its internalization. Proc Natl Acad Sci U S A **104:**16904-9.

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

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

15. **Kim, H. J., and D. Bar-Sagi.** 2004. Modulation of signalling by Sprouty: a developing story. Nat Rev Mol Cell Biol **5:**441-50.

16. **Kintscher, C., S. Wuertenberger, R. Eylenstein, T. Uhlendorf, and Y. Groemping.** 2010. Autoinhibition of GEF activity in Intersectin 1 is mediated by the short SH3-DH domain linker. Protein Science **19:**2164-74.

17. **Lao, D. H., S. Chandramouli, P. Yusoff, C. W. Fong, T. Y. Saw, L. P. Tai, C. Y. Yu, H. F. Leong, and G. R. Guy.** 2006. A Src homology 3-binding sequence on the C terminus of Sprouty2 is necessary for inhibition of the Ras/ERK pathway downstream of fibroblast growth factor receptor stimulation. J Biol Chem **281:**29993-30000.

18. **Levkowitz, G., H. Waterman, S. A. Ettenberg, M. Katz, A. Y. Tsygankov, I. Alroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, S. Lipkowitz, and Y. Yarden.** 1999. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell **4:**1029-40.

19. **Marmor, M. D., and Y. Yarden.** 2004. Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene **23:**2057-70.

20. **Martin, N. P., R. P. Mohney, S. Dunn, M. Das, E. Scappini, and P. O'Bryan J.** 2006. Intersectin Regulates Epidermal Growth Factor Receptor Endocytosis, Ubiquitylation, and Signaling. Mol Pharmacol **70:**1643-53.

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

22. **Martinez, N., C. A. Garcia-Dominguez, B. Domingo, J. L. Oliva, N. Zarich, A. Sanchez, S. Gutierrez-Eisman, J. Llopis, and J. M. Rojas.** 2007. Sprouty2

binds Grb2 at two different proline-rich regions, and the mechanism of ERK inhibition is independent of this interaction. Cell Signal **19:**2277-85.

23. **Miller, S. L., E. Malotky, and J. P. O'Bryan.** 2004. Analysis of the role of ubiquitin-interacting motifs in ubiquitin binding and ubiquitylation. J Biol Chem **279:**33528-37.

24. **Mohney, R. P., M. Das, T. G. Bivona, R. Hanes, A. G. Adams, M. R. Philips, and J. P. O'Bryan.** 2003. Intersectin activates Ras but stimulates transcription through an independent pathway involving JNK. J Biol Chem **278:**47038-45.

25. **O'Bryan, J. P.** 2010. INTERSECTINg Pathways in Cell Biology. Sci Signal **3:**re10.

26. **Oldham, C. E., R. P. Mohney, S. L. Miller, R. N. Hanes, and J. P. O'Bryan.** 2002. The ubiquitin-interacting motifs target the endocytic adaptor protein epsin for ubiquitination. Curr Biol **12:**1112-6.

27. **Rubin, C., V. Litvak, H. Medvedovsky, Y. Zwang, S. Lev, and Y. Yarden.** 2003. Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops. Curr Biol **13:**297-307.

28. **Schmidt, M. H., and I. Dikic.** 2005. The Cbl interactome and its functions. Nat Rev Mol Cell Biol **6:**907-19.

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

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

31. **Taketomi, T., D. Yoshiga, K. Taniguchi, T. Kobayashi, A. Nonami, R. Kato, M. Sasaki, A. Sasaki, H. Ishibashi, M. Moriyama, K. Nakamura, J. Nishimura, and A. Yoshimura.** 2005. Loss of mammalian Sprouty2 leads to enteric neuronal hyperplasia and esophageal achalasia. Nat Neurosci **8:**855-7.

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

33. **von Schwedler, U. K., M. Stuchell, B. Muller, D. M. Ward, H. Y. Chung, E. Morita, H. E. Wang, T. Davis, G. P. He, D. M. Cimbora, A. Scott, H. G. Krausslich, J. Kaplan, S. G. Morham, and W. I. Sundquist.** 2003. The protein network of HIV budding. Cell **114:**701-13.

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

35. **Wong, E. S., C. W. Fong, J. Lim, P. Yusoff, B. C. Low, W. Y. Langdon, and G. R. Guy.** 2002. Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. Embo J **21:**4796-808.

36. **Wong, K. A., and J. P. O'Bryan.** 2011. Bimolecular Fluorescence Complementation. J Vis Exp**:**e2643.

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

38. **Yigzaw, Y., L. Cartin, S. Pierre, K. Scholich, and T. B. Patel.** 2001. The C terminus of sprouty is important for modulation of cellular migration and proliferation. J Biol Chem **276:**22742-7.

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

40. **Zamanian, J. L., and R. B. Kelly.** 2003. Intersectin 1L Guanine Nucleotide Exchange Activity Is Regulated by Adjacent src Homology 3 Domains That Are Also Involved in Endocytosis. Mol Biol Cell **14:**1624-37.

Figure Legends:

Figure 1. ITSN1 binds Spry2. (A) Endogenous ITSN1 (red) co-localizes with endogenous Spry2 (green) in IMR-5 neuroblastoma cells (top panels). As controls (middle and bottom panels), cells were stained with both fluorescently-labelled secondary antibodies (Cy5-labelled donkey anti-rabbit and FITC-labelled donkey anti-mouse) but only a single primary antibody as indicated in the panels. (B) Spry2 binds both ITSN1 isoforms. FLAG-tagged Spry2 was co-expressed with either HA-tagged ITSN1-S or ITSN1-L. Both isoforms are detected in FLAG immunoprecipitates. (C) Schematic of Spry constructs. (D) HEK293T cells were transiently transfected with the constructs indicated at the top of the gels. ITSN1 SH3A-E was epitope tagged with a Myc epitope whereas the Spry2 constructs were tagged with FLAG. Proteins were immunoprecipitated with either Myc or FLAG antibodies as indicated on the left. Western blots of the immunoprecipitates were then probed with the antibodies indicated on the right. Expression of the various proteins is indicated in the Western blots of cell lysates shown in the bottom two panels. The migration of Spry2 WT (WT), Spry2N (N), and Spry2C (C) are shown by arrows.

Figure 2. Mapping Spry2-ITSN1 interactions. (A) GST-tagged constructs of the individual SH3 domains of ITSN1 were co-expressed in HEK293T cells along with FLAG-Spry2. Only the SH3A domain of ITSN1 co-precipitates with Spry2. Control, GST alone, is not visible in the cell lysates blot due to its smaller size. (B) Mutation of Pro304 disrupts ITSN1 binding. AU5-tagged Spry2 wild type (WT) or point mutants containing Pro-Ala substitutions at the indicated amino acids were co-expressed with GST-SH3A in

HEK293T cells. Following purification of the SH3A domain from cell lysates using Glutathione beads, Western blots were performed to detect association of Spry2 proteins. Mutation of P304A disrupted ITSN1 SH3A binding whereas the other Pro mutations had little to no effect.

Figure 3. Interaction of Spry2 and ITSN1 by BiFC. (A) VN-tagged Spry2 WT or various Spry2 mutants were co-expressed with VC-ITSN1 in COS cells. CFP was included at one-fifth the amount of DNA as a transfection control. pep, a non-specific peptide control fused to VC. (B) Interaction of Spry2 and ITSN1 was quantified as described (36). WT, Y55A, and P59A Spry2 proteins interacted with VC-ITSN1 whereas Spry2 P304A mutant was impaired in the interaction. Experiments were performed in duplicate. Data is expressed as the average fluorescence intensity per cell +/- SEM. Asterisks indicate that the values for these Spry2 mutants were significantly different from wild type Spry2 ($p<0.05$) (C) Western blot of lysates from the BiFC experiments demonstrates equivalent expression of the tagged proteins.

Figure 4. The NH_2-terminus of ITSN1 negatively regulates binding to Spry2. (A) Schematic of ITSN1 NH_2-truncation mutants. (B) HEK293T cells were co-transfected with FLAG-tagged wild type Spry2 along with full-length ITSN1-S or various NH_2-terminal truncations. Top panel: FLAG-tagged Spry2 was immunoprecipitated using M2 beads to identify the ITSN1 truncations that interact with Spry2. Middle panel, 10 x longer exposure of the same blot as seen in the top panel. Bottom panel, the level of protein expression from the various transfected gene constructs was similar. (C)

549 Immunoprecipitation of Pro-rich Spry2 peptides with various NH_2-terminal truncations of
550 ITSN-S. HEK293T cells were transfected with full-length ITSN1 or various NH_2-terminal
551 truncations. Cell lysate were incubated with either biotinylated Pro-rich Spry2 peptides
552 or control peptides. Top panel: biotin-labeled peptides were immunoprecipitated using
553 streptavidin Sepharose beads. Precipitates were analyzed by immunobloting with Myc
554 antibodies to detect the ITSN1 proteins. Bottom panel shows the level of expression of
555 the various Myc-tagged ITSN1 proteins in cell lysates.
556
557 **Figure 5. ITSN1 disrupts Spry2-Cbl interaction.** (A) Spry2-Cbl interaction was
558 measured by BiFC. ITSN1 expression leads to a dose-dependent decrease in Spry2-
559 Cbl interaction. Co-expression of VN-Spry2 with VC-pep, a non-specific peptide control,
560 does not result in a BiFC signal. (B) Quantification of BiFC signal. Interaction of Spry2
561 and Cbl was quantified as described (36). Results are the average of three independent
562 experiments +/- SEM. Samples marked with asterisk were significantly different from
563 VN-Spyr2 + VC-Cbl sample ($p<0.05$). (C) Western blot demonstrates the expression of
564 the various proteins. Both ITSN1 and Cbl are HA tagged. The differences in Spry2-Cbl
565 interaction are not due to changes in the overall expression of these proteins. (D)
566 Overexpression of HA-epitope tagged ITSN1 dose-dependently disrupts the binding of
567 Spry2 WT to Cbl. HA-Cbl was immunoprecipitated from cells and the co-precipitation of
568 Spry2 was monitored by Western blot of Cbl precipitates. Top two panels: Western blot
569 of anti-Cbl precipitates with the indicated antibodies. Bottom two panels: Western blot of
570 cell lysates with the indicated antibodies.
571

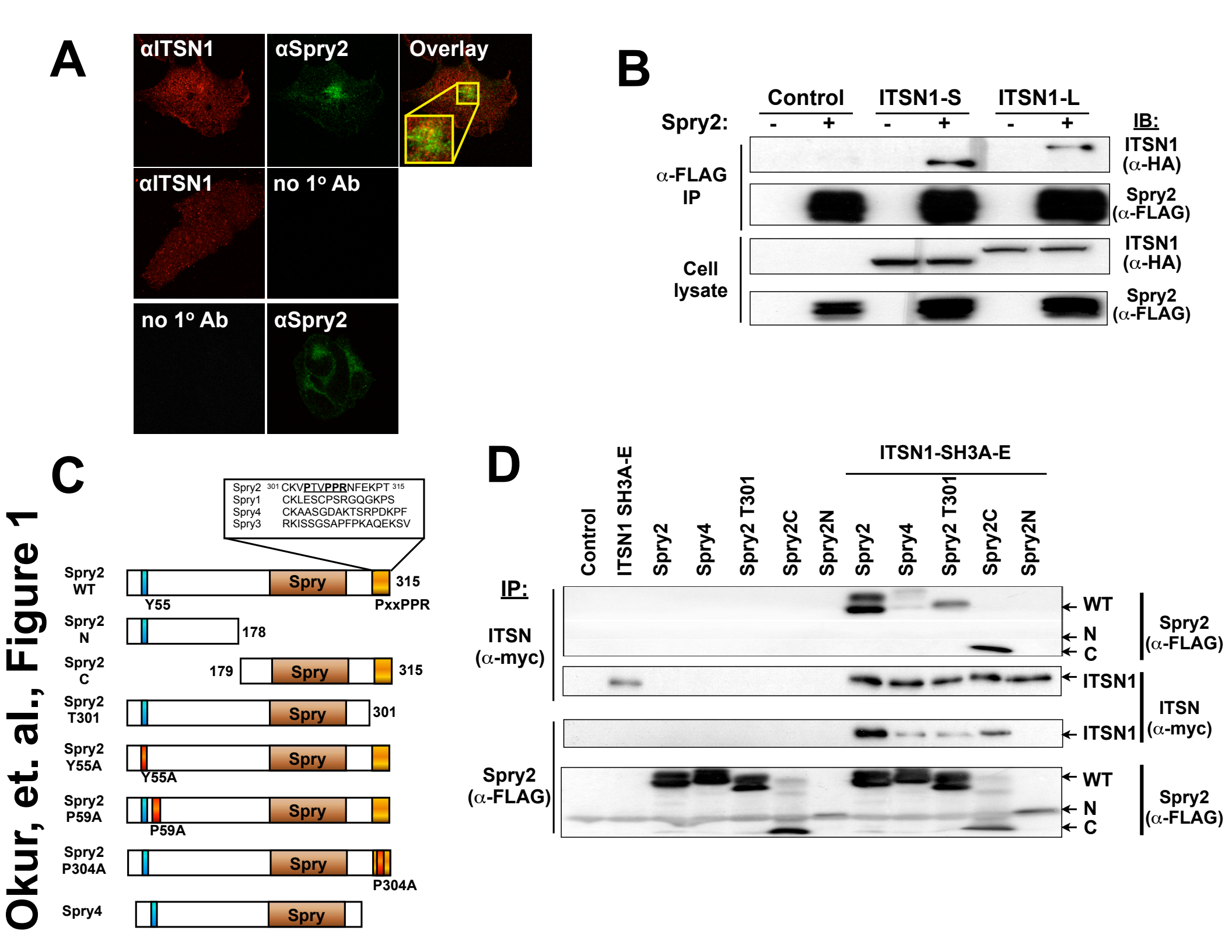
572

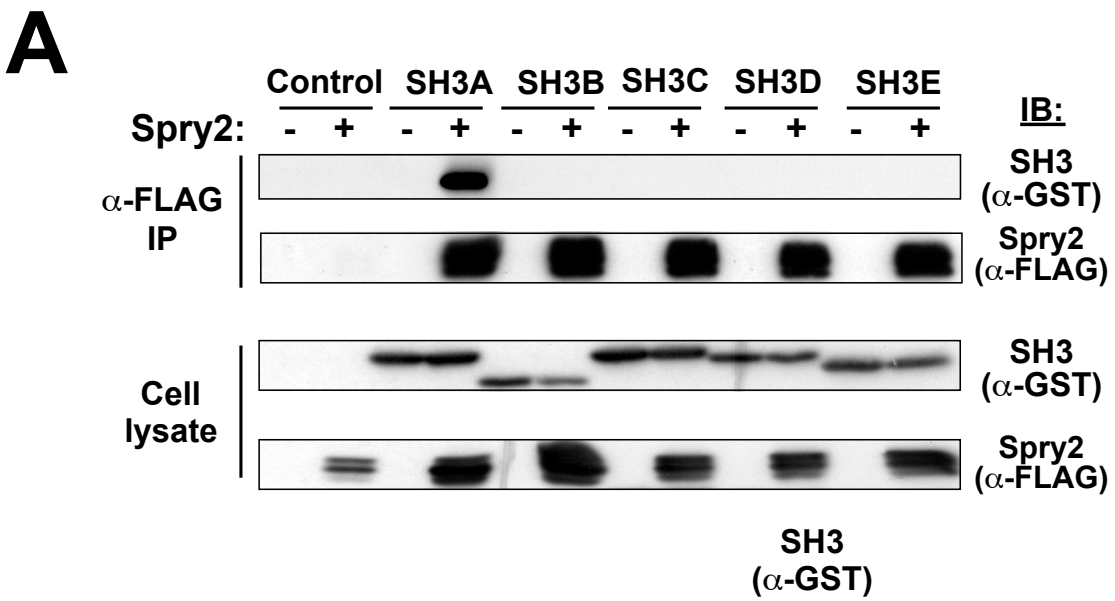
573 **Figure 6. ITSN1 overexpression reverses the inhibitory effects of Spry2 on Cbl-**
574 **mediated EGFR ubiquitylation.** Overexpression of Cbl in COS cells results in
575 enhanced EGFR ubiquitylation following EGF stimulation (compare lanes 2&3). Co-
576 expression of Spry2 with Cbl reduces EGFR ubiquitylation even though Cbl levels are
577 elevated even higher than in the absence of Spry2 overexpression (compare lanes
578 3&4). Co-expression of ITSN, however, reverses the effect of Spry2 resulting in
579 increased EGFR ubiquitylation. The ratio of ubiquitylated EGFR to total EGFR was
580 determined by densitometry and compared between samples. The results are shown in
581 the graph below the Western blots. These results are representative of three
582 independent experiments.

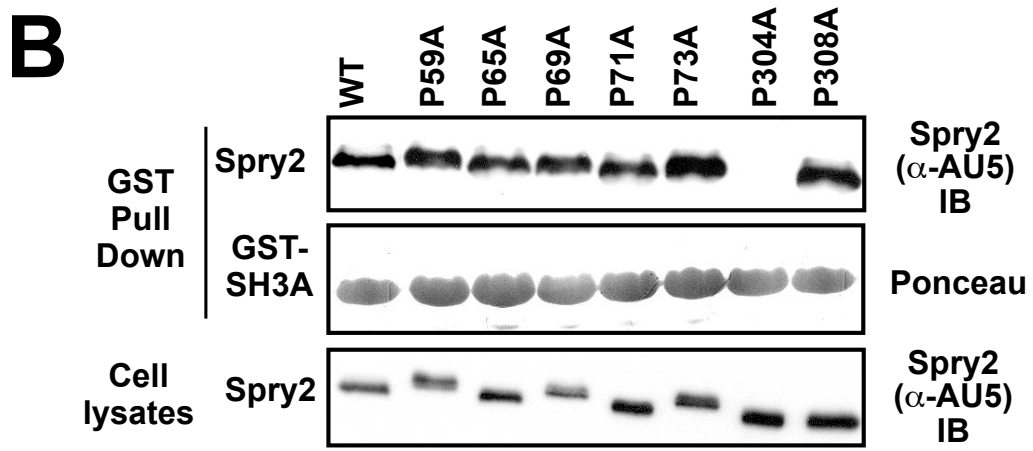
583

584 **Figure 7. ITSN1 binding to Cbl in the absence of Spry2 binding leads to enhanced**
585 **Spry2-Cbl interaction and decreased EGFR ubiquitylation.** (A) Interaction of Spry2
586 P304A with Cbl was measured by BiFC in the absence or presence of increasing ITSN1
587 levels as described in Fig. 5. ITSN1 overexpression results in enhanced binding of
588 Spry2 P304A to Cbl. (B) Quantification of BiFC signal. Interaction of Spry2 P304A and
589 Cbl was quantified as described (36). Results are the average of three independent
590 experiments +/- SEM. Samples marked with asterisk were significantly different from
591 VN-Spyr2 P304A + VC-Cbl sample ($p<0.05$). (C) Western blot demonstrates the
592 expression of the various proteins. Both ITSN1 and Cbl are HA tagged. The differences
593 in Spry2 P304A-Cbl interaction are not due to changes in the overall expression of
594 these proteins. (D) Overexpression of HA-epitope tagged ITSN1 dose-dependently

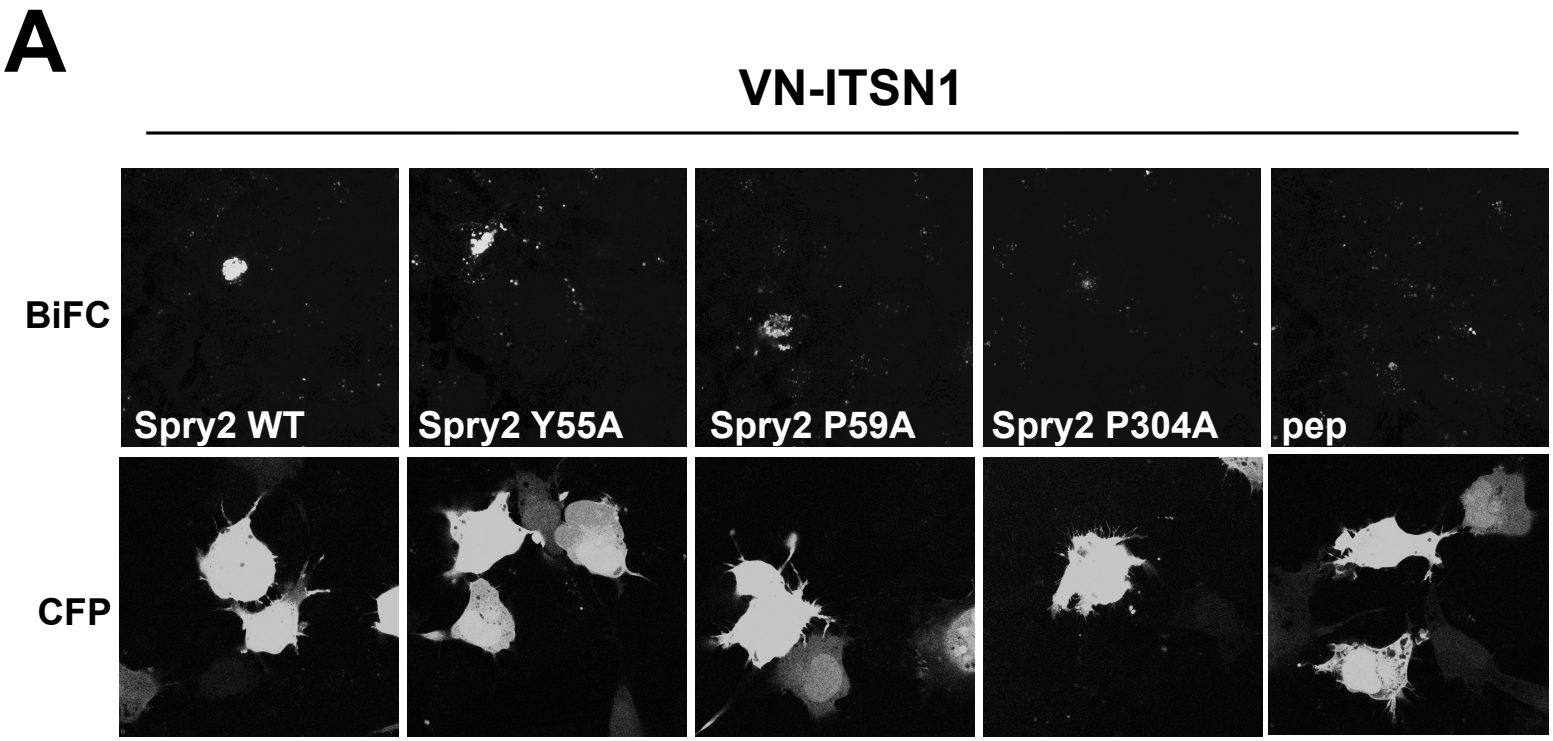
595 enhances the binding of Spry2 P304A mutant to Cbl. HA-Cbl was immunoprecipitated
596 from cells and the co-precipitation of Spry2 P304A was monitored by Western blot of
597 Cbl precipitates. Top two panels: Western blot of anti-Cbl precipitates with the indicated
598 antibodies. Bottom two panels: Western blot of cell lysates with the indicated antibodies.
599
600 **Figure 8. ITSN1 overexpression enhances the inhibitory effects of Spry2 P304A**
601 **on Cbl-mediated EGFR ubiquitylation.** Overexpression of Cbl in COS cells results in
602 enhanced EGFR ubiquitylation following EGF stimulation (compare lanes 2&3). Co-
603 expression of Spry2 P304A with Cbl reduces EGFR ubiquitylation (compare lanes 3&4).
604 Co-expression of ITSN1 enhanced the inhibitory effect of Spry2 P304A resulting in a
605 further decrease in EGFR ubiquitylation. The ratio of ubiquitylated EGFR to total EGFR
606 was determined by densitometry and compared between samples. The results are
607 shown in the graph below the Western blots. These results are representative of three
608 independent experiments.

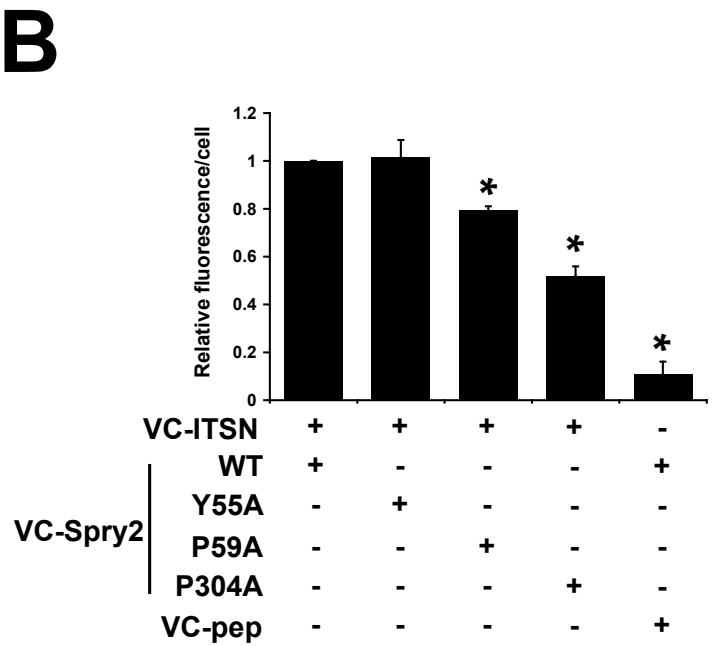

A
αITSN1
αSpry2
Overlay
αITSN1
no 1° Ab
no 1° Ab
αSpry2
B
Control
ITSN1-S
ITSN1-L
Spry2:
- + - + - +
IB:
α-FLAG IP
ITSN1 (α-HA)
Spry2 (α-FLAG)
Cell lysate
ITSN1 (α-HA)
Spry2 (α-FLAG)
C
Spry2 301 CKVPTVPPRNFEKPT 315
Spry1 CKLESCPSRGQGKPS
Spry4 CKAASGDAKTSRPDKPF
Spry3 RKISSGSAPFPKAQEKSV
Spry2 WT
Spry
315
Y55
PxxPPR
Spry2 N
178
Spry2 C
179
Spry2 T301
301
Spry2 Y55A
Y55A
Spry2 P59A
P59A
Spry2 P304A
P304A
Spry4
D
ITSN1-SH3A-E
Control
ITSN1 SH3A-E
Spry2
Spry4
Spry2 T301
Spry2C
Spry2N
IP:
ITSN (α-myc)
WT
N
C
Spry2 (α-FLAG)
ITSN1
ITSN (α-myc)
Spry2 (α-FLAG)
Okur, et. al., Figure 1

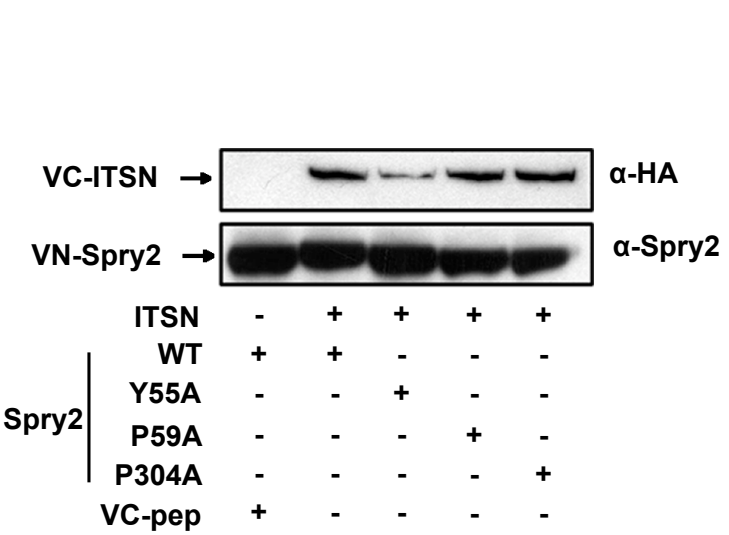




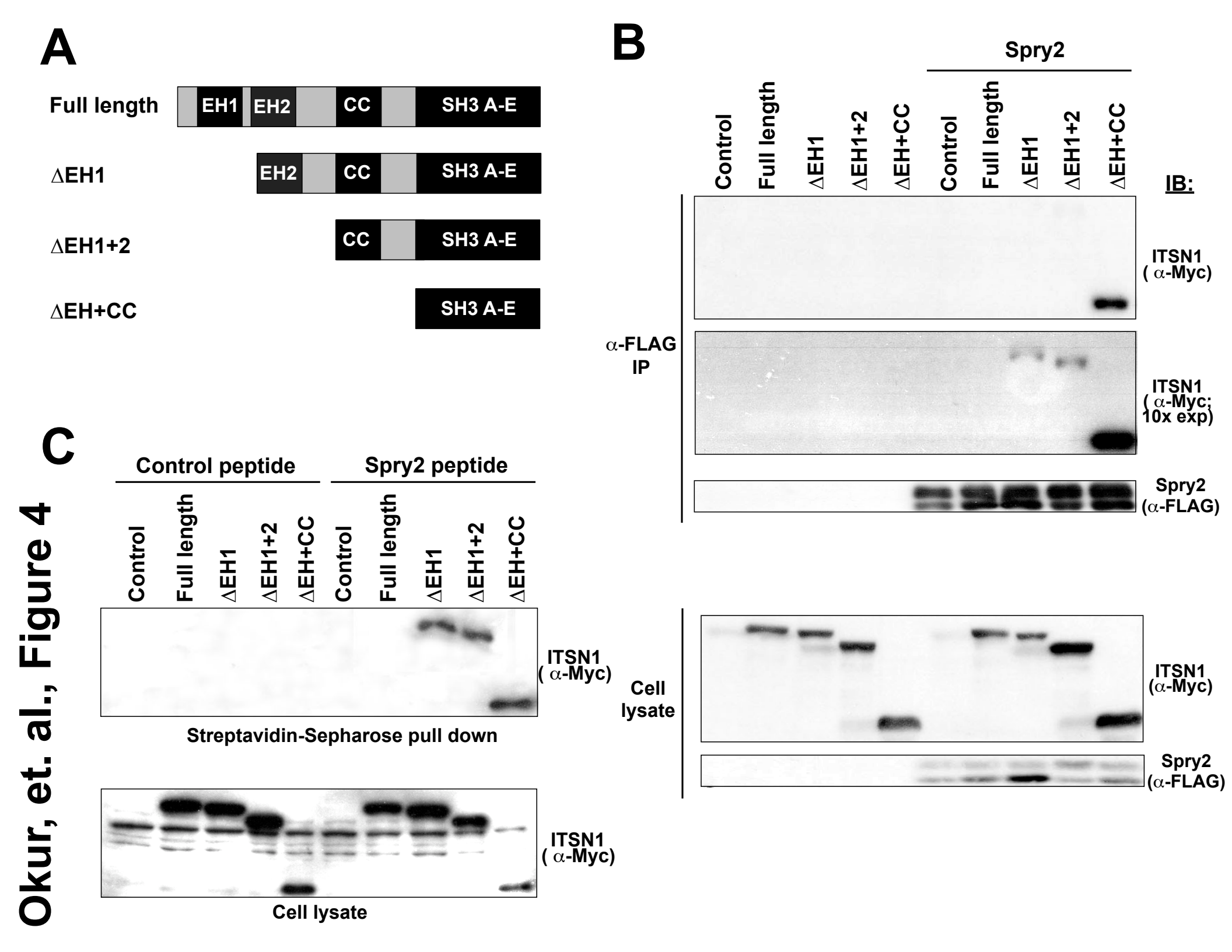
Okur, et. al., Figure 2



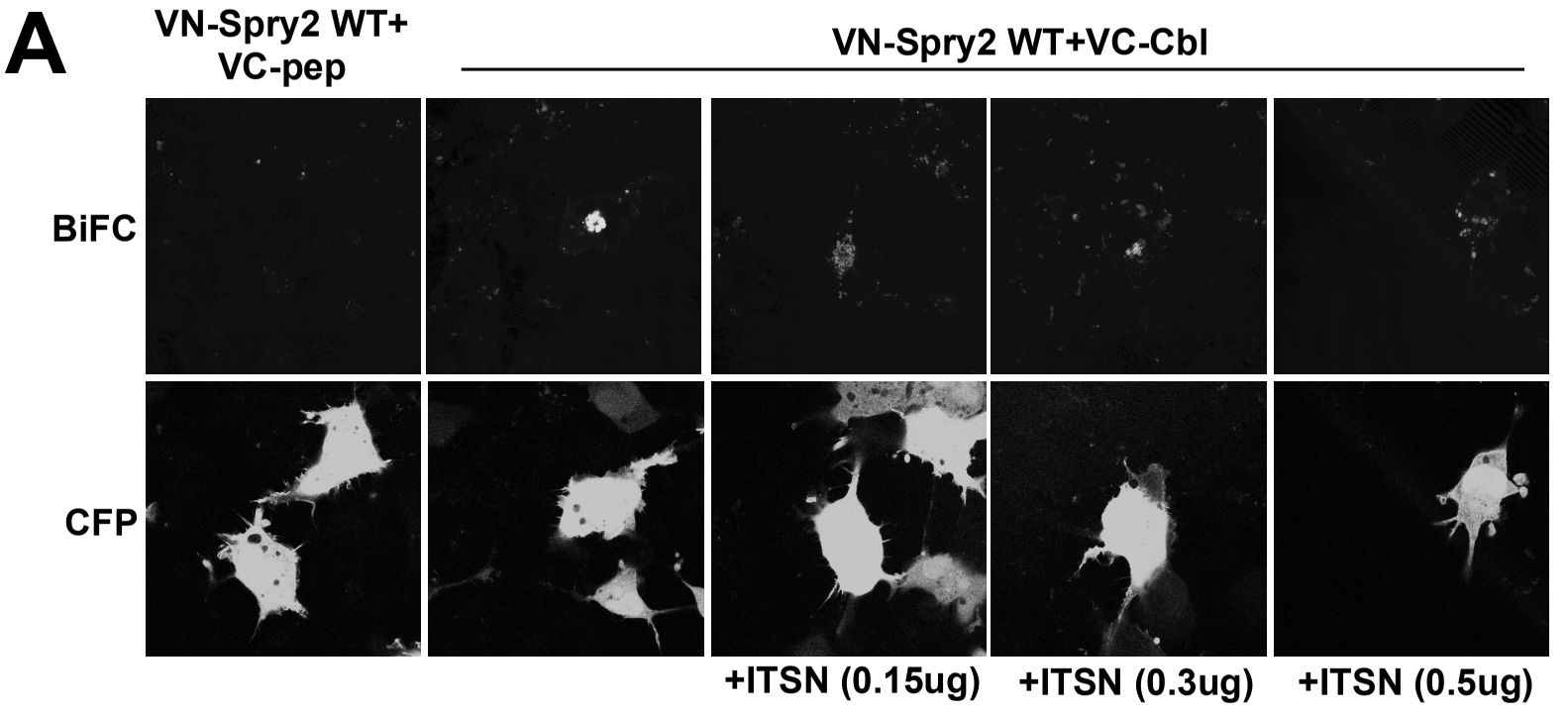


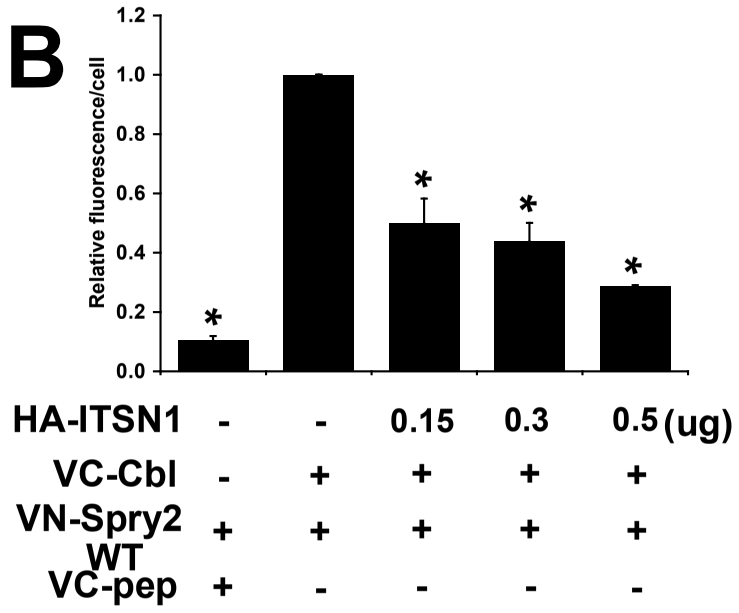


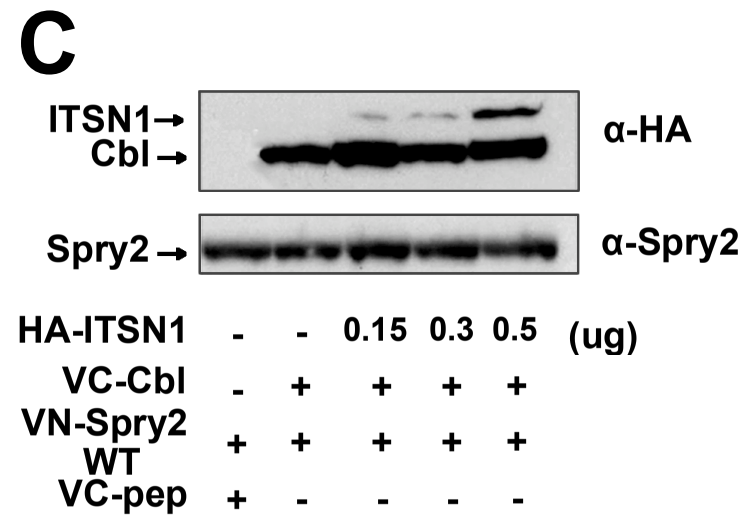
Okur, et. al., Figure 3

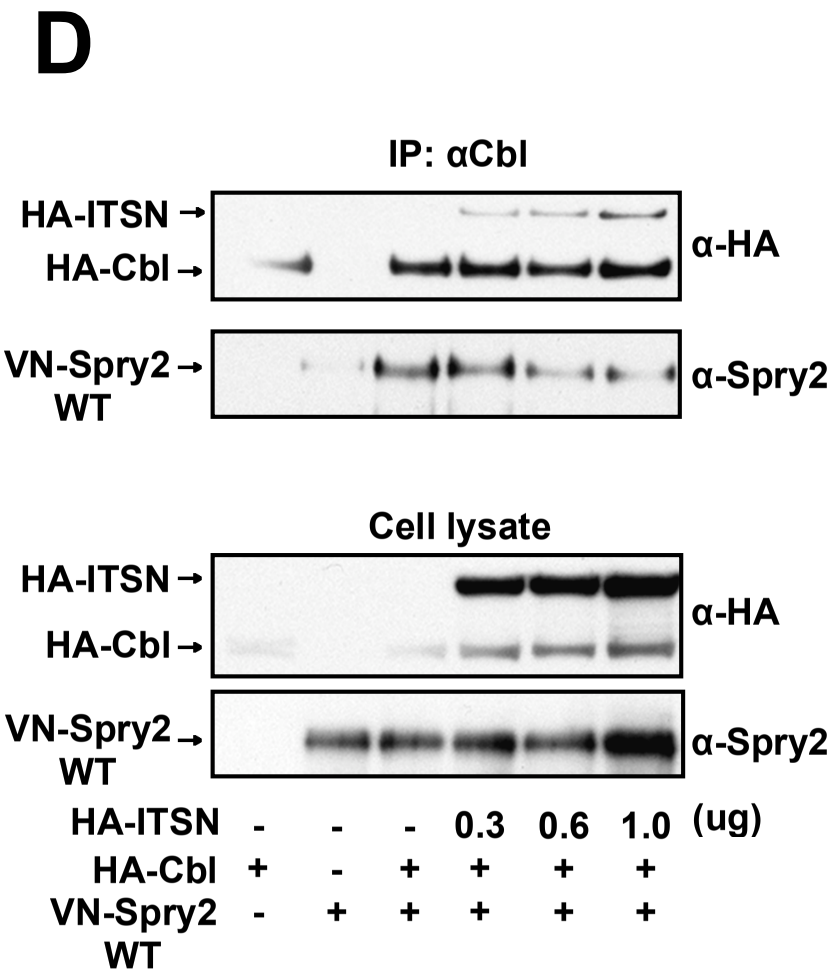

A
Full length
EH1
EH2
CC
SH3 A-E
ΔEH1
EH2
CC
SH3 A-E
ΔEH1+2
CC
SH3 A-E
ΔEH+CC
SH3 A-E
B
Spry2
Control
Full length
ΔEH1
ΔEH1+2
ΔEH+CC
Control
Full length
ΔEH1
ΔEH1+2
ΔEH+CC
IB:
α-FLAG IP
ITSN1 (α-Myc)
ITSN1 (α-Myc; 10x exp)
Spry2 (α-FLAG)
Cell lysate
ITSN1 (α-Myc)
Spry2 (α-FLAG)
C
Control peptide
Spry2 peptide
Control
Full length
ΔEH1
ΔEH1+2
ΔEH+CC
Control
Full length
ΔEH1
ΔEH1+2
ΔEH+CC
ITSN1 (α-Myc)
Streptavidin-Sepharose pull down
ITSN1 (α-Myc)
Cell lysate
Okur, et. al., Figure 4

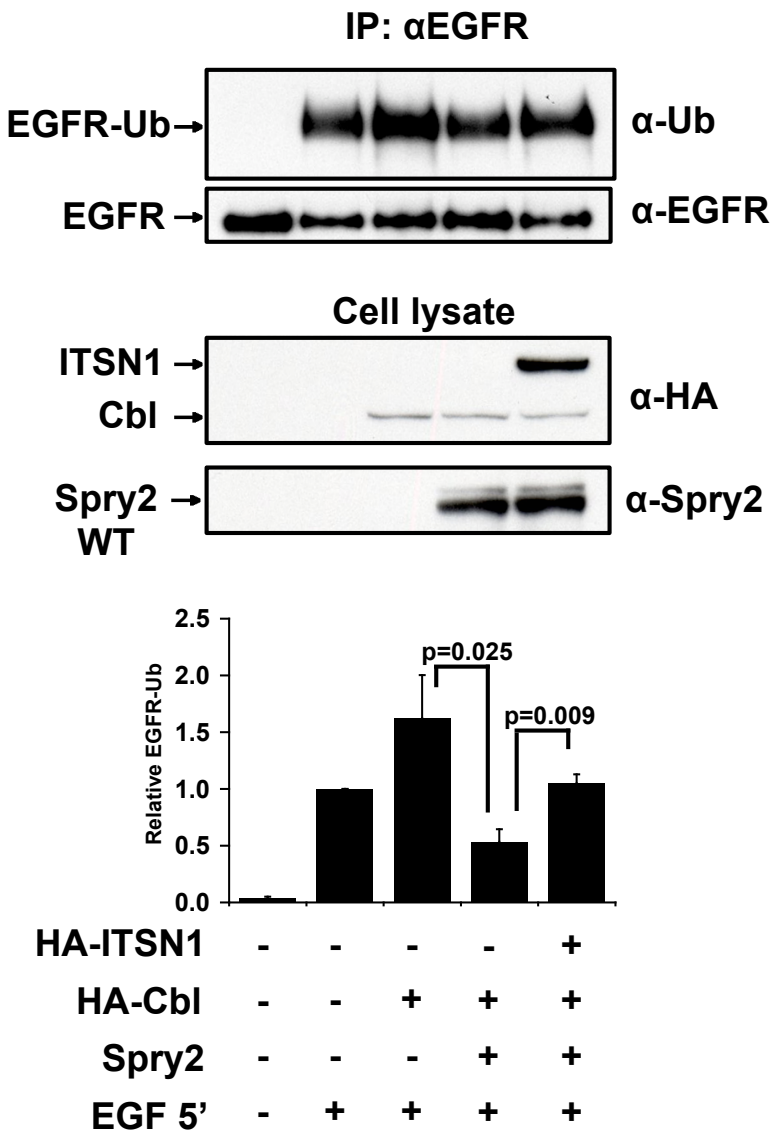
Okur, et. al., Figure 5





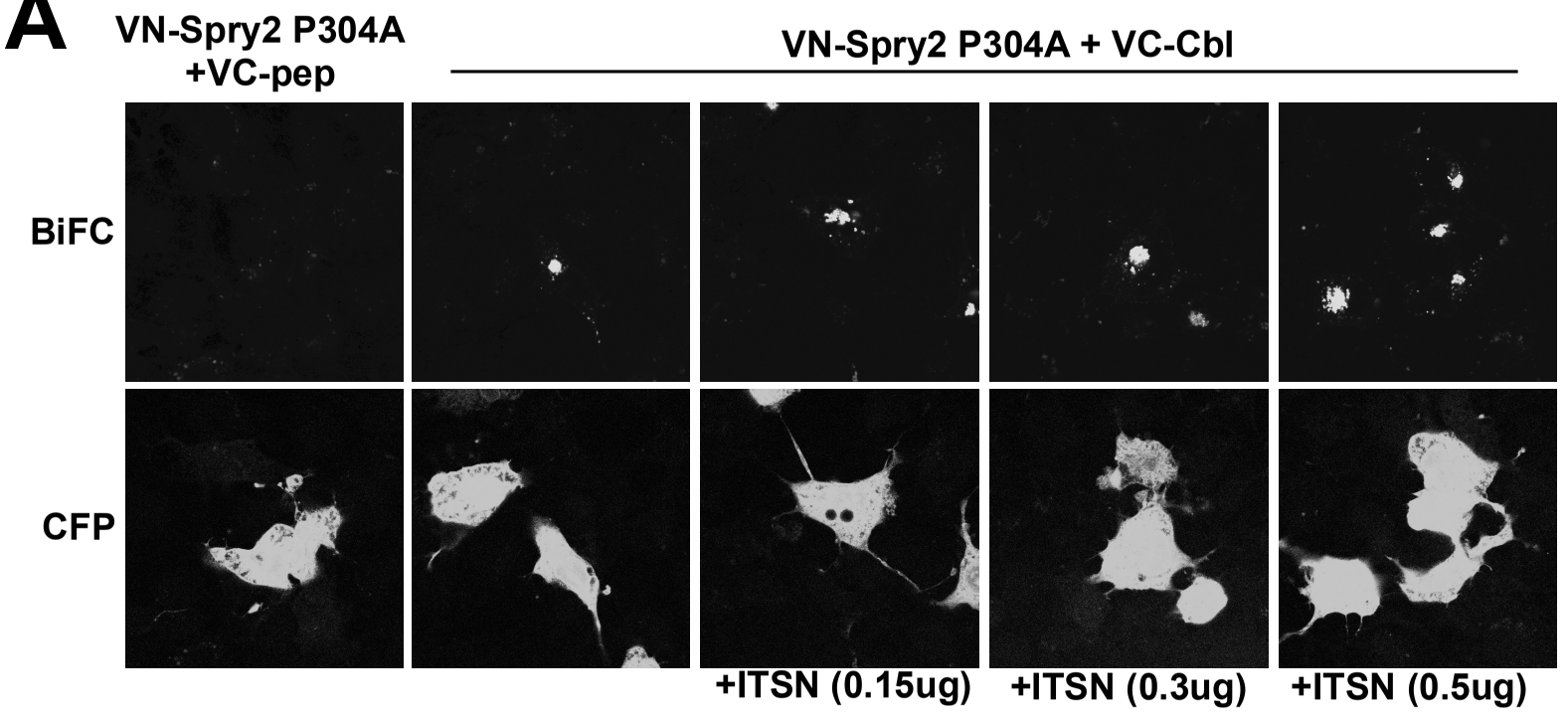


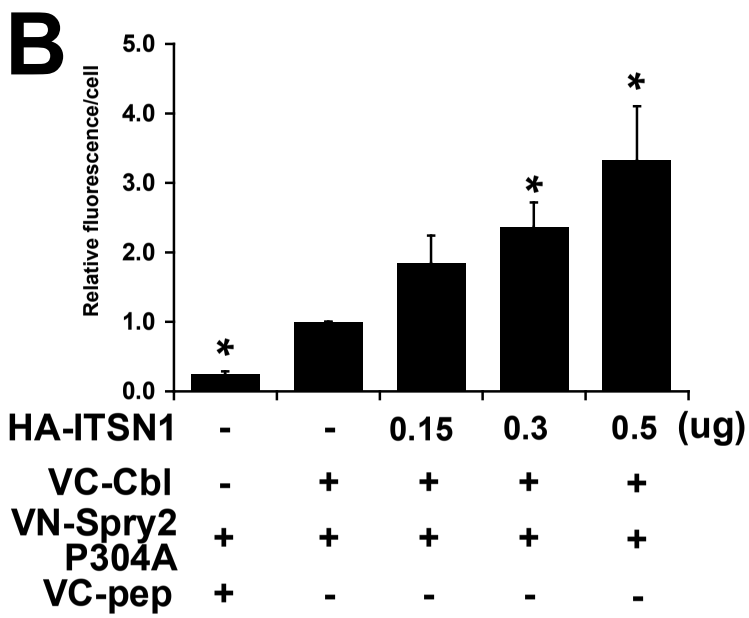


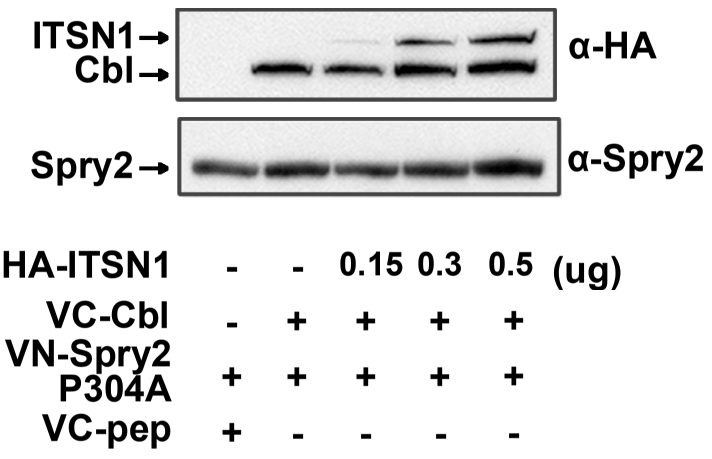


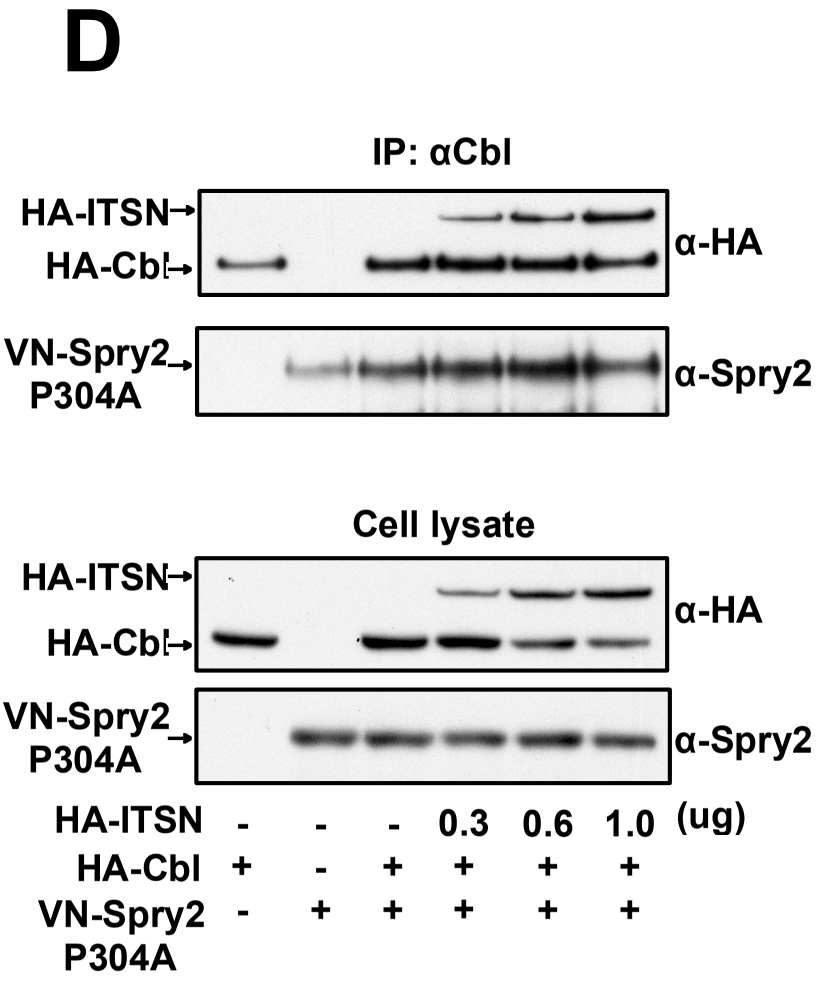
Okur, et. al., Figure 6

Okur, et. al., Figure 7









Okur, et. al., Figure 8