Intersectin (ITSN) Regulation of Epidermal Growth Factor Receptor (EGFR) Ubiquitylation

BY

MUSTAFA NAZIR OKUR

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THESIS

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Defense committee:

John P. O'Bryan, Advisor, Pharmacology
Nissim Hay, Biochemistry and Molecular Genetics
Karen J. Colley, Biochemistry and Molecular Genetics
Angela Tyner, Biochemistry and Molecular Genetics
Randal Skidgel, Pharmacology
Masuko Ushio-Fukai, Pharmacology

I am dedicating this thesis to my parents; Yasin Okur and Necla Muhup Okur.

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LIST OF ABBREVIATIONS

4H	Four-helix Bundle
AD	Alzheimer's Disease
C2	Ca^{2+} binding
CC	Coiled-coiled
CRD	Cysteine rich domain
COS-1	Monkey kidney cells
DH	Dbl homology
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant negative
DS	Down Syndrome
EF	A calcium-binding EF hand
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EH	Eps15 homology
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
HA	Hemagglutinin
HB-EGF	Heparin-binding EGF
HECT	Homologous to E6-associated protein C-terminus
HEK293T	Human kidney epithelial cells
ITSN	Intersectin
ITSN1-L	Intersectin1-Long
ITSN1-S	Intersectin1-Short
ITSN2-L	Intersectin2-Long
ITSN2-S	Intersectin2-Short
JMML	Sporadic juvenile myelomonocytic leukemia
JNK	c-Jun N-terminal kinase
L	A small linker domain
MAPK	Mitogen activated protein kinase
NB	Neuroblastoma
NSCLC	Non-small-cell lung cancer
p-Tyr	Phosphotyrosine
PDGFR	Platelet derived growth factor receptor
PH	Pleckstrin homology PI3K Class II alpha
PI3K	Phosphoinositide 3' kinase
Pro	Proline
PRD	Proline-rich domain
PTPs	Protein-tyrosine phosphatases
RF	A ring finger
RING	Really interesting new gene
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3

LIST OF ABBREVIATIONS (Continued)

Shps	SH2 domain containing phosphatases
SPRY	Sprouty
TGF-α	Transforming growth factor–α
TKB	Tyrosine kinase binding
UBA	Ubiquitin-associated
WNK	With-no-lysinekinases

SUMMARY

Ubiquitylation of receptor tyrosine kinases (RTKs) plays a critical role in regulating their trafficking and lysosomal degradation. Our laboratory 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 Cbl E3 ubiquitin ligase. However, the precise mechanism through which ITSN1 enhances Cbl activity is unclear. My dissertation work here revealed a novel interaction of ITSN1 with two proteins, Spry2 and Shp2, involved in this Cbl-mediated EGFR ubiquitylation mechanism. With this study, I discovered that ITSN1 recruits Shp2 to Spry2 to enhance Spry2 tyrosine dephosphorylation, thereby blocking Spry2 interaction with Cbl and Spry2 inhibiton of Cbl activity for EGFR ubiquitylation. In addition, I also found that disruption of ITSN1 binding to Spry2 through point mutation of the Pro-rich ITSN1 binding site in Spry2 resulted in decreased Shp2:Spry2 interaction and enhanced Spry2 tyrosine phosphorylation, probably due to increased Shp2 sequestration. Although I mostly analyzed the effect of forced expression of these proteins on the mechanism, results obtained from my work are mechanistically quite informative. This study demonstrates that ITSN1 enhances Cbl activity, in part, by modulating the interaction of Cbl with Spry2 through recruitment of Shp2 phosphatase to the Cbl-Spry2.

1.INTRODUCTION

1.1 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The ErbB family of proteins are RTKs involved in a wide variety of biological processes ranging from cell division to death, motility to adhesion. Four members (ErbB1, ErbB2, ErbB3 and ErbB4) of this family are all structurally-related transmembrane proteins and made up of an extracellular ligand-binding domain, a single hydrophobic transmembrane region and cytoplasmic tyrosine kinase domain. All ErbB receptors are monomeric but binding of its cognate ligand to the extracellular domain of the receptor results in homo- or hetero- dimerization of the ErbB family members in every possible combination. Unlike others, ErbB2 has no known ligand and it is minimally active in ligand binding. However, it still pairs with the other three receptors and it is considered the preferred heterodimerization partner of the ErbB members (Graus-Porta et al., 1997). Dimerization in turn leads to auto-phosphorylation and subsequent activation of the intrinsic tyrosine kinase domain, causing recruitment of downstream signaling proteins which initiate several signal transduction cascades regulating cellular proliferation, differentiation and survival.

EGFR also known as ErbB1/HER1 is one of the most studied and the first described member of ErbB family. It binds to multiple ligands including epidermal growth factor (EGF), transforming growth factor–α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin and epiregulin (Linggi and Carpenter, 2006). It is present on a wide variety of cells including epithelial, stromal and smooth muscle cells. Signaling from activated EGFR is involved in the regulation of several biological processes such

as mitogenesis, apoptosis, cell motility, and differentiation. However, elevated levels of EGFR results in intense signal generation and aberrant activation of downstream signaling pathways, causing cells to have more aggressive growth and tumorigenic characteristics. Indeed, EGFR is overexpressed in 40% to 80% of non-small-cell lung cancer (NSCLC) cases as well as other tumors types including breast cancer, renal cancer, ovarian and colon cancer (Fujino et al., 1996; Herbst and Shin, 2002).

Following growth factor stimulation, EGFR is internalized into early endosomes through clathrin-dependent and independent endocytosis. Once internalized, EGFR is either recycled from early endosomes to the plasma membrane or transported to late endosomes and lysosomes for degradation. One biochemical event having a role in deciding the destination of the receptor following endocytosis is ubiquitylation. Ubiquitylation is an event of labeling proteins with the covalent attachment of the small ubiquitin protein to lysine residues in the modified protein. It occurs in a three-step process involving different enzymes. First, ubiquitin is activated by an E1 ubiquitin-activating enzyme through formation of a thioester link between the C-terminal carboxyl group of ubiquitin and the sulfhydryl group of an E1 enzyme. ATP is used as energy source to catalyze this process. In the second step, ubiquitin is transferred from E1 enzyme to the cysteine site of an E2 ubiquitin-conjugating enzyme through trans-(thio)-esterification reaction. The final step requires the E3 ligase enzyme which recognize the substrate protein and catalyzes an isopeptide bond between the carboxyl group of the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue on a target protein. Compared to few E2s, there are possibly hundreds of E3s and they can

be divided into two large groups; HECT (homologous to E6-associated protein C-terminus) and RING (really interesting new gene) domain E3 ligases. HECT E3 ligases possess a conserved active site cysteine residue at the C-terminal of their HECT domain, which forms a thioester bond with ubiquitin and plays a direct catalytic role in the attachment of ubiquitin to substrate protein. However, unlike HECT, RING domain ligases promote ubiquitin attachment to substrate by bringing an E2 enzyme and a substrate into sufficiently close proximity so catalysis can occur.

During ubiquitylation, substrates are subjected to diverse types of ubiquitin modification including mono-ubiquitylation (addition of single ubiquitin to target); multiple mono-ubiquitylation (addition of single ubiquitin to multiple lysine residues); and polyubiquitylation (addition of multiple ubiquitins in a chain of diverse lengths to the target protein). The type of ubiquitin modification process leads to unique functional consequences for a target protein such as degradation, signal transduction, trafficking or transcription (Bergink and Jentsch, 2009; Haglund and Dikic, 2005; Hershko and Ciechanover, 1998; Raiborg and Stenmark, 2009). For example, polyubiquitylation targets modified proteins for proteasomal degradation whereas monoubiquitylation drives endocytosis of membrane-bound proteins (Hicke, 2001; Mosesson et al., 2003). Biochemical and genetic evidence indicate that EGFR is modified with multiple mono-ubiquitin or polyubiquitin chains, primarily linked through Lys63, regulating trafficking and sorting to ensure receptor endocytosis and degradation (Haglund et al., 2003; Huang et al., 2006; Mosesson et al., 2003). There are several E3 ubiquitin ligases that interact with EGFR and promote ubiquitylation of the receptor (de Melker et al., 2001;

Ray et al., 2011; Smith et al., 2013). The evolutionarily conserved Cbl protein is the first identified E3 ligase that is recruited to EGFR following cell stimulation and it catalyzes EGF receptor ubiquitylation (de Melker et al., 2001). However, regulation of this process is quite complex (Soubeyran et al., 2002). I and previous members of my laboratory discovered that Cbl-mediated EGFR ubiquitylation is regulated by several Cbl-binding proteins including Intersectin 1 (ITSN1), Spry2 and Shp2. In the following sections, I will provide an overview of the role of these proteins in regulation of EGFR ubiquitylation and control of cell signaling.

1.2 CBL E3 LIGASE

1.2.1 CBL ISOFORMS AND STRUCTURE

Cbl proteins are E3 ubiquitin ligases that ubiquitylate and target numerous proteins for degradation in the cell. They also function as adaptor proteins in the regulation of signal transduction pathways. Interestingly, the first family member of Cbl, v-Cbl was cloned from the Cas-NS-1 retrovirus and found to induce pre-B-cell lymphoma (Langdon et al., 1989). However, subsequent work revealed that this 355 amino acid oncogene was a truncated form of a larger 913 amino acid homologue known as c-Cbl, which did not seem to promote tumorigenesis (Blake et al., 1991). In addition to c-Cbl, mammals have two additional homologues called as Cbl-b and Cbl-3. *C. elegans* has one Cbl ortholog, called as SLI-1, whereas *Drosophila* possesses two isoforms, called short and long D-Cbl (Figure 1). All of these proteins including D-Cbl and SLI-1 have a highly conserved N-terminal region consisting of tyrosine kinase binding (TKB) domain,

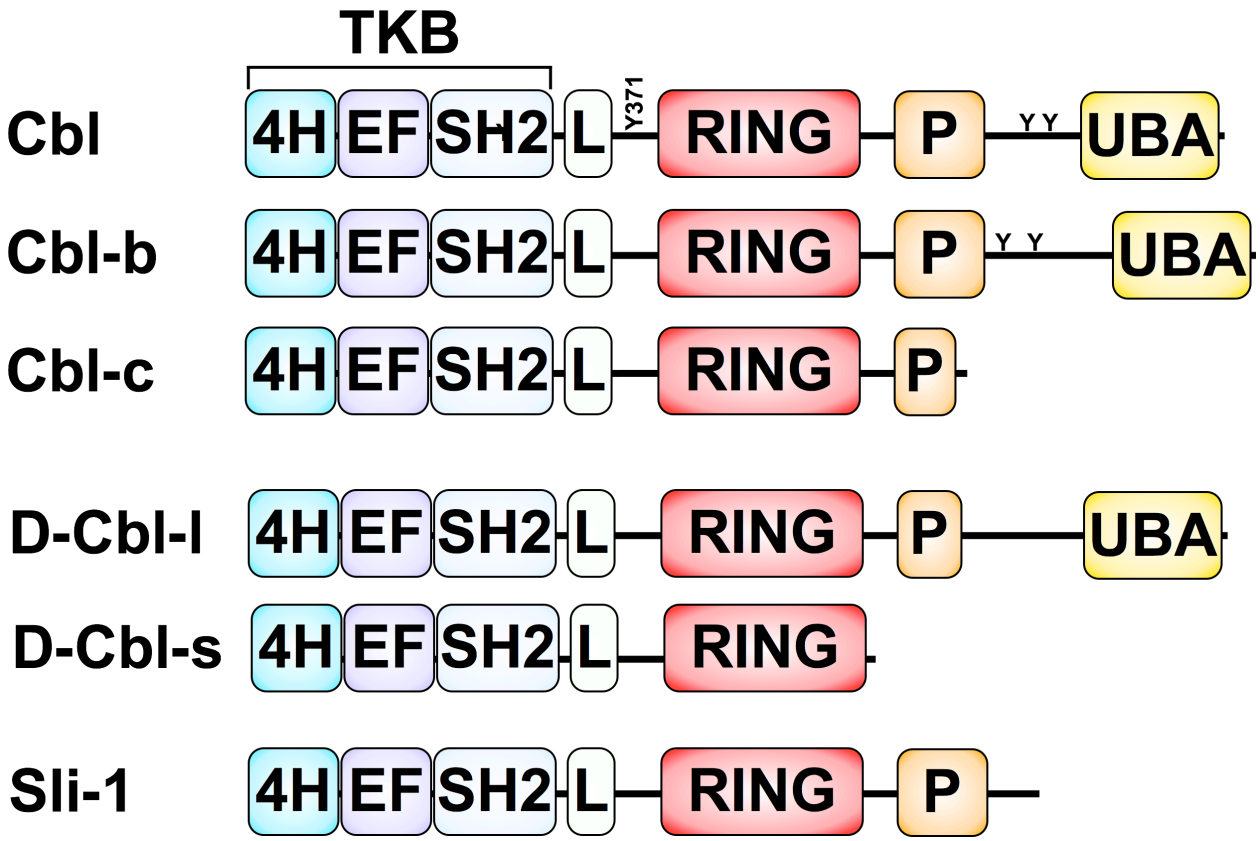

Figure 1. Cbl isoforms. A schematic view of domain structure of Cbl mammalian isoforms as well as Cbl orthologs in various species including *Drosophila* and *C.elegans.* In addition to Linker region (L) and Ring Finger domain (RING), all Cbl proteins contain a Tyrosine Kinases Binding (TKB) domain which is composed of a four-helix (4H) bundle, a calcium-binding EF hand (EF) and a variant Src homology 2 (SH2) domain. P stands for Pro-rich domain, Y stands for Tyrosine residue and UBA stands for Ubiquitin-associated domain. Phosphorylation of Tyr371 on c-Cbl leads to conformation change in Cbl and its activation.

a small linker domain (L) and a ring finger (RF) motif. The TKB domain is composed of four-helix (4H) bundle, a calcium-binding EF hand (EF) and a variant Src homology 2 (SH2) domain. All three domains in TKB region are required for recognition of specific phosphotyrosine residues on activated tyrosine kinases like EGFR and Zap-70 or other proteins like Spry2 (Meng et al., 1999). A short linker region connects TKB and RF domains and functions to regulate Cbl activity in a phosphorylation-dependent manner (Kassenbrock and Anderson, 2004). The RF domain interacts with E2s and mediates the E3 ubiquitin ligase activity of Cbl (Joazeiro et al., 1999; Levkowitz et al., 1999). This domain is also a site where Spry2 interacts and inhibits Cbl activity constitutively (Wong et al., 2001). Although C-terminal region of Cbl proteins is less conserved, all known Cbl proteins except D-Cbl-s contain Pro-rich motifs that are known to interact with SH3 domains of proteins. Furthermore, c-Cbl, Cbl-b and D-Cbl long isoforms additionally have conserved ubiquitin-associated (UBA) domain at the far end of their C-termini (Figure 1).

1.2.2 Cbl function

Cbl functions as a negative regulator of RTKs as initially shown by genetic studies in *C.elegans* (Jongeward et al., 1995). Following growth factor stimulation, activated EGF receptors are autophosphoylated at Tyr 1045 creating a docking site for Cbl binding through Cbl's TKB domain (Grovdal et al., 2004). Binding of Cbl to EGFR results in Cbl-mediated EGFR ubiquitylation. In addition, Fukazawa and colleagues demonstrated that Grb2 through its N-terminal SH3 domain binds to the Cbl Pro-rich region and recruits Cbl to EGFR suggesting that Cbl has multiple ways to interact with

and ubiquitylate EGFR. Once ubiquitylated, the EGFR attracts ubiquitin-binding proteins such as Epsin or Eps15 which are components of endocytic machinery, suggesting that ubiquitylation promotes receptor internalization. Indeed, Lee and colleagues reported that CSF-1 receptor internalization is delayed in c-Cbl-/- macrophages although it is not blocked completely (Lee et al., 1999). The requirement of ubiquitylation for receptor internalization is still a matter of controversy as Cbl overexpression or both c-Cbl and Cbl-b knock down by using RNA interference was shown to have no effect on EGFR internalization (Levkowitz et al., 1998; Pennock and Wang, 2008; Thien et al., 2001). In addition, Duan and collegues demontrated that Cbl-mediated EGFR ubiquitiylation is dispensible for receptor internalization in c-Cbl-/- mouse embryonic fibroblast cell lines although EGFR down-regulation is reduced (Duan et al., 2003). Interestingly, ubiquitylation still occurs following internalization. It is more commonly accepted that ubiquitylation marks receptors for degradation as well as preventing their recycling from early endosomes to the cell surface. Indeed, a recent report demonstrated that the hepatocyte growth factor-regulated tyrosine kinase substrate protein, Hrs sorts ubiquitylated membrane proteins into early endosomes, thereby preventing their recycling back to the cell surface, suggesting the role of ubiquitylation in trafficking (Hicke, 1999; Kolling and Hollenberg, 1994; Raiborg et al., 2002).

Due to their crucial role in cell signaling, Cbl functions are tightly regulated in the cell. In response to interaction with tyrosine kinases, Tyr 371 on c-Cbl is phosphorylated, resulting in a confirmation change in Cbl and its activation. Levkowitz and colleagues demonstrated that mutation at Tyr 371 resulted in loss of Cbl's ability to

enhance receptor ubiquitylation and degradation (Levkowitz et al., 1999). In addition to Tyr 371, there are 21 additional potential tyrosine phosphorylation sites in c-Cbl. Grossman and colleagues demonstrated that Syk, Fyn, and Abl kinases differentially phosphorylate c-Cbl at sites Tyr 700, Tyr 731 and Tyr 774, which, in turn alters the phosphotyrosine-dependent interactions of c-Cbl with other proteins (Grossmann et al., 2004). Cbl protein levels are also tightly regulated by various mechanisms. Several reports demonstrated that Nedd4 and Itch, both HECT domain containing E3 ligases, interact with Cbl, resulting in Cbl ubiquitylation and degradation (Lafont and Simons, 2001; Rotin et al., 2000). In addition, Cbl binding to a substrate such as Src, induces auto-ubiquitylation and subsequent degradation of Cbl, thereby providing a potential negative mechanism to prevent the activated Cbl from degrading other proteins (Bao et al., 2003; Yokouchi et al., 2001). Similar substrate-induced degradation of Cbl is also seen between EGFR and Cbl in a different mechanism. Following activated EGFR internalization, Cbl sustains its interaction with EGFR on endosomes and it is degraded at lysosomes along with its substrate. Furthermore, Feshchenko and colleagues showed that Sts-2 constitutively interacts with c-Cbl and its overexpression causes Cbl ubiquitylation and degradation (Feshchenko et al., 2004). In addition to its stability control, Cbl activity is also regulated through modulation of its TKB and Ring finger domains. A recent study reported that Cdc42, a small GTPase of the Rho family, interacts with Cbl and prevents it from binding to EGFR, resulting in decreased receptor ubiquitylation and degradation (Wu et al., 2003). Another protein that competitively inhibits Cbl for EGFR downregulation is Sprouty2 (Spry2), which is also phosphorylated in response to growth factor induction and competes with activated EGFR for Cbl

binding. Spry2 also competes with E2 enzymes for binding the ring finger domain of Cbl but this interaction is growth factor independent. My dissertation work revealed that ITSN1 disrupts Spry2 and Cbl interaction, thereby blocking Spry2 inhibition of Cbl. In the next section, I will give more detailed information about Spry2 function and Spry2 inhibition of Cbl.

1.3 SPROUTY (SPRY)

1.3.1 SPRY FAMILY MEMBERS

Spry genes were first discovered in *Drosophila* while screening for genes involved in shaping the developing trachea (Hacohen et al., 1998)**.** Subsequent work revealed dSpry as a widespread inhibitor of RTK/Ras/ERK pathway either at the level of Ras or at the level of Raf or MEK kinases (Casci et al., 1999; Reich et al., 1999). Unlike *Drosophila*, mammals have four Spry isoforms from different genes identified based on their similarities with *dSpry* (de Maximy et al., 1999; Hacohen et al., 1998). All Spry products have a conserved cysteine-rich domain (CRD), which is located at the C-termini and functions in the homo- or hetero- dimerization of Spry family members (Ozaki et al., 2005) (Figure 2) . Spry proteins, in unstimulated conditions, are distributed throughout the cytosol with the exception of Spry2, which is also localized to microtubules. Lim and colleagues reported that CRD of Spry promotes membrane localization of the protein upon growth factor stimulation (Lim et al., 2000; Lim et al., 2002). In addition, several studies reported that optimal Spry2 function requires the C-

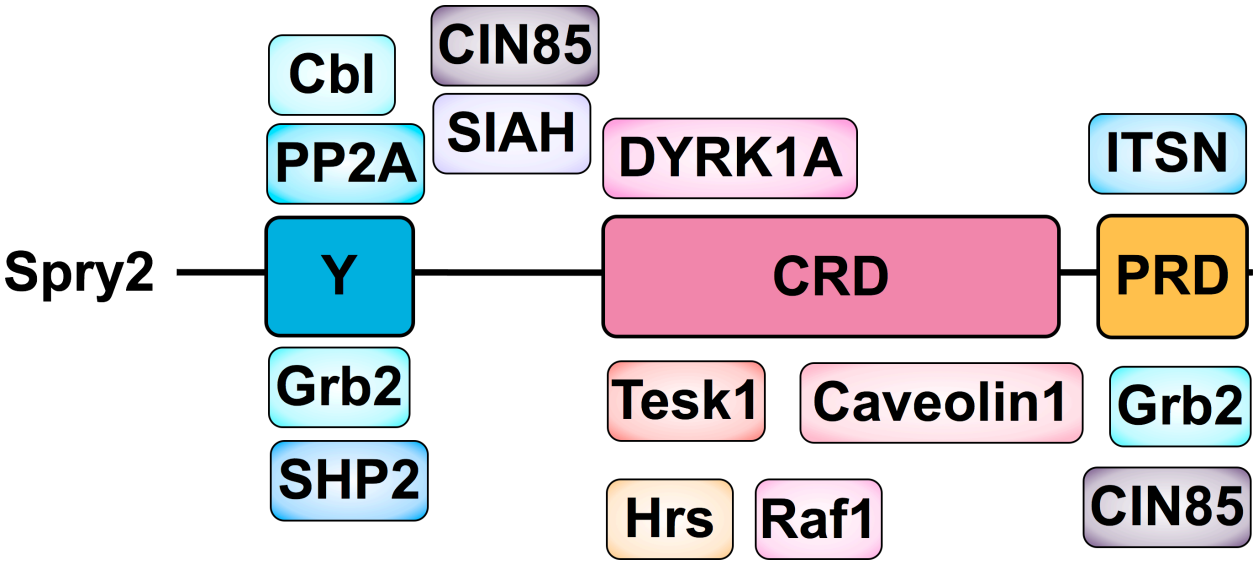

Figure 2. Spry2 domain structure and Spry2 binding proteins. A schematic view of Spry2 interaction with Grb2 (Gross et al., 2001), Shp2 (Hanafusa et al., 2004; Tefft et al., 2002), Cbl (Wong et al., 2001), PP2A (Lao et al., 2007), SIAH2 (Nadeau et al., 2007), Raf1 (Tefft et al., 2002), Tesk1 (Chandramouli et al., 2008), Caveolin1 (Cabrita et al., 2006), Hrs (Kim et al., 2007), ITSN1 (Okur et al., 2012), DYRK1A (Aranda et al., 2008), and CIN85 (Haglund et al., 2005) is shown in the figure. (Y) indicates tyrosine residue at N-terminus of Spry2 which is phosphorylated following growth factor stimulation. CRD indicates Cysteine-rich domain. PRD indicates Pro-rich domain which interacts with SH3 domain of other proteins.

terminus where CRD resides, as well as Spry2 recruitment to the membrane, suggesting a crucial regulatory role of CRD (Hanafusa et al., 2002; Wong et al., 2002). The CRD is also involved in Spry interaction with other proteins such as testicular protein kinase (TESK), dual-specificity tyrosine-phosphorylation-regulated protein kinase (DYRK) and Raf1 (Leeksma et al., 2002) (Figure 2). Compared to the C-terminus, the N-termini of Spry proteins are more variable but contain conserved short Serine-rich motifs as well as several isolated N-X-Y sequences. Several kinases and phosphatases target these motifs and conserved regions in N-terminus of Spry, playing a crucial role in function of the protein.

Although Spry genes are expressed ubiquitiously and present in many tissues including lung, brain, heart and kidney, Spry3 has more restricted distribution to the brain and the testis (Leeksma et al., 2002; Minowada et al., 1999). Among Spry family proteins, Spry2 is the most studied. It is a 35kDa, 315 amino acid polypeptide in humans and it shares highest homology with *dSpry*. Unlike other family members, Spry2 also has a Pro-rich domain, which enables it to interact with SH3-containing proteins such as CIN85, Grb2 and ITSN1 (Figure 2). During my work, I have focused on the Spry2 isoform and its functional interaction with ITSN1.

1.3.2 SPRY2 AND ITS FUNCTIONS

Similar to its ortholog *dSpry*, Spry2 is a regulator of RTK signaling pathways. However, Spry2's role in signaling seems to be more complicated in vertebrates. Several studies reported that Spry2 potentiates or has no effect on EGF-induced

ERK1/2 activation (Egan et al., 2002; Furthauer et al., 2001; Ozaki et al., 2005; Wong et al., 2002) although it still inhibits FGF, PDGF and VEGF induced MAPK signaling (Furthauer et al., 2001; Impagnatiello et al., 2001; Kajita et al., 2007; Ozaki et al., 2005; Yusoff et al., 2002), suggesting that Spry2 regulation of RTK signaling pathways is growth factor specific. However, It is still not clear how Spry2 discriminates between different growth factors. In addition to Grb2, Spry2 interacts with multiple components of the MAPK pathway including FRS2, Shp2, Raf1, Ras-GAP and Cbl. Post-translational modification of Spry2 has a crucial role in interaction of Spry2 with these proteins as well as its activation and function (Hanafusa et al., 2002; Sasaki et al., 2001; Tefft et al., 2002; Wong et al., 2001). Spry2 possesses a conserved N-terminal tyrosine (Tyr55 or Y55) residue, which undergoes phosphorylation by c-Src or a related kinase in response to growth factor stimulation (Li et al., 2004). Several studies reported that Tyr55 phosphorylation of Spry2 is required for its regulation of Ras/ERK/MAPK signaling cascade (Hanafusa et al., 2002). As mentioned before, one known consequence of Spry2 Tyr55 phosphorylation is that it binds to the TKB domain of c-Cbl E3 ubiquitin ligase and inhibits Cbl activity following growth factor stimulation of cells (Wong et al. 2001) (Figure 3). Binding of Cbl to phosphorylated Spry2 Tyr55 also results in ubiquitylation and subsequent degradation of Spry2 by the proteasome degradation pathway (Rubin et al., 2003). In addition to Cbl, the same region of Spry2 where Tyr55 resides is also a target of PP2A. Lao and colleagues reported that PP2A competes with Cbl to bind around amino acid 50-60 on Spry2 and dephosphorylate Spry2 at Ser115 and Ser118 following growth factor stimulation. They propose that Ser

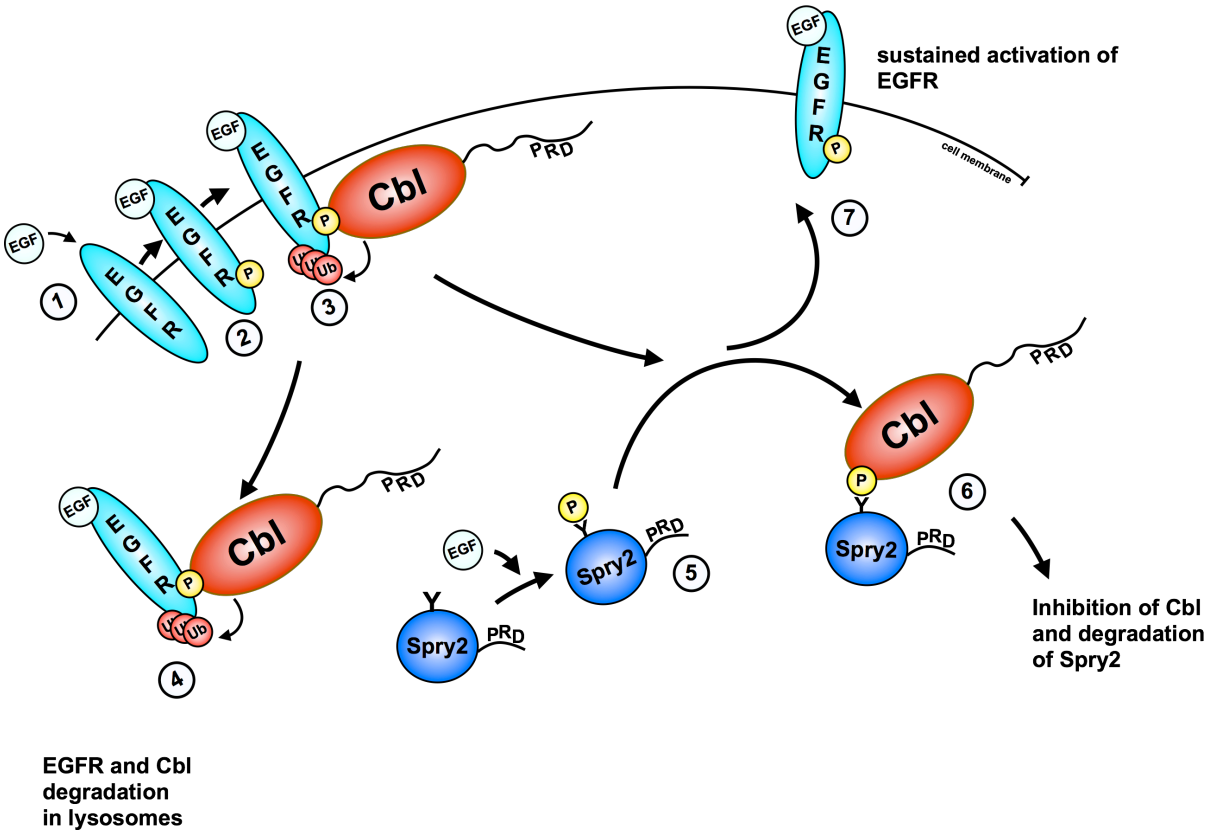

Figure 3. Spry2 inhibition of Cbl. EGF binds to extracellular domain of EGFR (1). Binding of EGF leads to autophosphorylation and activation of the receptor (2). Cbl interacts with phosphorylated receptor and mediates its ubiquitylation (3), resulting in EGFR internalization and degradation at lysosome along with Cbl. (4). EGF stimulation also leads to Spry2 phosphorylation (5). Then, modified Spry2 binds to Cbl (6) and acts as a positive regulator of signaling by preventing Cbl from continuously ubiquitylating EGFR, thereby leading to sustained activation of the receptor (7).

dephosphorylation likely cause a change in the tertiary structure of Spry2 enabling PXXPXR Pro-rich motif to be exposed for Grb2 binding which is crucial for the inhibitory action of Spry2 on the Ras/ERK pathway (Lao et al., 2007). In addition, DaSilva and colleagues demonstrated mitogen-activated protein kinase-interacting kinase 1 (Mnk1) enhances phosphorylation of Spry2 on Ser 112 and 121 which in turn antagonizes the phosphorylation of Spry2 on Tyr 55 and stabilizes the protein by interfering with proteasome pathway-mediated degradation (DaSilva et al., 2006). However, Mnk1 binding to Spry2 was not confirmed in this study. In contrast, Edwin and colleagues stated that Mnk2, not Mnk1, is the kinase responsible for phosphorylation of Ser 112 and 121 on Spry2 and this phosphorylation enhances Spry2 interaction with Nedd4, a HECT domain family E3 ubiquitin ligase, thereby causing Spry2 polyubiquitylation and degradation (Edwin et al., 2010). In addition to Cbl and Nedd4, another E3 ubiquitin ligase, human Seven-in-Absentia homolog-2 (SIAH2) also binds to Spry2, resulting in polyubiquitylation and degradation of Spry2 by the proteasome. However, SIAH2 and Spry2 interaction is constitutive rather than dependent on Spry2 tyrosine phosphorylation.

1.4 SHP2

1.4.1 SHP2 ISOFORMS, STRUCTURE AND FUNCTION

Src homology-2 (SH2) domain containing phosphatases (Shps) are members of the larger superfamily of protein-tyrosine phosphatases (PTPs) which possess a 240-250 amino acid ‘PTP domain’ with diverse regulatory sequences. They are highly conserved and found both in *Drosophila* and *C.elegans* as well as vertebrates. Although

Drosophila and *C.elegans* each have one Shp ortholog, Corkscrew and Ptp-2, respectively, there are two Shp isoforms, Shp1 and Shp2 in vertebrates. Shp2 is a ubiquitiously expressed protein tyrosine phosphatase. Although PTPs are generally considered to be tumor suppressors, several reports implicate Shp2 but not Shp1 as a proto-oncogene due to its promoting effect on Ras/MAPK pathway. Indeed, Tartaglia and colleagues demonstrated that patients with sporadic juvenile myelomonocytic leukemia (JMML) contain somatic gain of function mutations in the Shp2 coding gene, *PTP11*, with approximately 35% incidence rate, suggesting a role for Shp2 in tumorigenesis. Like Shp1, Shp2 is composed of two N-terminal SH2 (N-SH2 and C-SH2) domains, a PTP domain and a C-terminal tail (C-tail) with two tyrosine phosphorylation sites. In addition to those domains, Shp2 has an extra Pro-rich motif at the C-terminus, whereas Shp1 has a nuclear localization sequence (NLS). In the basal state, the close interactions of SH2 domains with PTP region keeps Shp2 in a closed conformation, suppressing its catalytic activity (Hof et al., 1998). However, upon stimulation of cells with growth factors or cytokines, binding of tyrosine phosphorylated ligands such as the insulin receptor substrate 1 (IRS-1) or the Grb2-associated binder 1 (Gab1) to SH2 domains of Shp2 releases the SH2 inhibition of PTP and switches the protein into open active-state conformation (Mohi and Neel, 2007; Neel et al., 2003). In addition, Lu and colleagues proposed an alternative model for Shp2 activation regulated by C-tail region of Shp2. By using non-hydrolyzable phosphotyrosine analogs at the sites of Tyr residues in Shp2, they suggested that Tyr-542 relieves basal inhibition of the PTPase by intramolecularly interacting with the N-terminal SH2 domain, whereas Tyr-580 phosphorylation interacts with the C-terminal SH2 domain and enhances

Shp2's PTPase activity. In addition, both Tyr-542 and Tyr-580 residues in Shp2 are contained in a canonical Grb2 SH2 binding sequence and are reported to interact with Grb2. Bennett and colleagues identified Tyr-542 as the major *in vivo* Shp2 tyrosine phosphorylation site and reported that tyrosine phosphorylated Shp2 binds and recruits Grb2 following PDGF stimulation, providing a mechanism for Ras activation by PDGFR (Bennett et al., 1994). On the other hand, Vogel and colleagues identified Tyr-580 as the major phosphorylation and Grb2-binding site, leading to EGF- or PDGF-mediated Ras activation (Vogel and Ullrich, 1996). Although these Shp2:Grb2 studies mentioned above implicate Shp2 as an adaptor protein in Ras/MAPK signaling, several reports demonstrated that Shp2 catalytic activity as a phosphatase is required for its regulation of Ras signaling, suggesting that Shp2 does not simply function as an adaptor protein (Cunnick et al., 2000; Deb et al., 1998).

One potential Shp2 substrate that is involved in Ras signalling is Gab1. Herbst and colleagues identified Dos, a Gab1 homolog, as a substrate of Csw, the SHP2 homolog in *Drosophila* through genetic and biochemical approaches (Herbst et al., 1996). A recent report by Montagner and colleagues suggested that Shp2 down-regulates interaction between Gab1 and RasGAP by dephosphorylating YXXP motifs on Gab1, thereby promoting efficient Ras activation in response to EGF stimulation (Montagner et al., 2005). In addition to Gab1, several studies identified both Spry1 and Spry2 isoforms as potential Shp2 substrates. Hanafusa and collegues demonstrated that Shp2 dephosphorylates Spry1/Spry2 in response to FGF both *in vivo* and *in vitro*, leading to Spry2 and Grb2 dissociation and reversing Spry1/Spry2 inhibition of MAP kinase

activation (Hanafusa et al., 2004). Jarvis and colleagues reported that Shp2 inactivation enhances Spry1 phosphorylation. Recently, Pan and colleagues proposed a model suggesting that Shp2 not only dephosphorylates Spry2 to inhibit its function but also activates Ras signaling to induce Spry transcription. My thesis work demonstrates that ITSN1 mediates Shp2 recruitment to Spry2, which in turn, enhances Spry2 dephoshporylation that leads to inhibition of Spry2 and thus enhancement of Cbl activity.

1.5 INTERSECTIN (ITSN)

1.5.1 ITSN GENES AND STRUCTURE

Intersectin (ITSN) is a highly conserved scaffolding protein with multiple domains. It is also known as Ese-1/2 in mouse (Sengar et al., 1999), EHSH-1/2 in rat (Okamoto et al., 1999) or Dap160 in *Drosophila* (Roos and Kelly, 1998). Mammals express two *ITSN* genes (*ITSN1* and *ITSN2*) that both have two major mRNA transcripts (Pucharcos et al., 2001; Tsyba et al., 2004) encoding short (ITSN-S) and long (ITSN-L) isoforms (Figure 4). There are also multiple minor splice variants encoding ITSN variants with altered binding affinity to several proteins (Okamoto et al., 1999; Pucharcos et al., 2001; Tsyba et al., 2004). ITSN1-S is ubiquitiously expressed throughout the body with the exception of neurons (Ma et al., 2003; Pucharcos et al., 2001) and it consists of two amino-terminal Eps15 homology domains (EH), a central coiled-coil domain (CC) and five-tandem Src homology 3 domains (SH3). EH domains, which interact Asp-Pro-Phe sequences, are present in several endocytic proteins (Santolini et al., 1999). CC

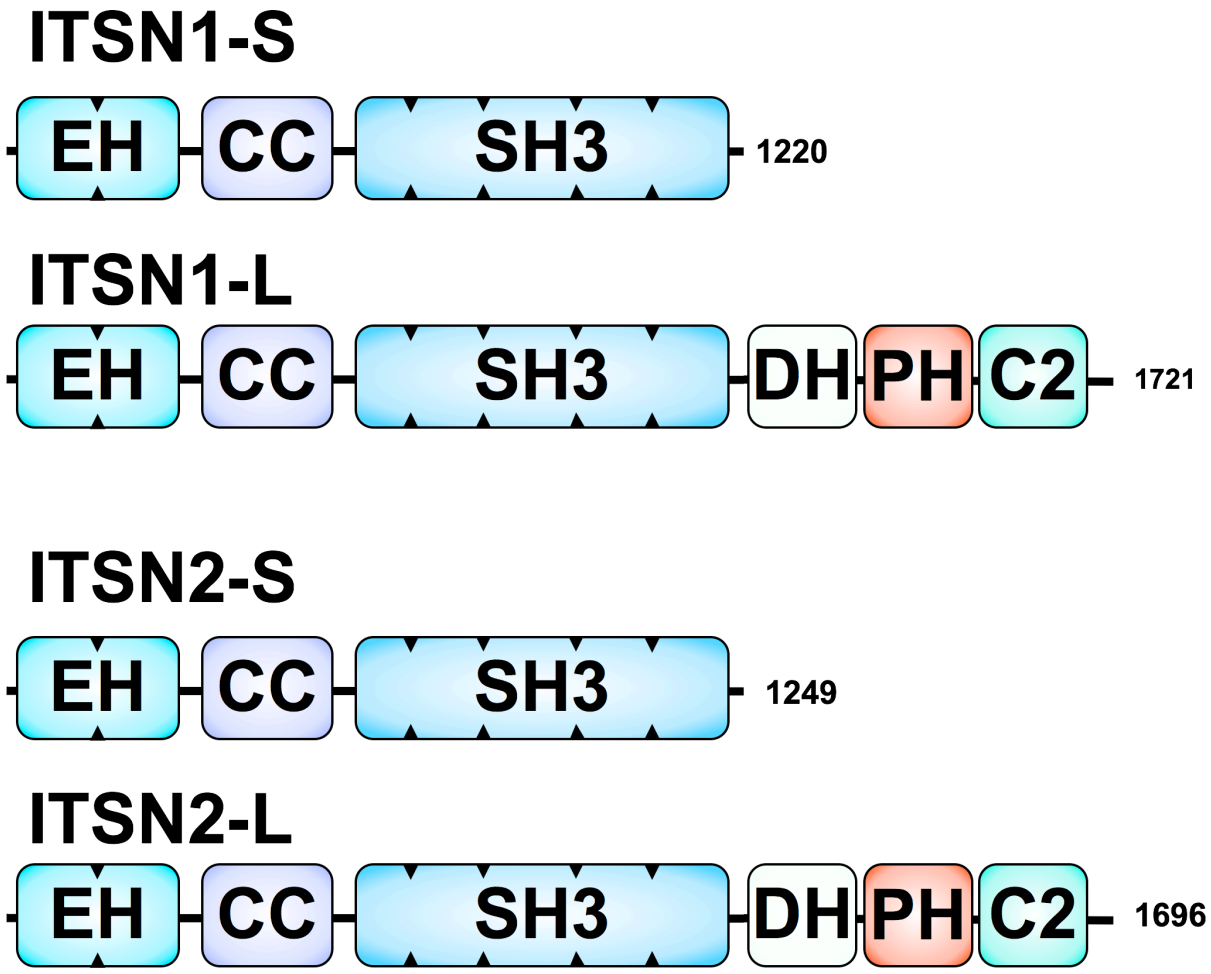

Figure 4. Schematic view of ITSN structure and mammalian isoforms. Each mammalian ITSN gene encodes two spliced products of short and long isoforms. Both ITSN1 and ITSN2 short isoforms are comprised of two Eps15 homology (EH) domains, a coiled-coil (CC) domain, and five Src homology 3 (SH3) domains. ITSN1 and ITSN2 long isoforms possess all these domains plus a C-terminal extension encoding DH, PH and C2 domain.

domains promote homo- or hetero-dimerization of ITSN with other CC-containing proteins (Lupas, 1996). Indeed, ITSN proteins have recently been shown to homo- and heterodimerize (Wong et al., 2012b). SH3 domains recognize Pro-rich sequences, which are usually found in proteins involved in cellular signaling (Mayer and Eck, 1995). In addition to these domains, ITSN-L isoforms also possesses a Dbl homology domain (DH), a pleckstin homology domain (PH) and a C2 domain. DH and PH domains in ITSN-L function in concert as a guanine nucleotide exchange factor (GEF) specifically for Cdc42 (Hussain et al., 2001; Zamanian and Kelly, 2003). ITSN-1 and ITSN-2 have similar structures with the same domains with the same order.

1.5.2 MULTIFUNCTIONAL ROLE OF ITSN

1.5.2.1 ITSN REGULATION OF ENDOCYTOSIS AND VESICLE TRAFFICKING

Possession of multiple domains enables ITSN proteins to interact with a plethora of proteins involved in several biological functions ranging from endocytic-exocytic membrane trafficking to cellular signaling. Initially, ITSN was thought to be an endocytic adaptor protein due to its localization on clathrin-coated pits via its EH domain (Hussain et al., 1999) and its interaction with multiple components of endocytic machinery such as Epsin 1 and 2, dynamin, Eps15, synaptojanin, secretory carrier membrane protein 1 (SCAMP1) and Stonin2. Recent studies confirmed the crucial role of ITSN in the process of endocytosis. Transient silencing of ITSN1 decreases EGFR uptake in human embryonic kidney cells (293T) and transferrin internalization in hippocampal neurons (Martin et al., 2006; Thomas et al., 2009). However, stable silencing of ITSN1 does not inhibit transferrin uptake in mouse neuroblastoma cells (Das et al., 2007). This could be

due to compensation by other ITSN isoforms or other endocytic proteins since ITSN is stably silenced in those cells or it could be cell-type specific differences. In addition, silencing ITSN in endothelial cells reduces caveolae-dependent endocytosis (Predescu et al., 2007; Predescu et al., 2003). Interestingly, ITSN overexpression is also found to inhibit endocytosis in multiple cell lines [reviewed in (O'Bryan, 2010; Tsyba et al., 2011)]. Its overexpression blocks clathrin-mediated endocytosis in COS cells as measured by transferrin receptor internalization (Thomas et al., 2009). However, this inhibition can be rescued by dynamin overexpression. Given that ITSN co-localizes with dynamin, which is well known to participate in endocytosis, ITSN might have role in dynamin recruitment to clathrin-mediated endocytosis (Roos and Kelly, 1998; Sengar et al., 1999). Similar effects of ITSN silencing and overexpression could be due to disruption of higher order protein complexes between ITSN and its binding partners, suggesting that regulation of ITSN concentration levels could have important consequences on biological functions in cell. In addition to endocytosis, the role of ITSN in vesicle trafficking should also be noted. In Drosophila, loss-of-function mutations in Dap160 resulted in aberrant enlargement of endocytic vesicles in addition to defects in vesicle recycling and fusion (Koh et al., 2004; Marie et al., 2004). Further, severe reduction in the levels of key endocytic proteins such as dynamin, synaptojanin, endophilin and AP180 were also observed in the site of synapses in Dap160 mutant flies (Koh et al., 2004; Marie et al., 2004). In mice lacking ITSN1, vesicle trafficking defects in neurons are also observed (Yu et al., 2008). Interaction of ITSN with several endocytic proteins and vesicle trafficking defects seen in ITSN null or mutant animals suggest that ITSN functions as a stabilizing scaffold protein in the process of endocytosis and trafficking by recruiting

endocytic proteins to the sites of membrane internalization and vesicles (Kay et al., 1999; Koh et al., 2004; Rose et al., 2007; Wang et al., 2008).

1.5.2.2 ITSN REGULATION OF RAS FAMILY GTPASES

Besides participating in endocytosis, ITSN also plays a crucial role in cell signaling pathways such as regulation of the Ras superfamily GTPases, stimulation of JNK-dependent signaling pathway and regulation of RTKs. Recent studies showed ITSN1 regulates activation of various Ras family GTPases including H-Ras, Cdc-42 and Rac1 [reviewed in (O'Bryan et al, 2010)]. Although overexpression of ITSN's SH3 domains inhibits growth factor mediated activation of Ras, full length ITSN1 overexpression results in H-Ras activation on perinuclear vesicles (Mohney et al., 2003; Tong et al., 2000a; Tong et al., 2000b). Interestingly, ITSN1 mediated H-Ras activation on vesicles does not seem to affect classical ERK or c-Jun N-terminal kinase (JNK) activation, suggesting that ITSN1-H-Ras stimulates a different signaling pathway (Mohney et al., 2003). Indeed, Wong and colleagues proposed a new mechanism involving the PI3K pathway (Wong et al., 2012a). Ras GTPases are guanosine nucleotide binding proteins that function as binary molecular switches to regulate cellular signaling pathways. They cycle between an "ON" and "OFF" form. In the "OFF" state, they are bound to GDP whereas in the "ON" state, they are bound to "GTP". Binding of guanine nucleotide exchange factors (GEFs) promotes the release of GDP and subsequent binding of GTP, causing activation of Ras. On the other hand, GTPase activating proteins (GAPs) enhance the intrinsic GTPase activity of Ras to hydrolyze its own bound GTP into GDP to inactivate Ras. Wong and colleagues discovered that nucleotide-free Ras

preferentially interacts with PI3KC2β, an isoform of the PI3 kinase family, and inhibits PI3KC2β's enzymatic activity. In addition, they proposed a model in which ITSN1 interaction with PI3KC2β results in dissociation of nucleotide-free Ras from PI3KC2β causing immediate GTP loading on Ras and its activation, thereby blocking Ras inhibition of PI3KC2β due to complex dissociation (Wong et al., 2012a). Furthermore, ITSN1-L isoform forms a complex with Cdc42 and functions as GEF specifically for this protein due to presence of a DH/PH module on ITSN1. In addition to activating the members of Ras family GTPases, ITSN is also shown to interact with regulators of this family of GTPases such as Sos, TRIO, cytohesin 1, TIAM1 as GEFs and CdGAP, the p85 subunit of PI3K and HRB/HRB-L (Jenna et al., 2002; Wong et al., 2012b) suggesting a general role for ITSN in Ras family GTPase pathways. Indeed, ITSN regulates Rac1 activation through its binding to and inhibition of the GTPase-activating protein CdGAP (Jenna et al., 2002). Additionally, Wong and colleagues reported that ITSN1-S interacts with Arf6 GTPase and its effector Arfaptin2 as well as Rab5 GTPase and its regulator Rabaptin-5, suggesting ITSN's involvement in the regulation of Arf6 and Rab5 GTPase pathways (Wong et al., 2012b).

1.5.2.3 ITSN REGUALTION OF RTKs

Growing evidence indicates a role for ITSN1 in the regulation of RTKs. One of the first links between ITSN1 and RTKs was described by Adams and colleagues (Adams et al., 2000). They demonstrated that ITSN1 cooperates with EGFR and synergistically enhanced Elk-1 transcriptional activation and transformation of cells. In addition, Martin and colleagues reported that ITSN1 interacts with c-Cbl and enhances Cbl-mediated

ubiquitylation of EGFR (Martin et al., 2006). They also demonstrated that transient ITSN1 silencing in HEK293T cells decreased EGFR internalization and ITSN1 overexpression enhanced EGFR trafficking and degradation at lysosomes, which could be due to enhanced receptor ubiquitylation since ubiquitylation is widely accepted as receptor marking for degradation in lysosomes following internalization (Haglund et al., 2003; Huang et al., 2006; Mosesson et al., 2003). Indeed, ITSN1 overexpression enhanced EGFR ubiquitylation in A431 cells and that activity of ITSN1 was dependent on Cbl E3 ligase (Martin et al., 2006). However, these results left unanswered the question of how the ITSN enhances Cbl activity for EGFR ubiquitylation.

1.5.3 ITSN AND DISEASES

ITSN is implicated in the pathogenesis of several diseases such as Down Syndrome, Alzeimer's Disease, Huntington Disease, Pseudohypoaldosteronism and cancer. (He et al., 2007; Hunter MP, 2011; Russo and O'Bryan, 2012; Scappini et al., 2007). Down Syndrome (DS) occurs when an individual has three copies of chromosomes 21 rather than two. This extra genetic material manifests itself numerous ways including delay in physical growth, mental retardation, heart defects, and increases risk of Alzheimer 's Disease (AD). One of the main focuses to cure this genetic disorder is to identify and understand the function of the genes on chromosome 21 that lead to the characteristics of DS. ITSN is one of the genes that is localized to the DS critical region and overexpressed in the brains of DS patients and in Ts65Dn, a mouse model for DS (Gardiner et al., 2003; Pucharcos et al., 1999). Among DS individuals, there is a common early incidence of Alzeimers's Diesase (AD) by their mid

40s, suggesting overexpression of genes due to trisomy 21 may be involved in AD pathology as well. Indeed, ITSN1 is reported to be one of the most highly induced transcripts in brains of AD patients (Blalock et al., 2004; Dunckley et al., 2006; Wilmot et al., 2008), suggesting that its overexpression in DS may contribute to the emergence of AD in these patients**.** Enlarged early endosomes are observed in both DS brains and sporadic AD suggesting the effect of endocytic and vesicle trafficking defects in both diseases (Cataldo et al., 2000). Interestingly, Yu and colleagues observed enlarged early endosomes and reduction in synaptic vesicle endocytosis in ITSN1 null brains (Yu et al., 2008).

ITSN is not only implicated in neurodegenerative diseases but also tumorigenesis. In addition to its regulation of compartmentalized activation of Ras proto-oncogene, ITSN activates the Elk-1 transcription factor and ITSN overexpression is sufficient to induce oncogenic transformation of rodent fibroblasts, suggesting that ITSN has a role in activation of mitogenic signaling pathways as well as tumorigenesis (Adams et al., 2000; Wong et al., 2012b). Indeed, Russo and colleagues reported that ITSN1-S is highly expressed in primary neuroblastoma (NB) tumors and cell lines. NB is a malignant tumor originating from neural crest. Although NBs express abundant ITSN1-S, neurons express little to no ITSN1-S (Russo and O'Bryan, 2012). Silencing ITSN1 in NB cells lines reduced anchorage-independent growth of these cells and tumor growth in a xenograft model, suggesting involvement of ITSN in tumorigenesis.

The main focus of my dissertation studies was to determine the functional role of ITSN in the regulation of RTK ubiquitylation. ITSN regulates multiple biochemical pathways as well as RTK signaling in the cell. Previous studies demonstrated that ITSN1 stimulates ubiquitylation of EGFR, through enhancing the activity of the Cbl E3 ubiquitin ligase (Martin et al., 2006). My studies revealed that the increase in Cbl activity is mediated by inhibiting the Cbl negative regulatory protein Spry2 (Okur et al., 2012). In next two chapters, I will demonstrate that ITSN1 recruits Shp2 to Spry2 and enhances Spry2 dephosphorylation, thereby disrupting Spry2 inhibition of Cbl activity and enhancing EGFR ubiquitylation.

2. INTERSECTIN 1 ENHANCES CBL UBIQUITYLATION OF EPIDERMAL GROWTH FACTOR RECEPTOR THROUGH REGUALTION OF SPROUTY2-CBL INTERACTION

(Portions of this chapter have been previously published in Molecular and Cellular Biology 2012, 32 (4):817. DOI: 10.1128/MCB.05647-11.)

2.1 INTRODUCTION

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 posttranslational modification targets the activated receptors for lysosomal degradation (Marmor and Yarden, 2004). 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 (Schmidt and Dikic, 2005). Although binding of Cbl to activated RTKs represents an important step in regulation of RTK ubiquitylation, Cbl activity is modulated by both posttranslational modifications as well as interactions with numerous proteins (Schmidt and Dikic, 2005). One such protein is the intersectin 1 (ITSN1) scaffold protein. Although initially identified as a regulator of clathrin-dependent endocytosis, ITSN1 regulates a number of additional biochemical pathways (O'Bryan, 2010). Recently, we demonstrated that ITSN1 enhances Cbl-dependent ubiquitylation of the epidermal growth factor receptor (EGFR), leading to enhanced degradation of the

activated receptor (Martin et al., 2006). 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 defined a novel role for ITSN1 in attenuating Cbl inhibition by Spry2, a negative regulator of Cbl (Guy et al., 2003; Kim and Bar-Sagi, 2004). Our results demonstrate that ITSN1 binds both Cbl and Spry2 and that ITSN1 releases Cbl from Spry2 inhibition, leading to enhanced EGFR ubiquitylation.

2.2 MATERIAL AND METHODS

2.2.1 CELL LINES AND REAGENTS

HEK293T human kidney epithelial cells and COS-1 monkey kidney cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Human IMR-5 neuroblastoma cells were grown in RPMI medium supplemented with 10% fetal bovine serum. All cells were grown at 37°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 (Santa Cruz), EGFR AB12 and EGFR AB13 antibodies (Thermo Scientific), and monoclonal antihemagglutinin (HA) antibody (Covance).

2.2.2 DNA CONSTRUCTS AND TRANSFECTION

An amino-terminal HA epitope-tagged full-length ITSN1 (mouse) in pCGN construct was previously described (Mohney et al., 2003). HA-tagged wild-type (WT) human c-Cbl was a gift from Yosef Yarden (Weizmann Institute of Science, Rehovot,

Israel) and was described previously (Levkowitz et al., 1999). The pHM6-HA-Spry2 and its empty vector, pHM6-HA, were kindly provided by Tarun Patel (Loyola University, Chicago, IL) and were described previously (Yigzaw et al., 2001). COS-1 cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) according to the protocol provided by manufacturer. Glutathione *S*-transferase (GST)-tagged SH3 domains of ITSN were created by subcloning the individual SH3 domains into the mammalian expression vector pEFG (Oldham et al., 2002). The Spry2 mutants containing single-amino-acid mutations (Y55F, P59A, P65A, P69A, P71A, P73A, P304A, and P308A) were generated from the plasmid pCEFL-KZ-AU5-Spry2 WT (de Alvaro et al., 2005; Martinez et al., 2007) 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 wild-type (WT), Y55F, P59A, and P308A fragments were subcloned into pHA-VC155, kindly provided by 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 (Lao et al., 2006). Spry2N and Spry2C were also previously described (Chow et al., 2009). Various truncation mutants of the short isoform of ITSN1 were generated using the reverse primer 5′CGGGGTACCCCGAGATGCAGGTCTGAGCACC3′ and the following forward primers: ΔEH1, 5′ATAAGAATGCGGCCGCTGTCATGAAACAGGCAACCAGTG3′; ΔEH1+EH2, 5′ATAAGAATGCGGCCGCTCAGCCACTGCCGCCCGTC3′; and ΔEH1+EH2+CC, 5′ATAAGAATGCGGCCGCTCATCAGGAGCCAGCTAAGCTG3′.

The N-terminal truncation mutants were cloned into pXJ40-Myc using NotI and KpnI sites.

2.2.3 IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Whole-cell extracts were prepared as described previously (Oldham et al., 2002). 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 (Martin et al., 2006). For detection of Spry2, EGFR, and HA-tagged proteins, standard protocols suggested by the manufacturers were used.

2.2.4 GST PULLDOWN ASSAYS

Samples were lysed in a Tris-based buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and a cocktail of protease inhibitors) and centrifuged at 13,200 rpm for 15 min at 4°C. Fifteen microliters of glutathione Sepharose4B beads (Amersham Biosciences, Buckinghamshire, United Kingdom) was added to the supernatant to precipitate the GST epitope. The resulting immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2.2.5 YEAST TWO-HYBRID SCREENING

Yeast two-hybrid screening analysis was performed through a contract with Myriad Genetics essentially as described previously (Das et al., 2007; von Schwedler et al., 2003) except that the various individual domains of mouse or human ITSN1 were

used as bait. Multiple mouse and human Spry2 clones were identified as binding to the first SH3 domain of ITSN1.

2.2.6 PEPTIDE SCREENING OF SH3 DOMAIN BLOTS

SH3 domain blots were obtained from Panomics, Inc. (Redwood City, CA). Biotinylated peptides (1 μg/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 phosphate-buffered saline (PBS) containing 0.01% Tween 20 (PBST) three times for 15 min each time and then incubated with streptavidin-conjugated horseradish peroxidase (1:100,000 in PBST). After three 15-min washes with PBST, an enhanced chemiluminescent system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) was used to detect bound peptides.

2.2.7 BIMOLECULAR FLOURESCENCE COMPLEMENTATION (BIFC)

BiFC was performed essentially as described previously (Wong and O'Bryan, 2011). Briefly, COS-1 cells were seeded on glass-bottom plates and in 6-well dishes and transfected with 0.5 μg of plasmids encoding proteins fused to pHA-VC155N and pFLAG-VN173N. At 24 h posttransfection, the glass bottom dishes were fixed on ice in 3.7% formaldehyde for 20 min, rinsed twice with PBS, and stored in PBS at 4°C in the dark. A Zeiss LSM 510 META confocal microscope was used to image samples. Cyan fluorescent protein (CFP)-positive cells were selected and imaged for BiFC signal in the yellow fluorescent protein channel. BiFC was quantified and expressed as average

fluorescence intensity per pixel using ImageJ, available from the National Institutes of Health (NIH) as described previously (Wong and O'Bryan, 2011). In parallel with imaging, cells in 6-well dishes were lysed and expression levels of transfected proteins were determined by Western blot analysis.

2.3 RESULTS

2.3.1 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., 2012b). The SH3A domain of ITSN1 (amino acids [aa] 730 to 816) isolated both human and mouse Spry2 clones as targets. The COOH terminus of Spry2 (Egan et al., 2002; Furthauer et al., 2001; Ozaki et al., 2005; Wong et al., 2002) contains a consensus Pro-rich sequence (PTVPPRN) resembling the ligand for ITSN1's first SH3 domain, SH3A (Das et al., 2007; Martin et al., 2006). 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*.

2.3.2 ITSN1 AND SPRY2 INTERACT IN CELLS.

Immunocytochemical staining of cells reveals that a portion of endogenous ITSN1 and Spry2 colocalizes in cells (Figure 5A). Although we were unable to coprecipitate endogenous ITSN1 and Spry2 from cells, possibly because the antibodies

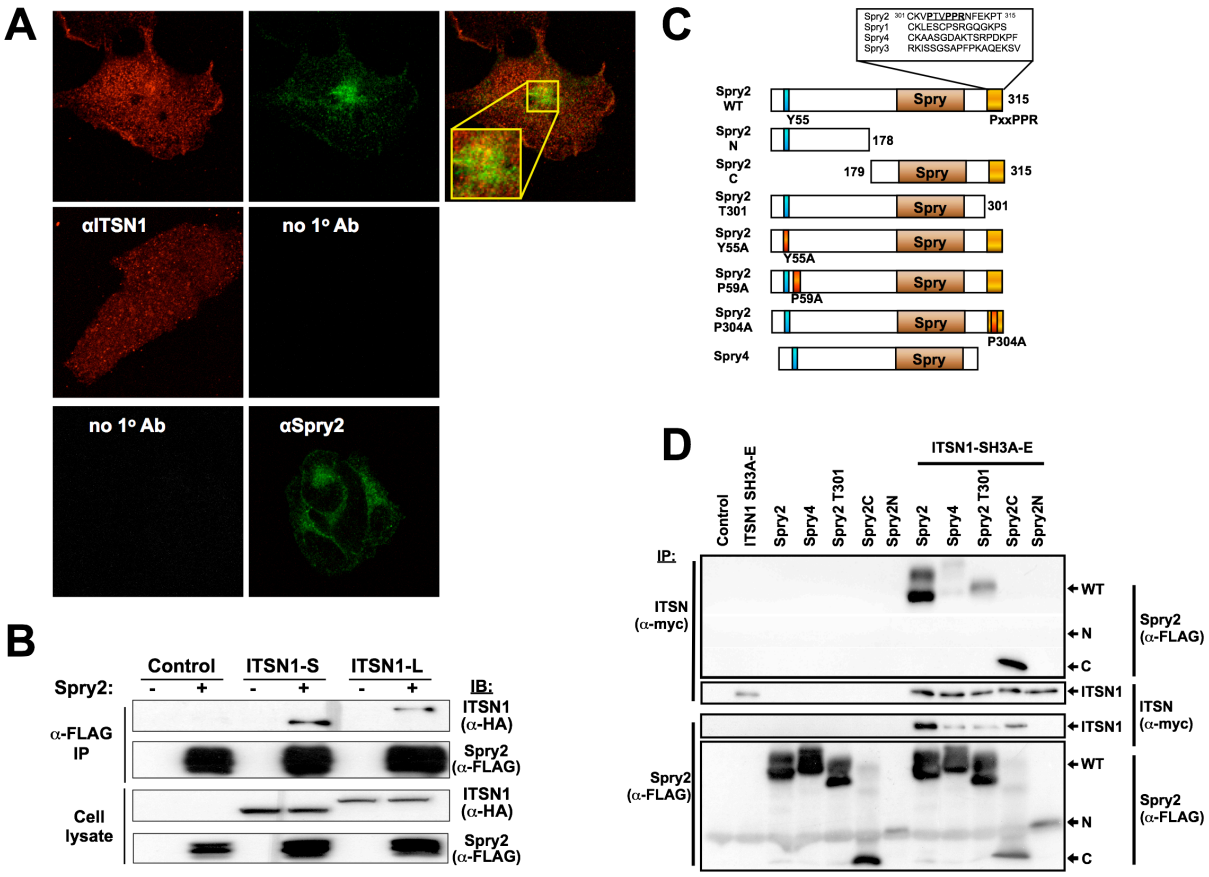

Figure 5. Endogenous ITSN1 binds Spry2. (A) Endogenous ITSN1 (red) colocalizes with endogenous Spry2 (green) in IMR-5 neuroblastoma cells (top row). As controls (middle and bottom rows), cells were stained with both fluorescently labeled secondary antibodies (Cy5-labeled donkey anti-rabbit and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse) but only a single primary antibody, as indicated in each panel. (B) Spry2 binds both ITSN1 isoforms. FLAG-tagged Spry2 was coexpressed 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 above 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. N, Spry2N; C, Spry2C. Experiments in Fig 5B, C, D were performed by laboratory of Dr. Graeme Guy, Institute of Molecular and Cell Biology, Singapore, Singapore.

target epitopes in the regions of interaction between the two proteins, we analyzed the interaction of epitope-tagged versions of the two proteins (Figure 5B). Using HA-epitope tagged versions of the major ITSN1 isoforms (O'Bryan, 2010), 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 (Figure 5B). Spry2 is a member of the Spry family of proteins, which consists of Spry1 to Spry4 (Kim and Bar-Sagi, 2004). To determine the specificity of ITSN1 for specific Spry members, we coexpressed ITSN1 with different Spry isoforms (Figure 5C and D). ITSN1 specifically interacted with full-length Spry2, and this binding was diminished by deletion of the COOH-terminal Pro-rich tail in the Spry2 T301 truncation mutant. Spry4, which lacks a comparable Pro-rich sequence, did not interact with ITSN1 (Figure 5C and D).

Using truncation mutants of ITSN1, we observed that ITSN1's SH3 domains mediated Spry2 binding (see Figure 8). Given the presence of five SH3 domains (domains A to E) 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 (Oldham et al., 2002) as described in Materials and Methods. These SH3 constructs were cotransfected 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 domains specifically interacted with full-length Spry2 (Figure 6A). Although Spry2 contains two Pro-rich stretches (aa 59-

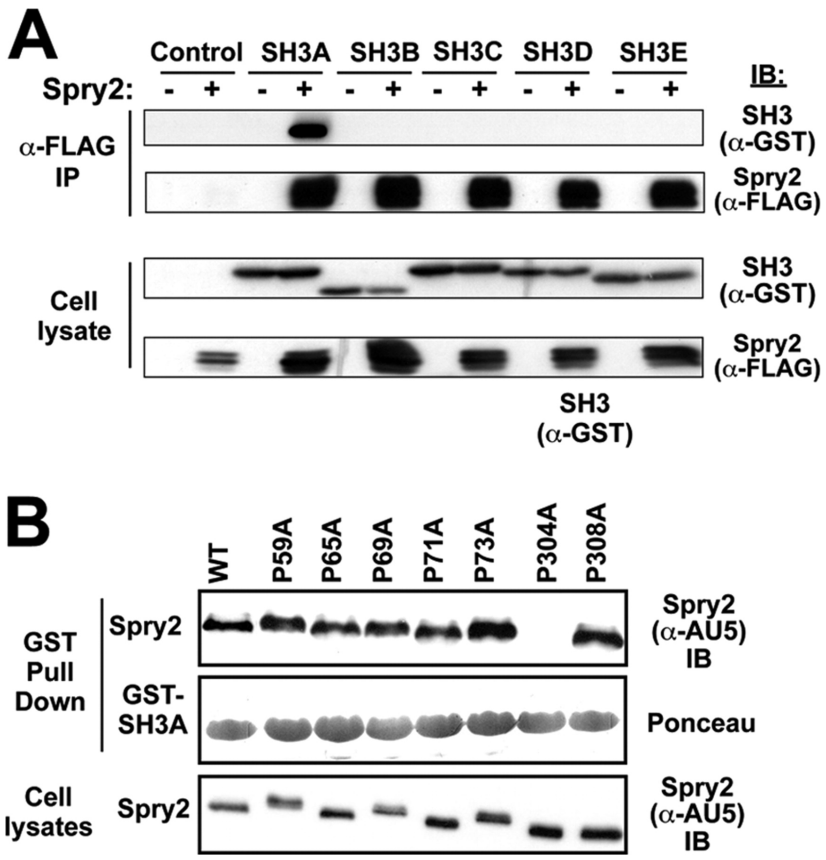

Figure 6. Mapping Spry2-ITSN1 interactions. (A) GST-tagged constructs of the individual SH3 domains of ITSN1 were coexpressed in HEK293T cells along with FLAG-Spry2. Only the SH3A domain of ITSN1 coprecipitates with Spry2. Control (GST alone) is not visible in the cell lysate blot due to its smaller 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 coexpressed 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 or no effect. Experiments in Fig. 6 A&B were performed by laboratory of Dr. Jose M. Rojas, Unidad de Biologia Celular, Área de Biología Celular y del Desarrollo, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain.

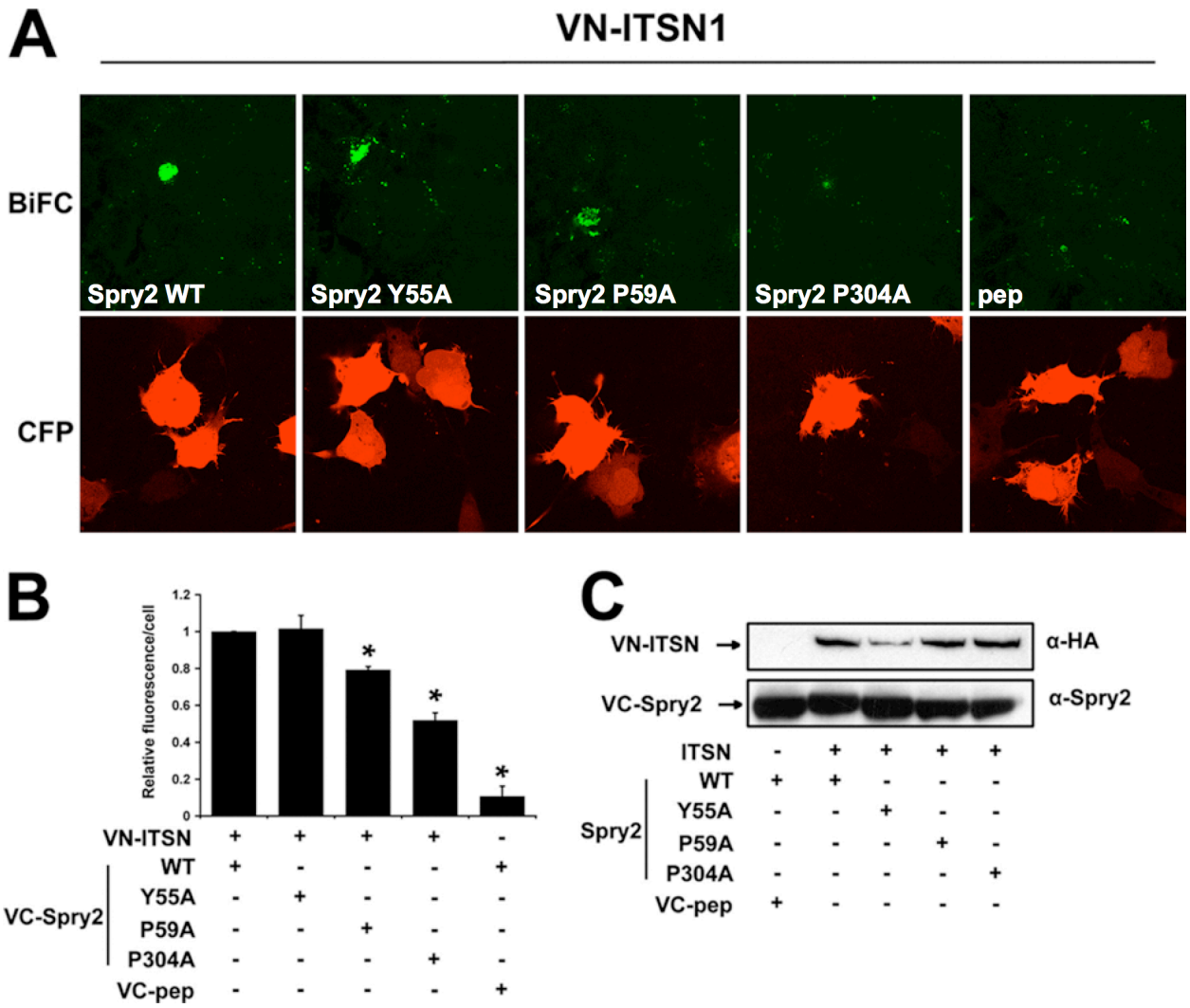

Figure 7. Interaction of Spry2 and ITSN1 by BiFC. (A) VC-tagged Spry2 WT or various Spry2 mutants were coexpressed with VN-ITSN1 in COS cells. CFP was included at one-fifth the amount of DNA as a transfection control. pep, a nonspecific peptide control fused to VC. (B) Interaction of Spry2 and ITSN1 was quantified as described previously (36). WT, Y55A, and P59A Spry2 proteins interacted with VN-ITSN1, whereas P304A Spry2 was impaired in the interaction. Experiments were performed in duplicate. Data are average fluorescence intensities per cell; error bars show standard errors of the means (SEM). Asterisks indicate that the values for these Spry2 mutants were significantly different from that for wild-type Spry2 ($P < 0.05$). (C) A Western blot of lysates from the BiFC experiments demonstrates equivalent expression of the tagged proteins.

PTVVPRP-65 and aa 304-PTVPPRN-310), only mutation of Pro304 to Ala (P304A) in the COOH-terminal Pro-rich sequence disrupted binding of ITSN1 (Figure 6B).

To examine the interaction of Spry2 and ITSN1 in whole cells, we utilized bimolecular fluorescence complementation (BiFC) (Figure 7). As seen with the individual SH3A domain of ITSN1, Spry2 interaction with full-length ITSN1 was disrupted by the P304A mutation but not by the Y55A mutation (Figure 7A and B). Mutation of P59A resulted in slight but significant reduction in ITSN1 interaction. These differences in BiFC signal were not due to differences in expression of the various Spry2 mutants and thus likely reflect true differences in the affinity of ITSN1 for these mutants (Figure 7C). These findings demonstrate that ITSN1 specifically interacts with the COOH-terminal Pro-rich sequence in Spry2.

2.3.3 SH3 BINDING TO TARGETS IS NEGATIVELY REGULATED BY ITSN1'S EH AND CC DOMAINS.

During the course of our investigations, we observed that Spry2 interacted better with the isolated SH3A domain than with full-length ITSN1 (data not shown). One possible explanation for these results is that the regions NH_2-terminal to the SH3 domains, i.e., the EH and CC domains, may sterically hinder SH3 binding to targets such as Spry2. To test this possibility, we created a series of ITSN1 mutants with NH_2-terminal truncations which were tested for interaction with Spry2 (Figure 8A). Myc-tagged full-length or truncated ITSN1 proteins were coexpressed with Spry2 in HEK293T cells. Immunoprecipitation of Spry2 revealed increased binding to ITSN1 with

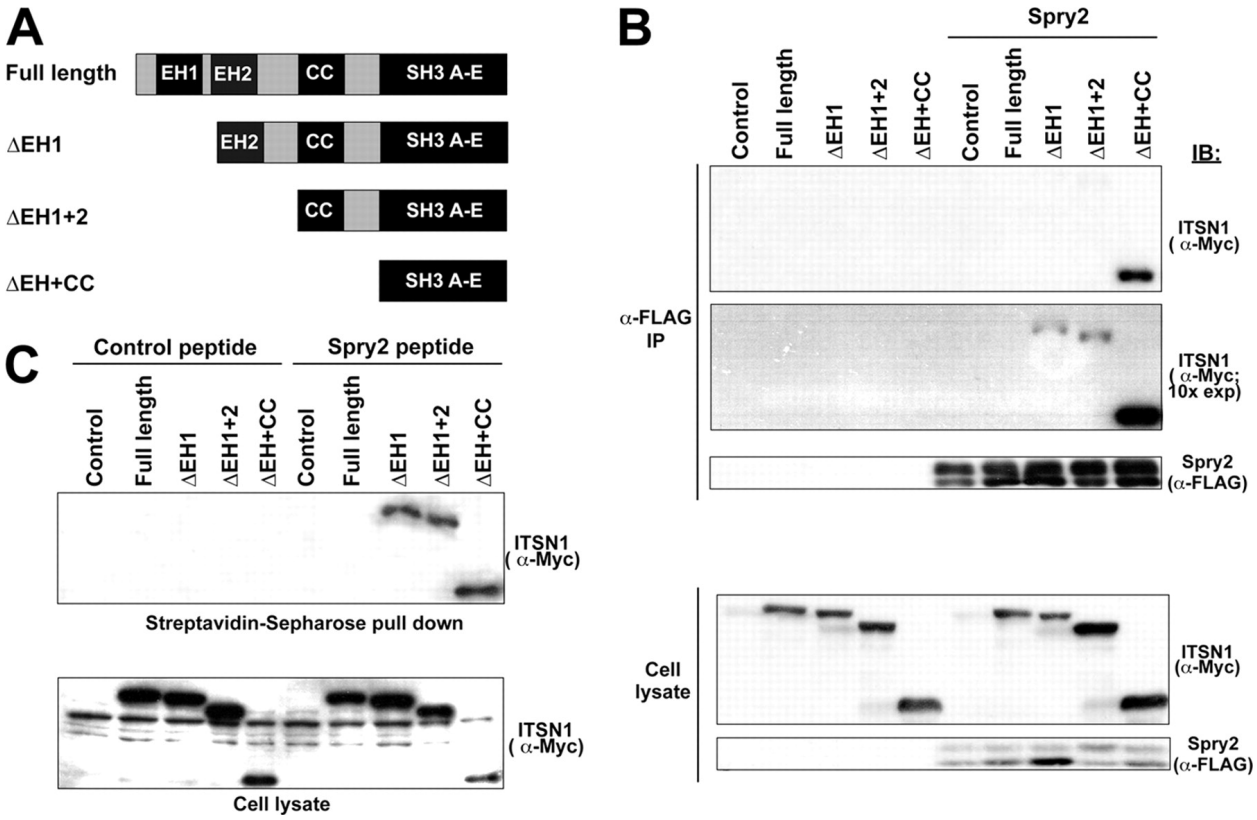

Figure 8. The NH_2 terminus of ITSN1 negatively regulates binding to Spry2. (A) Schematic of ITSN1 NH_2-terminal-truncation mutants. (B) HEK293T cells were cotransfected with FLAG-tagged wild type Spry2 along with full-length ITSN1-S or ITSN1 with various NH_2-terminal truncations. (Top) FLAG-tagged Spry2 was immunoprecipitated using M2 beads to identify the ITSN1 truncations that interact with Spry2. (Middle) A 10×-longer exposure of the blot in the top panel. (Bottom) Levels of protein expression from the various transfected gene constructs were similar. (C) Immunoprecipitation of Pro-rich Spry2 peptides with various NH_2-terminal truncations of ITSN-S. HEK293T cells were transfected with full-length ITSN1 or ITSN1 with various NH_2-terminal truncations. Cell lysates were incubated with either biotinylated Pro-rich Spry2 peptides or control peptides. (Top) Biotin-labeled peptides were immunoprecipitated using streptavidin Sepharose beads. Precipitates were analyzed by immunoblotting with Myc antibodies to detect the ITSN1 proteins. (Bottom) Levels of expression of the various Myc-tagged ITSN1 proteins in cell lysates. Experiments shown in this figure were performed by laboratory of Dr. Graeme Guy, Institute of Molecular and Cell Biology, Singapore, Singapore.

progressive truncation of the NH_2 terminus (Figure 8B). Deletion of the EH1 domain enhanced Spry2 binding to ITSN1 compared to its binding to full-length ITSN1. Although not visible on the gel in Figure 8B, full-length ITSN1-S and ITSN1-L do indeed interact with Spry2 by coimmunoprecipitation (Figure 5B). 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 (Figure 6A), 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 (Hussain et al., 2001; Zamanian and Kelly, 2003). 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 pulldown 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 preincubated with streptavidin-conjugated Sepharose beads and then mixed with cell lysates. Consistent with the results in Figure 8B, 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 (Figure 8C).

2.3.4 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 (Martin et al., 2006). Since ITSN1 did not affect Cbl binding to EGFR, Cbl phosphorylation, or Cbl stability (Martin et al., 2006), 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 (Figure 9A and B). The loss of Spry2-Cbl BiFC signal was not due to changes in the expression of VN-Spry2 or VC-Cbl (Figure 9C). Using epitope-tagged versions of these proteins instead of BiFC, we also demonstrated that ITSN1 dose-dependently decreased the coimmunoprecipitation of Spry2 with Cbl, thus corroborating the BiFC data (Figure 10).

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 coexpression of Spry2 with Cbl inhibited this effect (Figure 11, compare lanes 2, 3, and 4) (Egan et al., 2002; Rubin et al., 2003; Wong et al., 2002). However, addition of

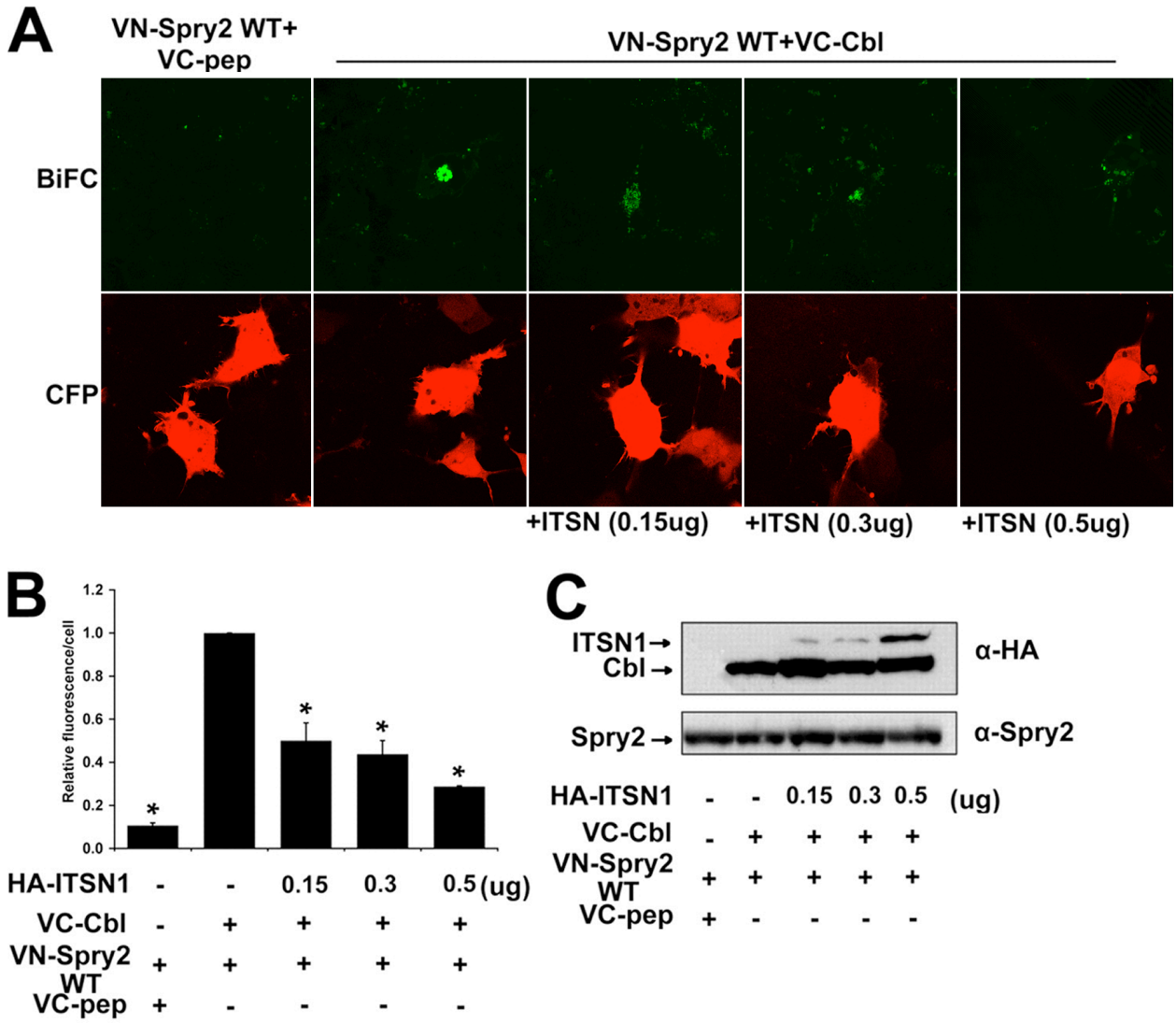

Figure 9. ITSN1 disrupts Spry2-Cbl interaction. (A) Spry2-Cbl interaction was measured by BiFC. ITSN1 expression leads to a dose-dependent decrease in Spry2-Cbl interaction. Coexpression of VN-Spry2 with VC-pep, a nonspecific peptide control, does not result in a BiFC signal. (B) Quantification of BiFC signal. Interaction of Spry2 and Cbl was quantified as described previously (36). Results are the averages and SEM from three independent experiments. Asterisks indicate significant differences from the result for the VN-Spyr2+VC-Cbl sample ($P < 0.05$). (C) Western blotting demonstrates the expression of the various proteins. Both ITSN1 and Cbl were HA tagged. The differences in Spry2-Cbl interaction are not due to changes in the overall expression of these proteins.

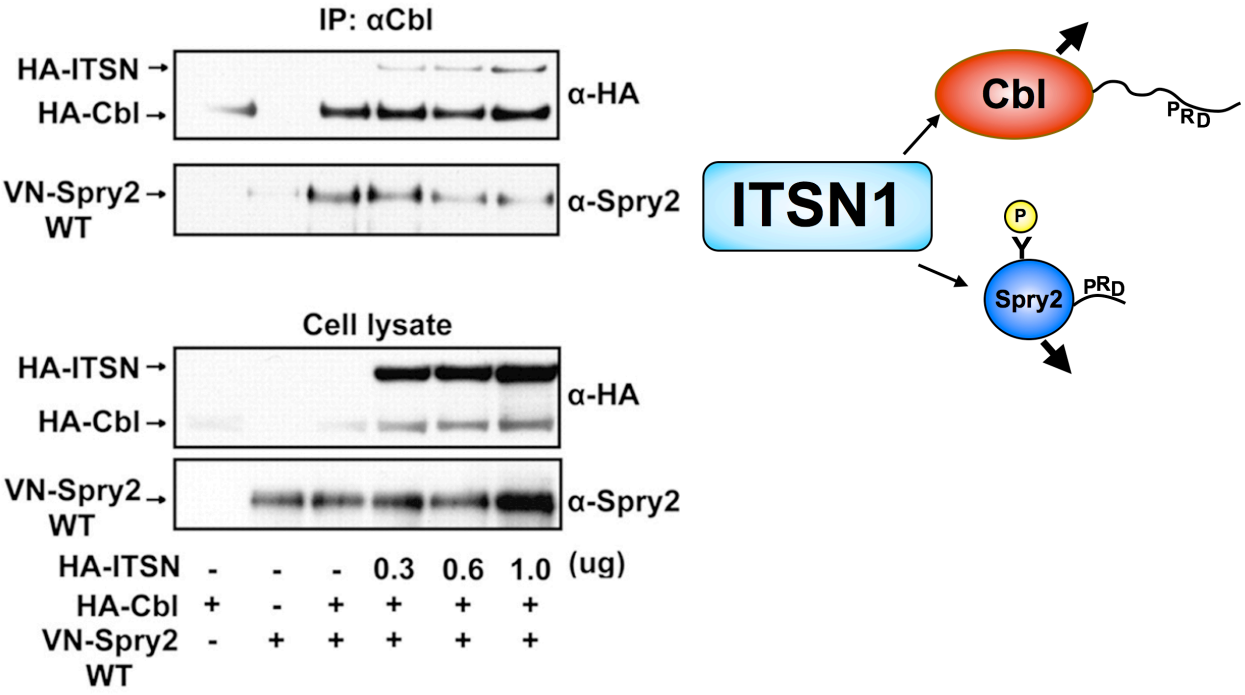

Figure 10. Overexpression of HA epitope-tagged ITSN1 dose dependently disrupts the binding of Spry2 WT to Cbl. HA-Cbl was immunoprecipitated from cells and the coprecipitation of Spry2 was monitored by Western blot of Cbl precipitates. (Top) Western blots of anti-Cbl precipitates with the indicated antibodies. (Bottom) Western blots of cell lysates with the indicated antibodies. Cartoon figure illustrates the disturbing effect of increasing ITSN1 levels on Spry2 and Cbl interaction.

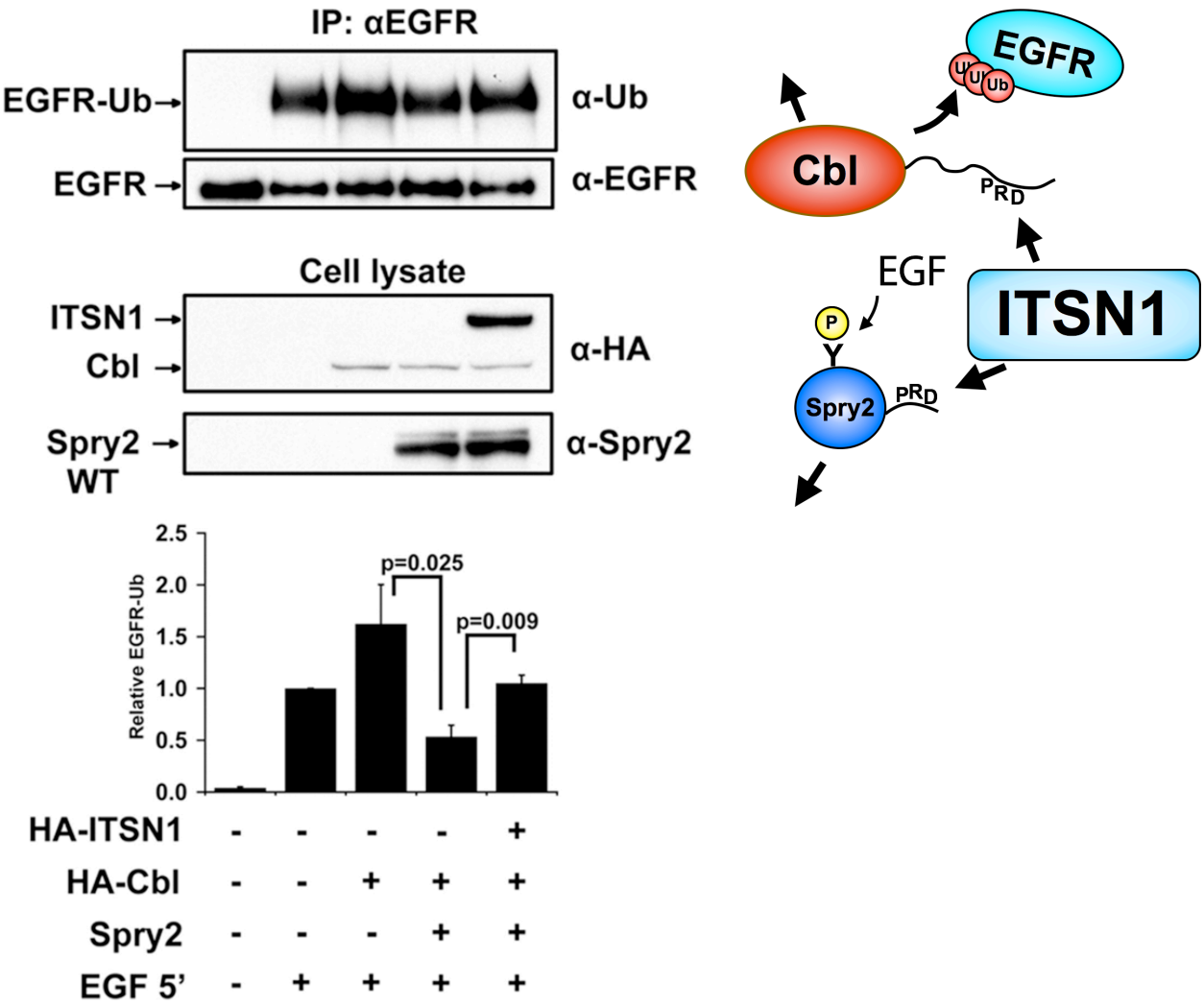

Figure 11. ITSN1 overexpression reverses the inhibitory effects of Spry2 on Cbl-mediated EGFR ubiquitylation. Overexpression of Cbl in COS cells results in enhanced EGFR ubiquitylation following EGF stimulation (compare lanes 2 and 3). Coexpression of Spry2 with Cbl reduces EGFR ubiquitylation even though Cbl levels are elevated even higher than in the absence of Spry2 overexpression (compare lanes 3 and 4). Coexpression of ITSN1, however, reverses the effect of Spry2 resulting in increased EGFR ubiquitylation. The ratio of ubiquitylated EGFR to total EGFR was determined by densitometry and compared between samples. The results are shown in the graph below the Western blots. These results are representative of three independent experiments. Cartoon figure illustrates the promoting effect of ITSN1 on Cbl activity for EGFR ubiquitylation by disturbing Spry2 and Cbl interaction.

ITSN1 reversed the inhibitory effect of Spry2 on Cbl, leading to enhanced ubiquitylation of endogenous EGFR (Figure 11, compare lanes 4 and 5). These results demonstrate that ITSN1 overexpression disrupts Spry2-Cbl binding, resulting in enhanced Cbl activity for the activated EGFR.

2.3.5 ITSN1 ENHANCES THE INHIBITORY EFFECT OF SPRY2 P304A MUTANT

ITSN1's SH3 domains bind the Pro-rich tail of Cbl (Martin et al., 2006) as well as Spry2 (Figure 5 and 8). 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 (Figure 12A, leftmost panels). Surprisingly, increasing ITSN1 expression resulted in increased interaction between Cbl and Spry2 P304A in a dose-dependent manner (Figure 12A and B). The increase in the Spry2-Cbl BiFC signal was not due to changes in the expression of VN-Spry2 or VC-Cbl (Figure 12C). To confirm these BiFC results, we again used epitope-tagged versions of these proteins and tested the effect of ITSN1 on coprecipitation of Spry2 P304A with Cbl. In agreement with the BiFC results, we observed that ITSN1 dose dependently increased association of Spry2 P304A with Cbl (Figure 13).

Given this increased interaction between Spry2 P304A and Cbl in the presence of ITSN1, we next tested the consequence on EGFR ubiquitylation. Coexpression of Spry2 P304A with Cbl inhibited Cbl activity and thus decreased EGFR ubiquitylation (Figure 14, compare lanes 3 and 4). These results are comparable to results with WT Spry2 (Figure 11, compare lanes 3 and 4). However, in contrast to the results with wild-

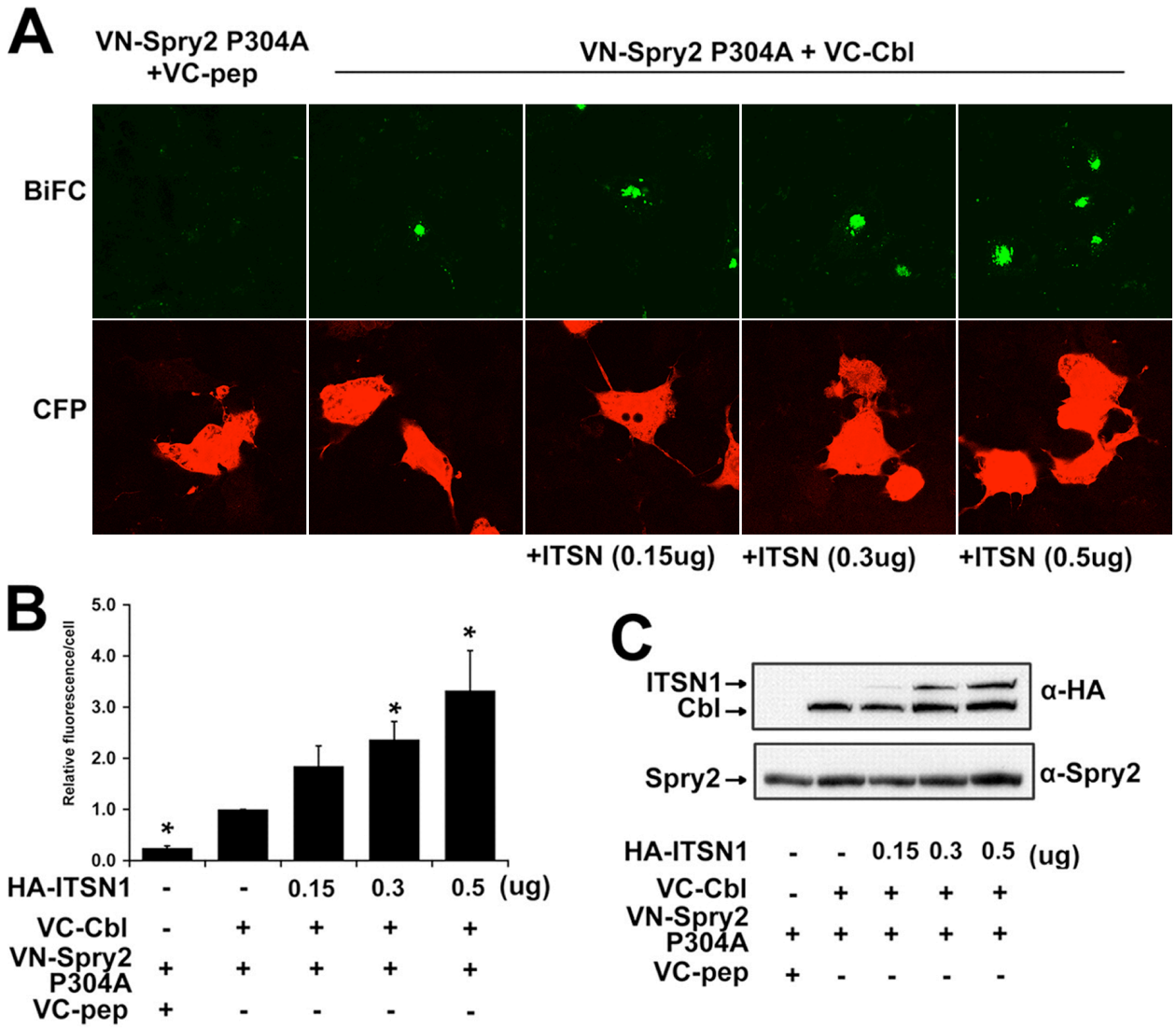

Figure 12. ITSN1 binding to Cbl in the absence of Spry2 binding leads to enhanced Spry2-Cbl interaction and decreased EGFR ubiquitylation. (A) Interaction of Spry2 P304A with Cbl was measured by BiFC in the absence or presence of increasing ITSN1 levels as described in the legend to Figure 9. ITSN1 overexpression results in enhanced binding of Spry2 P304A to Cbl. (B) Quantification of BiFC signal. Interaction of Spry2 P304A and Cbl was quantified as described previously (36). Results are the averages and SEM from three independent experiments. Asterisks indicate significant differences from the value for the VN-Spyr2 P304A+VC-Cbl sample ($P < 0.05$). (C) Western blotting demonstrates the expression of the various proteins. Both ITSN1 and Cbl are HA tagged. The differences in Spry2 P304A-Cbl interaction are not due to changes in the overall expression of these proteins.

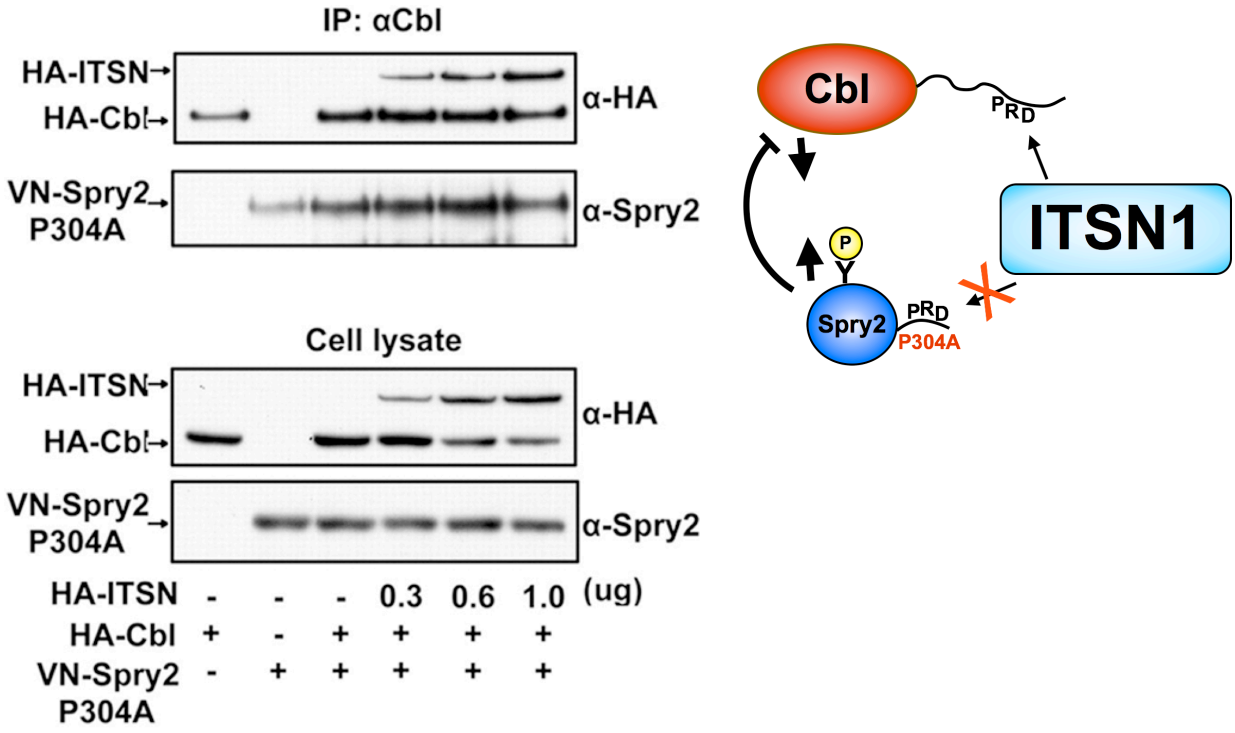

Figure 13. Overexpression of HA epitope-tagged ITSN1 dose dependently enhances the binding of the Spry2 P304A mutant to Cbl. HA-Cbl was immunoprecipitated from cells, and the coprecipitation of Spry2 P304A was monitored by Western blot of Cbl precipitates. (Top) Western blots of anti-Cbl precipitates with the indicated antibodies. (Bottom) Western blots of cell lysates with the indicated antibodies. Cartoon figure illustrates the promoting effect of increasing ITSN1 levels on Spry2-P304A and Cbl interaction.

type Spry2, coexpression 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 (Figure 14, compare lanes 4 and 5).

2.4 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 demonstrate that Spry2, 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 to 310). Indeed, this Pro-rich sequence conforms to previously identified ITSN1 binding sites (Das et al., 2007; Martin et al., 2006; Yamabhai et al., 1998).

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 (Martin et al., 2006). 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 posttranslational modifications as well as association of Cbl with numerous activators and inhibitors (Schmidt and Dikic, 2005). Although ITSN1's ability to activate

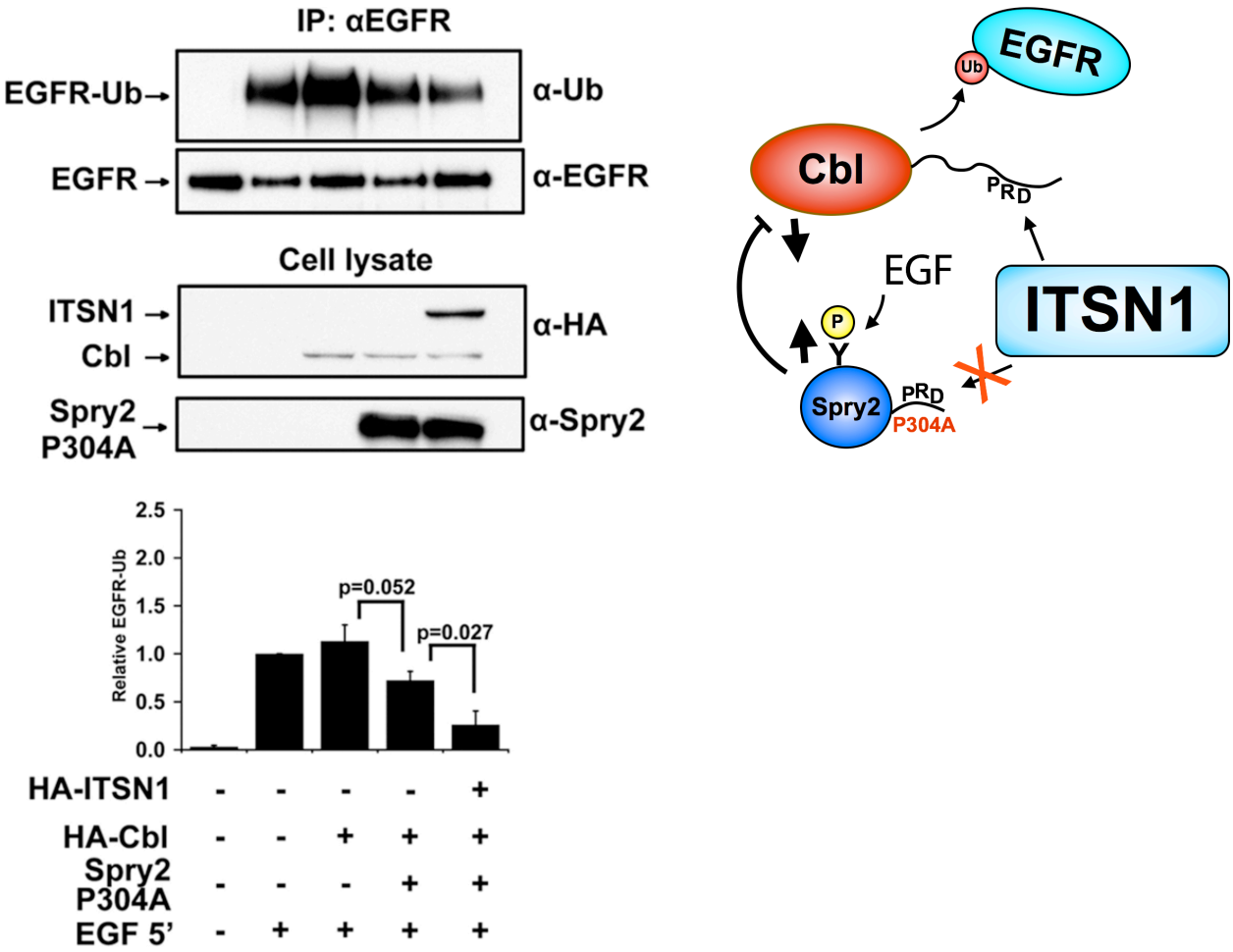

Figure 14. ITSN1 overexpression enhances the inhibitory effects of Spry2 P304A on Cbl-mediated EGFR ubiquitylation. Overexpression of Cbl in COS cells results in enhanced EGFR ubiquitylation following EGF stimulation (compare lanes 2 and 3). Coexpression of Spry2 P304A with Cbl reduces EGFR ubiquitylation (compare lanes 3 and 4). Coexpression of ITSN1 enhanced the inhibitory effect of Spry2 P304A, resulting in a further decrease in EGFR ubiquitylation. The ratio of ubiquitylated EGFR to total EGFR was determined by densitometry and compared between samples. The results are shown in the graph below the Western blots. These results are representative of three independent experiments. Cartoon figure illustrates the inhibitory effect of ITSN1 on Cbl activity for EGFR ubiquitylation by enhancing Spry2 and Cbl interaction.

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 (Martin et al., 2006). 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 or bodies for degradation in the lysosome (Eden et al., 2012; Huang et al., 2007). 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 likely underestimated the effects of these proteins on EGFR ubiquitylation, since we measured ubiquitylation of endogenous EGFR in the total population of cells yet were able to transfect only approximately 50% of cells. This approach allows us to measure the effects on endogenous receptors using endogenous ubiquitin and therefore avoids problems of uneven expression of epitope-tagged ubiquitin between samples (Miller et al., 2004). 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 ubiquitin ligase activity to regulate EGFR ubiquitylation. Both Cbl and Spry2 possess Pro-rich motifs that bind ITSN1 (Figure 5D) (Martin et al., 2006). Surprisingly, disrupting the binding of ITSN1 to Spry2 enhanced interaction between Cbl and Spry2 P304A, leading to decreased EGFR ubiquitylation (Figure 11). This result suggests that ITSN1 binding to the Pro-rich tail of Cbl may promote a conformational change that enhances the interaction of Spry2 with Cbl. While Spry2 binds Cbl through phosphotyrosine-dependent and -independent mechanisms (Kim and Bar-Sagi, 2004), ITSN1 overexpression does not alter the tyrosine phosphorylation of Cbl following growth factor stimulation (Martin et al., 2006). Thus, we do not believe that the enhanced interaction of Spry2 P304A with Cbl is due to altered phosphorylation of Cbl. However, it is unclear whether ITSN1 overexpression alters the phosphorylation of Spry2 to facilitate interaction with Cbl.

ITSN1 regulates numerous biological processes, including endocytosis and cellular signaling (O'Bryan, 2010). The modular structure of ITSN1 allows interaction with a variety of targets. Furthermore, intra- and intermolecular interaction of these domains appears to play an important role in ITSN1 function. For example, overexpression of ITSN1's SH3 domains inhibits the formation of clathrin-coated pits as well as ITSN1-regulated signaling pathways (Sengar et al., 1999; Simpson et al., 1999;

Tong et al., 2000a), indicating that SH3 domain availability must be strictly regulated to maintain proper ITSN1 function. Our data suggest that the EH and CC domains may negatively regulate SH3 domain availability as progressive NH_2-terminal deletions in ITSN1 enhanced binding to Spry2 as well as N-WASP. This regulation of SH3 binding may also have important implications for Cdc42 regulation by the long 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 (Kintscher et al., 2010). Furthermore, interaction of ITSN1-L with N-WASP relieves this inhibition (Hussain et al., 2001). Thus, EH binding to endocytic proteins, such as epsin (Sengar et al., 1999), stonin (Martina et al., 2001), SCAMP1 (Fernandez-Chacon et al., 2000), FCHo proteins (Henne et al., 2010), AP180 (Wang et al., 2008), and Dab (Wang et al., 2008), 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 (O'Bryan, 2010). 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, where it participates in vesicle assembly (Henne et al., 2010). However, this recruitment may also allow cross talk with RTK-associated Cbl and regulation of receptor ubiquitylation. In addition, EH domain binding to components of the JNK MAPK pathway (Adams et al., 2000; Mohney et al., 2003) 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 (Das et al., 2007; Dierssen et al., 2001; Keating et al., 2006; Yu et al., 2008). There is a high comorbidity of the obstructive gastrointestinal disorder Hirschsprung disease in Down syndrome. Hirschsprung disease 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 (Hanafusa et al., 2002). Moreover, Spry2-deficient mice develop enteric nerve hyperplasia (Taketomi et al., 2005). 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.

3. RECEPTOR TYROSINE KINASE UBIQUITYLATION INVOLVES THE DYNAMIC REGULATION OF CBL-SPRY2 BY INTERSECTIN 1 AND THE THE SHP2 TYROSINE PHOSPHATASE

(Portions of this chapter have been previously published in Molecular and Cellular Biology 2013 with the title: "Receptor tyrosine kinase ubiquitylation involves the dynamic regulation of Cbl-Spry2 by intersectin 1 and the Shp2 tyrosine phosphatase.")

3.1 INTRODUCTION

ITSN1 stimulates ubiquitylation of the epidermal growth factor receptor (EGFR) tyrosine kinase, through enhancing the activity of the Cbl E3 ubiquitin ligase (Martin et al., 2006). The work in chapter 2 demonstrates that the increase in Cbl activity is mediated by inhibiting the Cbl negative regulatory protein Spry2 (Okur et al., 2012). However, the precise mechanism through which ITSN1 regulates Cbl and Spry2 interaction remains unclear. Regulation of Cbl E3 ligase activity involves a complex interplay between Cbl and its many interacting partner (Schmidt and Dikic, 2005). Upon activation of RTKs with growth factor, Cbl binds to tyrosine phosphorylated receptors through its SH2-like tyrosine kinase binding (TKB) domain and mediates covalent attachment of ubiquitin to the activated receptors (Schmidt and Dikic, 2005). However, Cbl activity is modulated through its interaction with various proteins. For example, SH3-containing proteins such as ITSN1 and CIN85 bind Cbl's Pro-rich domain to stimulate Cbl activity and enhance RTK ubiquitylation (Martin et al., 2006; Okur et al., 2012; Petrelli et al., 2002; Soubeyran et al., 2002). However, Cbl activity is also negatively regulated by interaction with Spry2. Following growth factor stimulation (e.g., EGF), Spry2 is tyrosine phosphorylated by Src family kinases thereby creating a consensus

binding site for Cbl's TKB domain. Tyrosine phosphorylated Spry2 binds Cbl thereby inhibiting its interaction with activated RTKs and decreasing EGFR ubiquitylation (Edwin et al., 2009; Guy et al., 2009).

Although Spry2 negatively regulates RTK ubiquitylation suggesting it would enhance RTK signaling, the role of Sprouty proteins in signal transduction is more complex. Depending on the specific receptor activated, Spry2 may play either an inhibitory or activating role in ERK-MAPK signaling [see reviews (Edwin et al., 2009; Guy et al., 2009) and references therein]. Although it is currently not known how Spry2 accomplishes such contrasting roles, results from Bar-Sagi and colleagues suggest that binding of Spry2 to Cbl prevents Spry2 from inhibiting ERK activation by EGF (Egan et al., 2002). These studies illustrate the bimodal activity of Spry2 in both enhancing and inhibiting signaling depending on the context of Spry2 engagement.

Shp2 is an SH2-containing non-receptor tyrosine phosphatase that plays a critical role in cell signaling, specifically mediating the activation of the ERK-MAPK pathway by RTKs (Grossmann et al., 2010; Li et al., 2012). A number of mechanisms have been described by which Shp2 may regulate ERK activation including dephosphorylation of RasGAP binding sites on RTKs, activation of Src through direct dephosphorylation of inhibitory tyrosine phosphorylation sites or indirect regulation of Csk, and dephosphorylation of Spry2 [reviewed in (Grossmann et al., 2010)]. However, Shp2 has also been implicated in Cbl regulation. Following IL-6 stimulation, Shp2 recruits Cbl to activated gp130 receptor subunits to promote their ubiquitylation (Tanaka et al., 2008). In addition, several studies have implicated Shp2 in the dephosphorylation

of Sprouty proteins (Hanafusa et al., 2004; Jarvis et al., 2006; Pan et al., 2010) suggesting a potential role for Shp2 in the regulation of Cbl activity.

Recently, we demonstrated that ITSN1 interacts with both Spry2 and Cbl through binding of their Pro-rich domains by ITSN1's SH3 domains (Martin et al., 2006; Okur et al., 2012). This interaction leads to disruption of the Cbl-Spry2 complex, loss of Cbl inhibition by Spry2, and increased EGFR ubiquitylation (Okur et al., 2012). However, our study also revealed that when ITSN1 binding of Spry2 was inhibited by mutations of the ITSN1 binding site in Spry2, ITSN1 expression enhanced interaction of the Spry2 mutant with Cbl leading to reduced Cbl activity (Okur et al., 2012). These findings suggested that ITSN1 regulation of the Cbl-Spry2 complex may involve additional components. Here, we identified a novel interaction of ITSN1 with Shp2 which regulates the phosphorylation status of Spry2 and thus its ability to inhibit Cbl. Given that Shp2 dephosphorylates Sprouty proteins (Hanafusa et al., 2004; Jarvis et al., 2006; Pan et al., 2010), we propose that EGF stimulation leads to ITSN1 recruitment of Shp2 to the Cbl-Spry2 complex resulting in tyrosine dephosphorylation of Spry2, decreased Spry2 binding by Cbl, and increased Cbl activity.

3.2 MATERIALS AND METHODS

3.2.1 CELL LINES AND REAGENTS

COS-1 monkey kidney cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified chamber with 10% CO_2 and 90% air. The antibodies used in this study were N-Spry2 and ubiquitin P4D1

antibodies (Santa Cruz), EGFR AB12 and EGFR AB13 antibodies (Thermo Scientific), monoclonal anti-phosphotyrosine antibody (4G10) (Millipore), monoclonal anti-hemagglutinin (HA) antibody (Covance). The polyclonal rabbit anti-ITSN1 antibody (SH3) has been previously described (Martin et al., 2006). The polyclonal rabbit Shp2 antibody was kindly provided by Gen-Sheng Feng (University of California at San Diego, San Diego, CA).

3.2.2 DNA CONSTRUCTS AND TRANSFECTION

Spry2 constructs were previously described (Okur et al., 2012). FLAG-tagged Shp2 constructs (wild type, D61A constitutively active mutant, and C463S substrate trapping, dominant-negative mutant were kindly provided by Gen-Sheng Feng (University of California at San Diego) (He et al., 2013). VN-Shp2 truncation mutants were kindly provided by Graeme Carnegie and consist of the following amino acids of Shp2: N-SH2, 1-103; C-SH2, 111-218; N+C-SH2, 1-218; C-term, 219-597. ITSN1 truncation mutants were previously described (Martin et al., 2006). An amino-terminal HA epitope-tagged full-length ITSN1-S (mouse), HA-tagged wild-type (WT) human c-Cbl, pHM6-HA-Spry2 were previously described (Levkowitz et al., 1999; Mohney et al., 2003; Yigzaw et al., 2001). All experiments involving exogenous introduction of ITSN1 utilized the ITSN1-S isoform. COS-1 cells were transfected with polyethylenimine (PEI) (Polysciences). Briefly, a 1 mg/ml stock of PEI was prepared by resuspending 10 mg PEI in 3 ml of 100% ethanol, mixing til dissolved, and then adding 7 ml H_20. The stock solution was then sterile filter through 0.2 µm syringe filter and aliquots frozen at -80^oC. COS-1 cells were transfected with a PEI:DNA ratio of 3:1. COS cells were plated and

incubated for overnight in DMEM containing 10% FBS. On the following day, DNAs were added to 1 ml serum-free DMEM, mixed with 1 ml of serum-free media containing PEI, and the incubated 20 mins at room temperature. Following washing cells 2x with serum-free media, the DNA:PEI mix was added drop-wise to cells. Media was replaced with complete media after 5 hours of incubation at 37^{o}C degree.

3.2.3 IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Whole-cell lysates were prepared in PLC-LB as described previously (Oldham et al., 2002). For analysis of endogenous Shp2 and ITSN1 interaction, we prepared lysates adult brains from wild-type and ITSN1 knockout mice in PLC-LB. The ITSN1 mice were initially provided by Melanie Pritchard (Monash University) and have been previous described (Yu et al., 2008). Briefly, these mice possess a genomic deletion which eliminates expression of both the long and short isoforms of ITSN1 (Yu et al., 2008). For experiments measuring EGFR ubiquitylation, lysis buffer was supplemented with 5 mM N-ethylmaleimide for detection of endogenous ubiquitin. EGFR immunoprecipitation and ubiquitylation were determined as previously described (Martin et al., 2006). Tyrosine phosphorylation was detected as described previously (Sawasdikosol, 2010). Standard protocols suggested by the manufacturers were used to detect Shp2, Spry2, EGFR, FLAG- and HA-tagged proteins.

3.2.4 BIMOLECULAR FLUORESCENCE COMPLEMENTATION (BiFC)

BiFC allows for the monitoring of protein:protein interactions in live cells by fusing two proteins of interest to non-fluorescent NH_2-terminal and COOH-terminal fragments

of a fluorescent protein (Shyu and Hu, 2008; Wong and O'Bryan, 2011). Association of the two proteins leads to reformation of the fluorescent protein which can be detected by conventional fluorescence microscopy. COS-1 cells were plated on glass-bottom plates as well as in 6 well dishes and grown overnight. On the following day, cells were transfected with 0.5 ug of plasmids encoding proteins fused to pHA-VC155N and pFLAG-VN173N. ITSN1-S, Cbl, and Spry2 BiFC constructs have been previous described (Das et al., 2007; Okur et al., 2012). The VC-Shp2 construct encoding wild type Shp2 was kindly provided by Graeme Carnegie (University of Illinois at Chicago) and has been previously described (Burmeister et al., 2012). Following 24h, the glass bottom dishes were treated with 3.7% formaldehyde for 20 min to fix cells, washed with PBS twice, and stored in PBS at 4°C in the dark. To determine levels of protein expression in BiFC experiments, cells on a parallel 6 well-plate were lysed and protein expression determined by Western blot analysis. A Zeiss LSM 510 META confocal microscope was used to image samples. ImageJ, available from the National Institutes of Health (NIH) was used to quantify BiFC as average fluorescence intensity per pixel as described previously (Wong and O'Bryan, 2011). For all BiFC experiments, reconstituted Venus fluorescence was pseudo-colored green and CFP pseudo-colored red.

3.3 RESULTS

3.3.1 ITSN1 INTERACTS WITH SHP2

Previous findings demonstrated that Spry2 phosphorylation at Tyr55 mediates its binding to Cbl and inhibition of Cbl activity (Fong et al., 2003). Recently, we showed that

ITSN1 disrupts the interaction of Spry2 with Cbl suggesting that ITSN1 might regulate Spry2 phosphorylation. Given that Shp2, an SH2-containing protein-tyrosine phosphatase, dephosphorylates and inactivates Spry2 (Hanafusa et al., 2004; Jarvis et al., 2006; Pan et al., 2010), we speculated that ITSN1 may have a role in Shp2-mediated Spry2 dephosphorylation and therefore examined ITSN1 association with Shp2. Indeed, Shp2 co-immunoprecipitates with ITSN1 from brain lysates of wild type mice suggesting that endogenous ITSN1 and Shp2 interact in vivo (Figure 15A). These ITSN1-reactive bands were absent from Shp2 immunoprecipitates from brains of ITSN1 knock out mice (Yu et al., 2008). In addition, a control immunoprecipitation using non-specific rabbit IgG co-precipitated only relatively low amounts of ITSN1 suggesting that ITSN1 precipitation by Shp2 antibody is not due to non-specific binding (Figure 15A). To determine the regions of ITSN1 and Shp2 important for this interaction, we co-expressed full-length versions of either protein with truncation mutants of the other protein (Figure 15B). ITSN1's coiled-coil region co-precipitated with Shp2. Although the SH3 domains exhibited a slight interaction with Shp2, the EH domains did not interact. These results suggest that the coiled-coil region is sufficient for binding Shp2. Reciprocal experiments in which we expressed full-length ITSN1 with either the N-SH2, C-SH2 or the isolated catalytic domain did not result in interaction of any of these truncation mutants with ITSN1 (data not shown).

To further examine ITSN1-Shp2 association, we analyzed the interaction of epitope-tagged versions of the two proteins. As shown in Figure 16A, FLAG-tagged Shp2 co-immunoprecipitated with HA-ITSN1 following growth factor stimulation. Similar

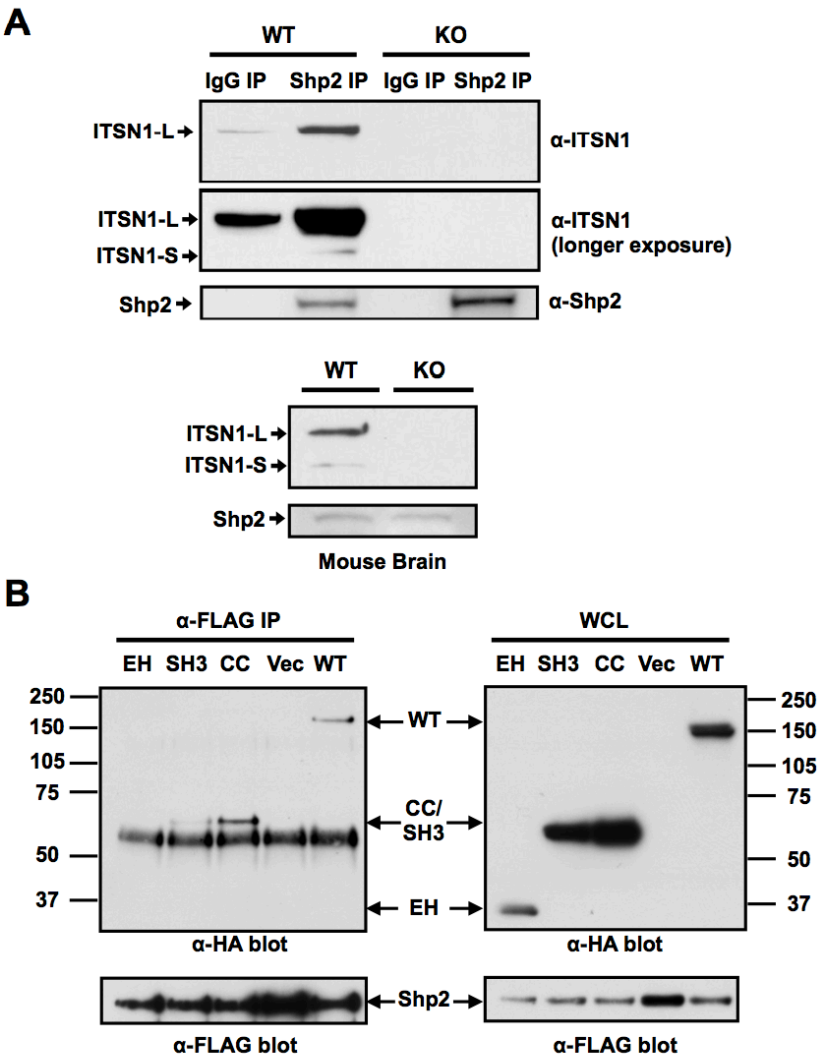

Figure 15. ITSN1 and Shp2 interaction. (A) Shp2 immunoprecipitates from brain lysates of wild type (WT) and ITSN1 knock out (KO) mice were fractionated on a 4-12% gradient gel, transferred to PVDF membrane and probed with antibodies to Shp2 (third panel from top) and ITSN1 (top two panels with longer exposure). Non-specific IgG was used as a negative immunoprecipitation control. Bottom panels shows endogenous Shp2 and ITSN1 levels in mouse brain lysates. The two ITSN1 bands represent positions of ITSN1-S and ITSN1-L marked with an S or L, respectively. Shp2 interacts with both ITSN1 isoforms. (B) FLAG-tagged VN-Shp2 was co-expressed with HA-tagged ITSN1 constructs encoding either full-length (WT) or the indicated truncation mutants. Following FLAG immunoprecipitation, samples were fractionated on gels and then probed for the presence of the ITSN1 proteins (top left panel). Levels of FLAG-Shp2 in the immunoprecipitation are indicated in the bottom left panel. Expression of the ITSN1 constructs and FLAG-Shp2 in the whole cell lysates (WCL) is shown in the panels to the right. Results are representative of three independent experiments. WT, full-length ITSN1; EH, Eps15 homology regions of ITSN1; CC, coiled-coil region; SH3, region encoding the five SH3 domains of ITSN1. Figure 15B is performed by Angela Russo, Ph.D., Laboratory of John O'Bryan, Department of Pharmacology, University of Illinois at Chicago, Chicago, Il, USA.

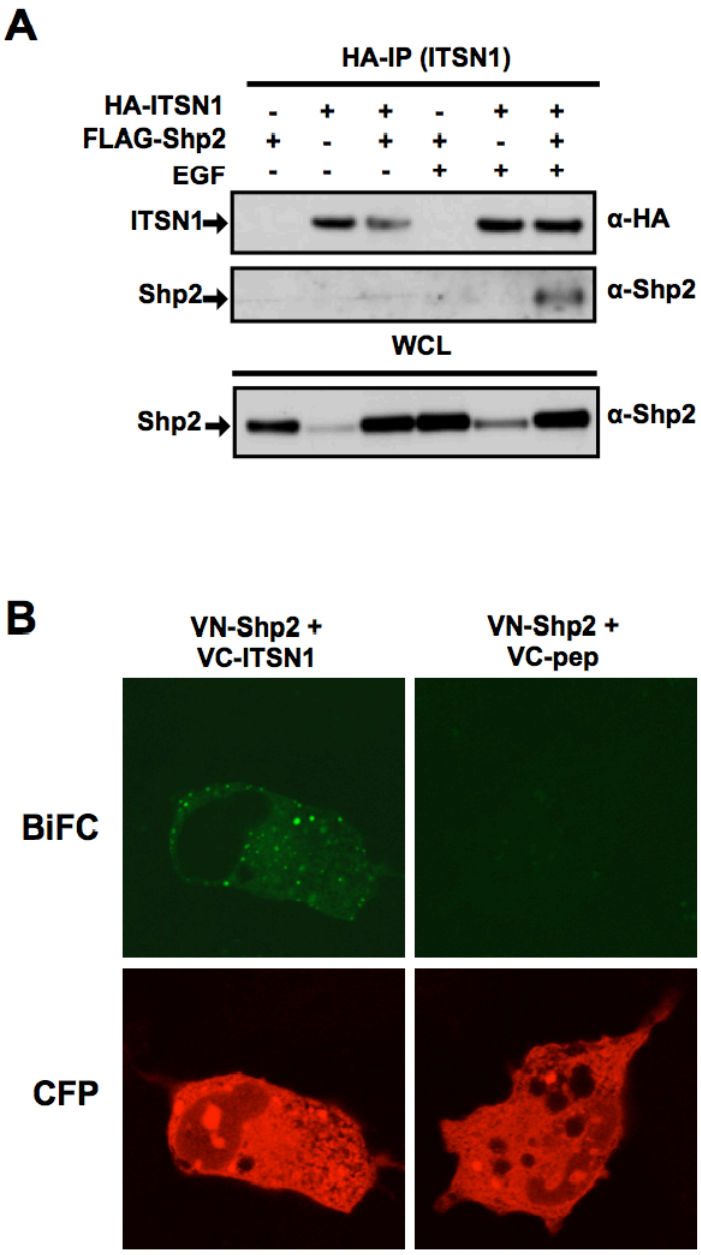

Figure 16. Interaction of ectopically expressed ITSN1 and Shp2. (A) COS cells were transfected with HA-ITSN1, FLAG-Shp2, or both constructs. Following EGF stimulation (100 ng/ml for 10 min), cells were lysed, and HA-tagged ITSN1 was immunoprecipitated with an α-HA antibody to examine association with FLAG-Shp2. Bottom panel reflect the expression of FLAG-Shp2 in the whole cell lysates (WCL). The band in lanes 2 and 5 of the WCL represents endogenous Shp2. (B) Bimolecular fluorescence complementation assay (BiFC) was performed to examine the interaction of VN-Shp2 with VC-ITSN1 in COS cells. Venus signal was pseudo-colored green and CFP pseudo-colored red. CFP was co-transfected at 5-fold lower levels to mark transfected cells (Wong and O'Bryan, 2011). VC-pep, expresses a non-specific peptide and serves as a negative control for BiFC as previously described (Okur et al., 2012).

results were seen with VN-Shp2 and HA-ITSN1 (data not shown). We also analyzed ITSN1 and Shp2 interaction in live cells. Using bimolecular fluorescence complementation (BiFC) (Shyu and Hu, 2008; Wong and O'Bryan, 2011), we observed that VN-Shp2 and VC-ITSN1 physically interact with one another in cells (Figure 16B). In contrast, VN-Shp2 and VC-pep [a non-specific peptide used as a negative control (Das et al., 2007)] do not interact indicating that the BiFC signal for VN-Shp2 and VC-ITSN1 is specific.

3.3.2 ITSN1-SHP2 INTERACTION IS CRUCIAL FOR SHP2-SPRY2 ASSOCIATION.

Given that Shp2 is a Spry2 phosphatase (Hanafusa et al., 2004; Jarvis et al., 2006; Pan et al., 2010) and that Cbl interaction with Spry2 is mainly regulated by Spry2 phosphorylation (Fong et al., 2003; Hall et al., 2003; Mason et al., 2004; Rubin et al., 2003; Wong et al., 2001), we speculated that interaction of ITSN1 with Shp2 may lead to dephosphorylation of Spry2, thereby disrupting Spry2 and Cbl interaction. To test this possibility, we first examined whether the ITSN1 binding site of Spry2 is crucial to Spry2 and Shp2 interaction by performing BiFC. Spry2 interaction with full length Shp2 is significantly reduced (~50%) by mutation of the COOH-terminal Pro-rich sequence (Spry2-P304A) (Figure 17A&B). These data suggest that interaction of ITSN1 with Spry2 regulates Spry2 binding to Shp2. As a control, we tested Shp2 interaction with Spry2-Tyr55Phe (Spry2-Y55F) and observed this mutant is also impaired in Shp2 interaction as demonstrated in earlier studies (Quintanar-Audelo et al., 2011). Western blot analysis of the various tagged proteins indicates that differences in BiFC signal are not due to differences in expression of the various Spry2 mutants (Figure 17C).

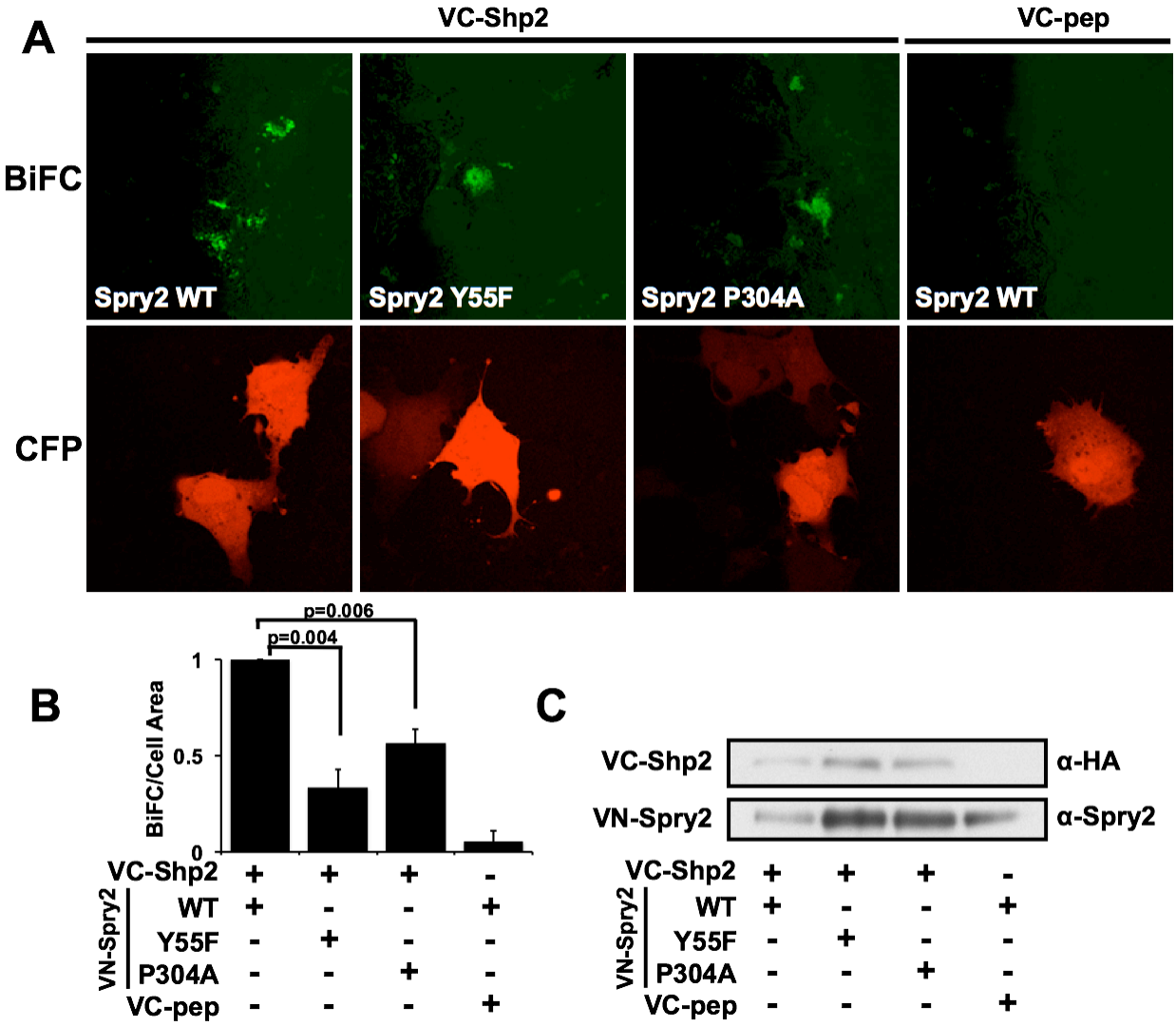

Figure 17. Spry2 binding to ITSN1 is crucial for Spry2 and Shp2 interaction. (A) Various VN-tagged Spry2 mutants were individually co-expressed with VC-Shp2 in COS cells and examined for interaction by BiFC as in Fig. 16. CFP was transfected at 5-fold lower concentrations to mark transfected cells and to determine the edges of cells for BiFC signal quantification as described (Okur et al., 2012; Wong and O'Bryan, 2011). (B) Quantification of BiFC signal using NIH ImageJ program (Wong and O'Bryan, 2011). Mutation of the ITSN1 binding site in Spry2 (P304A) decreased the interaction of Spry2 with Shp2. Spry2-Y55F mutation also decreased Spry2 and Shp2 interaction as consistent with earlier studies (Quintanar-Audelo et al., 2011). Transfection of VN-Spry2 with VC-peptide was used as a negative control. Over 25 cells were randomly imaged for BiFC quantification per experiment and experiments were performed in triplicate. The differences in BiFC signal between the indicated sample and the WT controls (first column) were significantly different as indicated by the p-values using a Student's t-test. (C) Expression of Spry2 mutants and Shp2 in whole cell lysates demonstrates that the differences in BiFC signal are not due to expression level fluctuations.

3.3.3 ITSN1 MEDIATES SPRY2 DEPHOSPHORYLATION BY RECRUITING SHP2

Given that ITSN1 interacts with both Spry2 (Okur et al., 2012) and Shp2 (Figures 15-17), we next tested whether all three proteins could be found in a complex with one another. Co-expression of CFP-ITSN1 with VN-Spry2 and VC-Shp2 demonstrates that the 3 proteins can be found in a complex in cells (Figure 18). The co-localization of ITSN1, Shp2 and Spry2 combined with the results demonstrating the importance of the ITSN1 binding region of Spry2 for Shp2:Spry2 interaction prompted us to examine whether ITSN1 contributes to recruitment of Shp2 to Spry2. To address this question, we tested the effect of titrating ITSN1 levels on the interaction of Shp2 and Spry2. Using BiFC to monitor Shp2-Spry2 interaction, we observed that increasing ITSN1 levels resulted in increased association of Shp2 and Spry2 in a dose dependent manner (Figure 19A-C). Our previous work demonstrated that interaction of Spry2 with Cbl inhibits Cbl's ubiquitin ligase activity and that ITSN1 disrupts this inhibition by blocking Spry2-Cbl interaction (Okur et al., 2012). However, the mechanism by which ITSN1 regulated Spry2-Cbl interaction was not defined. Given the ability of ITSN1 to disrupt Spry2-Cbl interaction coupled with the ability of Shp2 to dephosphorylate Spry2 at Tyr55, we next examined whether increasing ITSN1 levels affected Spry2 dephosphorylation. ITSN1 overexpression decreased EGF-stimulated Spry2 tyrosine phosphorylation (Figure 20). We did not detect any tyrosine phosphorylation of Spry2-Y55F mutant (data not shown) suggesting that the decrease in α-pTyr reactivity is due to specific loss of phosphorylation at Tyr55. Since Cbl mainly interacts with Spry2 following phosphorylation at Tyr55 (Fong et al., 2003; Hall et al., 2003; Mason et al.,

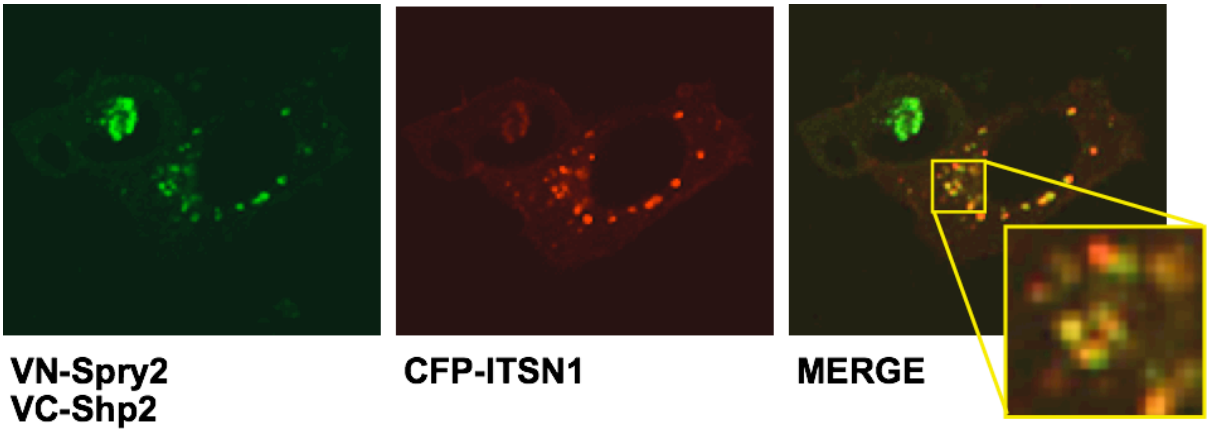

Figure 18. Co-localization of Spry2, Shp2, and ITSN1. VN-Spry2 and VC-Shp2 were co-expressed with CFP-ITSN1 in COS cells and then cells imaged by confocal microscopy as previously described (Okur et al., 2012). Venus fluorescence was pseudo-colored green and CFP fluorescence was pseudo-colored red for ease of visualization of the co-localized complexes. Co-localization of the VN-Spry2:VC-Shp2 BiFC complex with CFP-ITSN1 is indicated in yellow in the far right panel and the inset highlighting the region of the image.

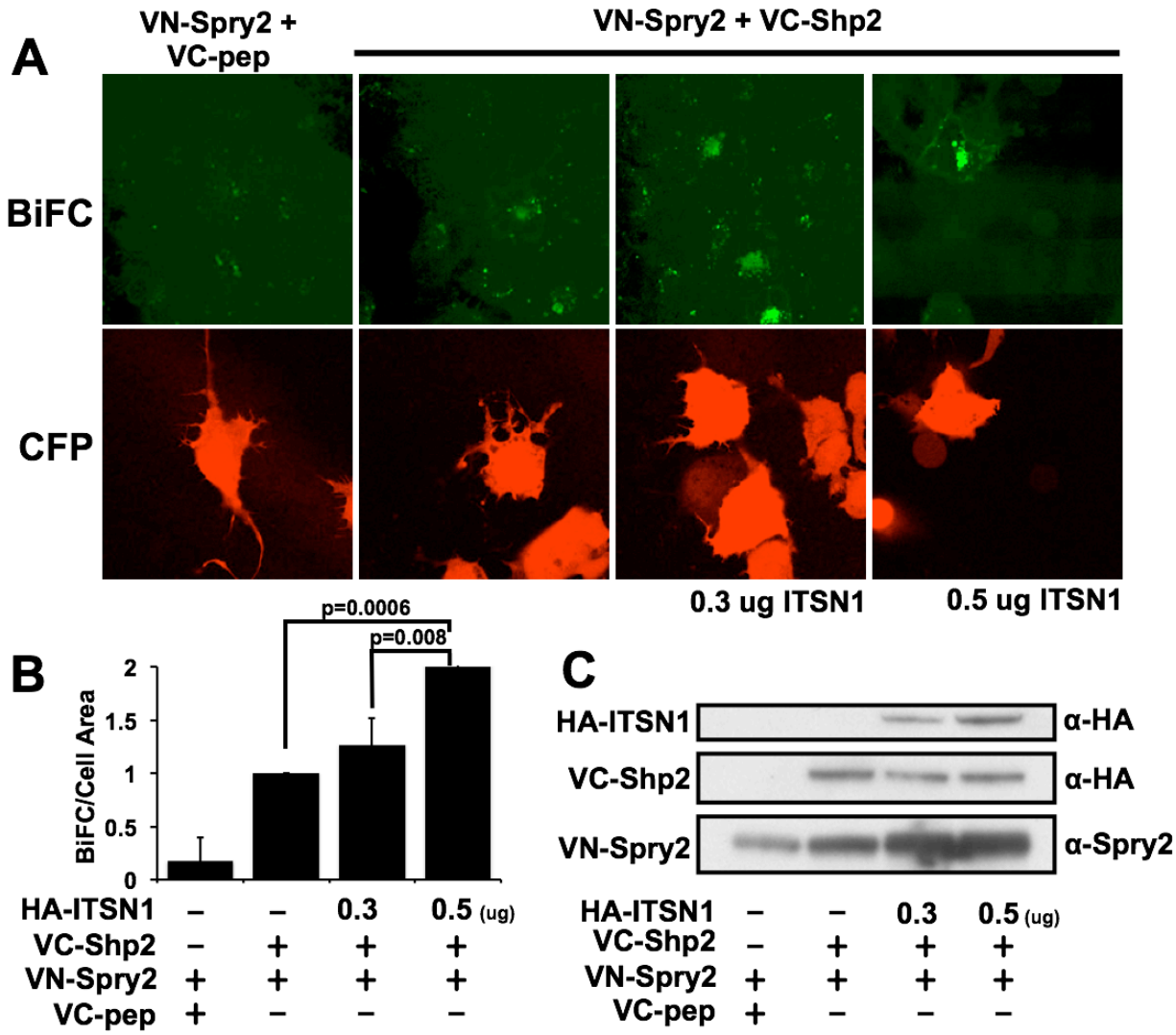

Figure 19. ITSN1 regulates Shp2-Spry2 association. (A) ITSN1 dose dependently enhanced Shp2-Spry2 interaction as measured by BiFC. COS cells were transfected with VN-Spry2 and VC-Shp2 along with increasing concentrations of HA-ITSN1. VC-pep was used as a negative control as described in Fig. 16. (B) Quantification of BiFC signal between Shp2-Spry2 in the presence of increasing ITSN1 levels. Graph represents the average BiFC signal intensity per cell area as described in Fig. 17B. Results are the average of three independent experiments. The differences in BiFC signal between the indicated sample and the WT controls were significantly different as indicated by the p-values using a Student's t-test. As a negative control, VC-pep, a non-specific peptide control, was co-expressed with VN-Spry2 which does not result in a BiFC signal. (C) Expression of ITSN1 (top panel), Spry2 (middle panel) and Shp2 (bottom panel) in whole cell lysates demonstrates that the differences in BiFC signal are not due to expression level fluctuations of Shp2 and Spry2 between samples.

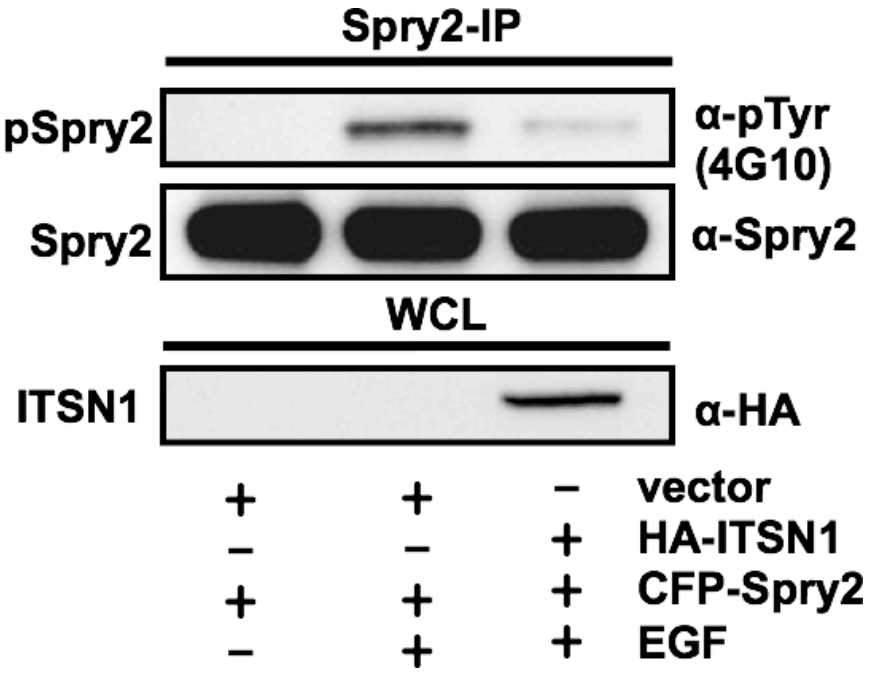

Figure 20. ITSN1 regulates Spry2 Tyr phosphorylation. Spry2 WT was co-expressed with or without HA-ITSN1. Following EGF stimulation (5 mins, 100 ng/ml), COS cells were harvested and Spry2 was immunoprecipitated with α-Spry2 antibody. EGF stimulation enhanced Tyr phosphorylation of WT Spry2. However, ITSN1 overexpression decreased Spry2 WT tyrosine phosphorylation. Bottom panel, whole cell lysate levels of ITSN1.

2004; Rubin et al., 2003; Wong et al., 2001), these data suggest that ITSN1 disrupts Spry2 and Cbl interaction due to ITSN1-mediated decrease in Spry2 tyrosine phosphorylation.

Next, we examined whether Shp2 is crucial for ITSN1-mediated Spry2 dephosphorylation. To address this possibility, we overexpressed ITSN1 and Spry2 in COS cells along with wild type (WT) Shp2 or a catalytically inactive mutant Shp2 (C463S) and examined Spry2 phosphorylation following EGF stimulation. Whereas expression of Shp2 WT enhanced Spry2 dephosphorylation, expression of Shp2-C463S reversed the effect of ITSN1 on Spry2 dephosphorylation (Figure 21).

3.3.4 ITSN1 MODULATION OF SPRY2 PHOSPHORYLATION DEPENDS ON ITS INTERACTION WITH SPRY2

Since ITSN1 interacts with Spry2 to regulate Cbl-Spry2 interaction (Okur et al., 2012), we next examined the consequence of increasing ITSN1 levels on the interaction of Shp2 and Spry2 when ITSN1 binding to Spry2 was disrupted. For these experiments, we used Spry2-P304A mutant which is impaired in ITSN1 binding (Okur et al., 2012). Surprisingly, increasing ITSN1 levels dose-dependently decreased Shp2 and Spry2-P304A interaction, suggesting that increasing ITSN1 levels may sequester Shp2 away from Spry2-P304A since ITSN1 no longer interacts with Spry2 (Figure 22A-C). Given this decreased interaction between Spry2-P304A and Shp2 in the presence of ITSN1,

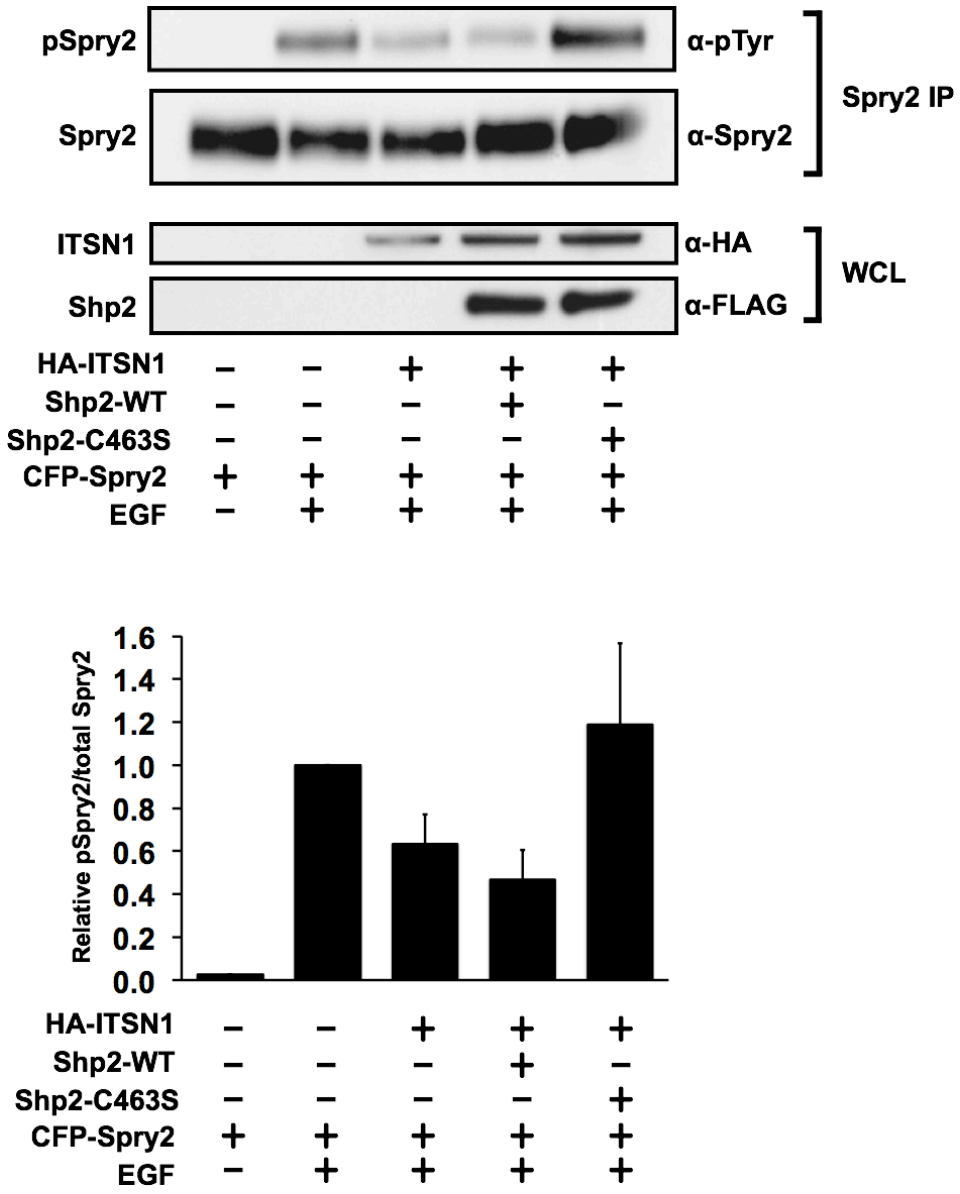

Figure 21. Shp2 mediates ITSN1-stimulated Spry2 dephosphorylation. (A) HA-ITSN1 was co-expressed with CFP-Spry2 in COS cells along with Shp2- WT or Shp2-C463 mutant. Cells were harvested following 5 mins EGF stimulation (100 ng/ml). Spry2 was immunoprecipitated with α-Spry2 antibody and levels of Spry2 phosphorylation examined using α-phosphotyrosine antibody (4G10). Expression of catalytically inactive Shp2-C463S mutant reversed ITSN1-mediated Spry2 dephosphorylation whereas expression of Shp2-WT increased dephosphorylation of Spry2. (B) Quantification of Spry2 phosphorylation levels. pSpry2 levels relative to total Spry2 expression was determined by densitometric analysis using NIH ImageJ. Results were normalized to the Spry2+EGF values. Data are expressed as the mean of three independent experiments +/- SEM. The effect of ITSN1 expression (lane 3 vs 2) on Spry2 phosphorylation slightly beyond statistical significance (p=0.058). However, the effect of ITSN1+Shp2 (lane 4 vs 2) was significant (p=0.031). The difference between lanes 2 and 5 was not significant (p=0.132). A Student's t-test was used to determine p values.

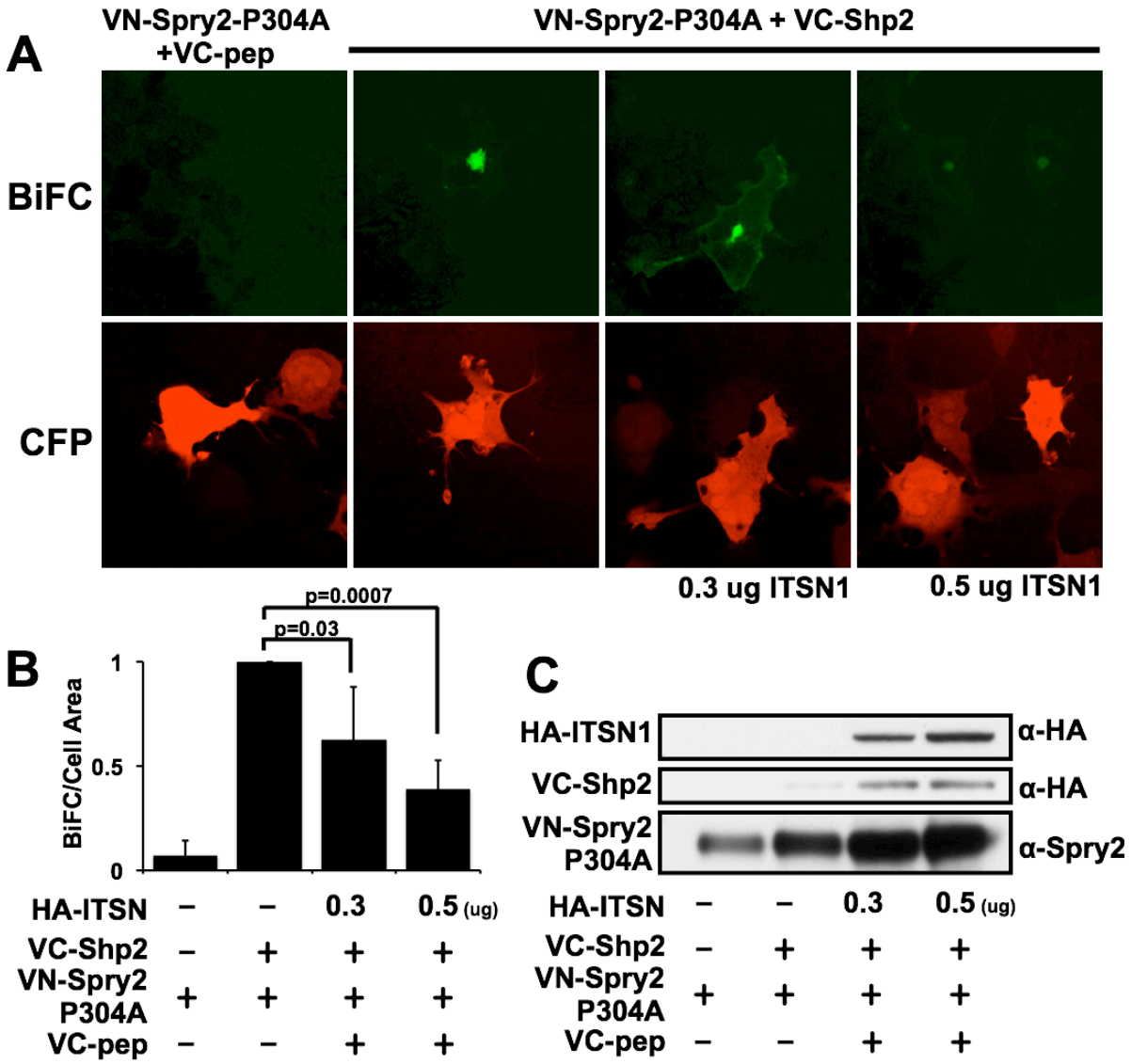

Figure 22. ITSN1 modulation of Spry2:Shp2 interaction depends on ITSN1's interaction with Spry2. (A) Shp2 interaction with Spry2-P304A by BiFC in the presence of increasing ITSN1. ITSN1 overexpression decreased binding of Spry2-P304A to Shp2. (B) Quantification of BiFC signal between Shp2-Spry2-P304A as in Fig. 19. in the presence of increasing ITSN1 levels. Results are the average of three independent experiments. Significance is indicated as in Fig. 19. (C) Expression of HA-ITSN1, VC-Shp2, and VN-Spry-P204A in whole cell lysates

we next tested the consequence on Spry2 phosphorylation. In contrast to the effect of ITSN1 expression on decreasing EGF-induced Spry2 tyrosine phosphorylation (Figure 20), co-expression of ITSN1 with Spry2-P304A enhanced EGF-stimulated Spry2-P304A tyrosine phosphorylation. These results are consistent with the notion that ITSN1 inhibits Shp2-mediated dephosphorylation of Spry2-P304A by sequestration of Shp2 away from Spry2 leading to increased tyrosine phosphorylation of Spry2-P304A (Figure 23).

3.3.5 SHP2 AND ITSN1 ENHANCE CBL-MEDIATED EGFR UBIQUITYLATION

Phosphorylation of Spry2 at Tyr55 results in binding of Spry2 to the NH_2-terminus of Cbl thereby inhibiting Cbl-dependent ubiquitylation of RTKs such as EGFR. Given that Shp2 interacts with and dephosphorylates Spry2 (Hanafusa et al., 2004), we next tested whether Shp2 regulates Spry2 inhibition of Cbl activity, and hence EGFR ubiquitylation. Expression of Shp2-WT or Shp2-D61A reversed Spry2 inhibition of Cbl activity thereby increasing EGFR ubiquitylation (Figure 24, compare lane 4 to lanes 5 & 6). In contrast, addition of Shp2-C463S decreased Cbl-mediated EGFR ubiquitylation (Figure 24, compare lanes 6 and 7).

Our prior results demonstrated that ITSN1 disrupts Spry2 interaction with Cbl to enhance EGFR ubiquitylation (Okur et al., 2012). Given our current findings that ITSN1 recruits Shp2 to dephosphorylate Spry2 thereby blocking Spry2 inhibition of Cbl, we next tested the effect of combined overexpression of Shp2 and ITSN1 on EGFR ubiquitylation. Consistent with earlier data, Shp2 overexpression reversed Spry2 inhibition of Cbl activity and enhanced EGFR ubiquitylation (Figure 25, compare lanes 4

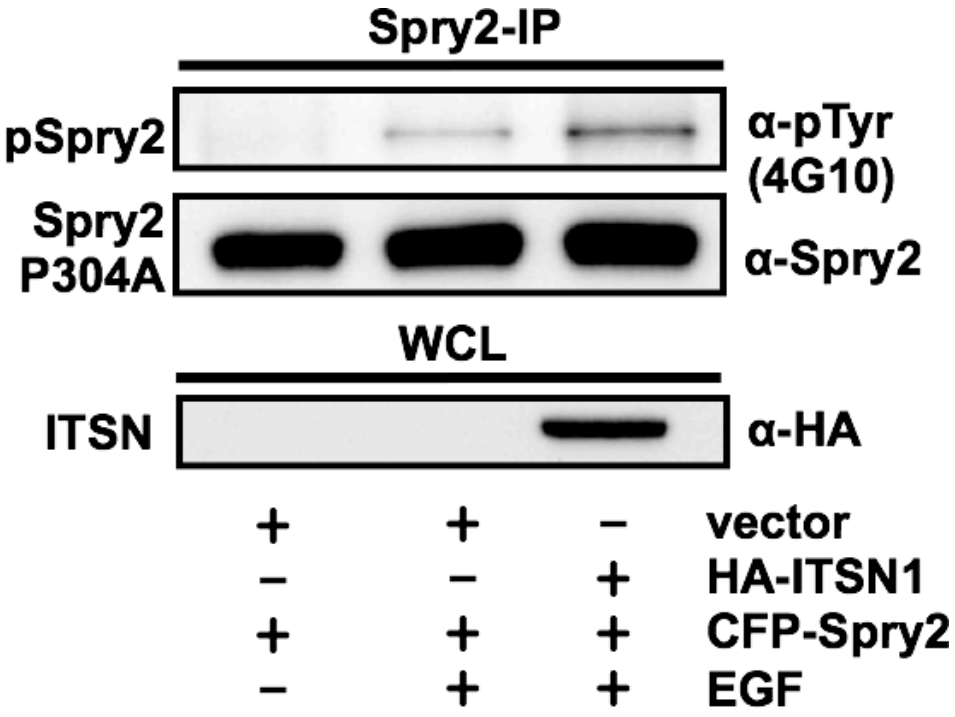

Figure 23. ITSN1 enhances Spry2-P304A Tyr phosphorylation following EGF stimulation. Spry2-P304A was co-expressed with or without ITSN1. COS cells were stimulated with EGF and harvested. Spry2 was immunoprecipitated from lysates with Spry2 antibody and then analyzed by Western blot with either 4G10 (top panel) or α-Spry2 (bottom panel).

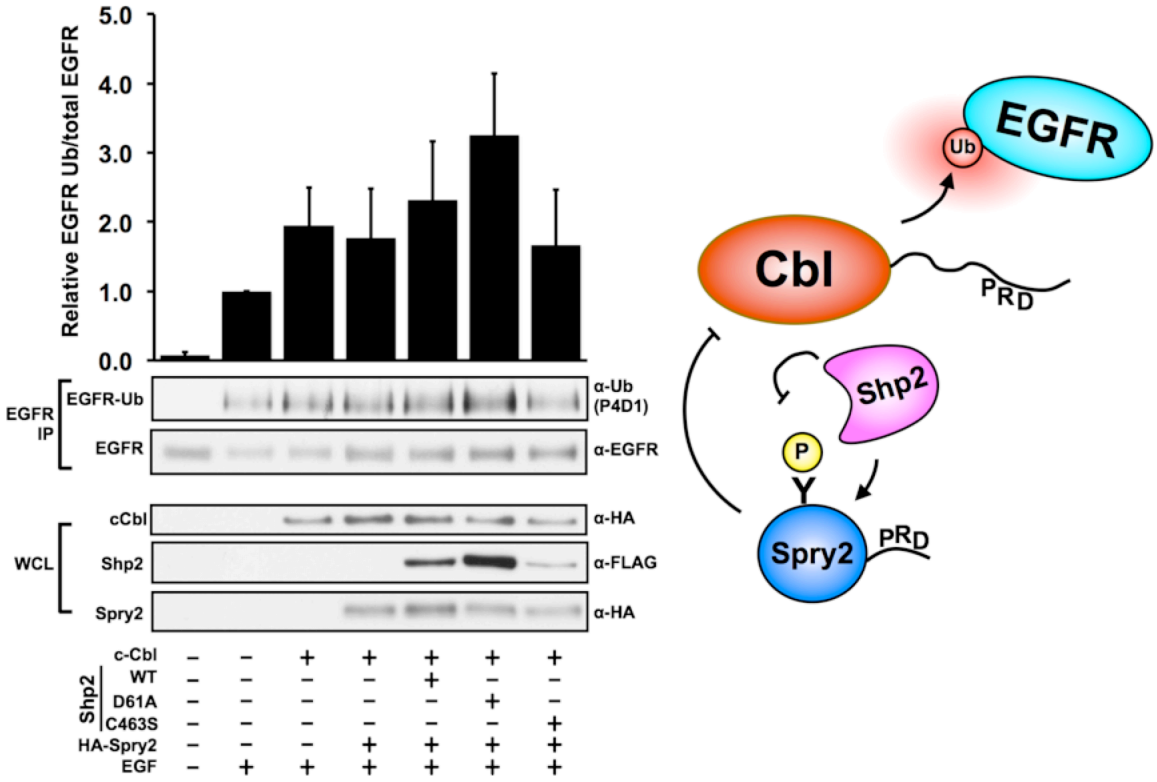

Figure 24. Shp2 enhances EGFR ubiquitylation. HA-Cbl, WT-Shp2, CA-Shp2, DN-Shp2 along with HA-Spry2 were co-expressed in COS cells in different combinations. Following EGF stimulation (5 mins, 100 ng/ml), cells were harvested, endogenous EGFR immunoprecipitated, and then precipitates were fractionated on gels, transferred to PVDF membranes and probed for levels of ubiquitylated EGFR. Whole cell lysates were also probed for level of expression of the various proteins. Overexpression of Shp2-WT or Shp2-D61A in COS cells reversed Spry2 inhibition of Cbl activity and enhanced EGFR ubiquitylation (compare lanes 4&5, p=0.029 or lanes 4&6, p=0.019). However, expression of Shp2-C463S had the opposite effect resulting in decreased Cbl-mediated EGFR ubiquitylation (compare lanes 4&7, p=0.29). A Student's t-test was used to determine p values.

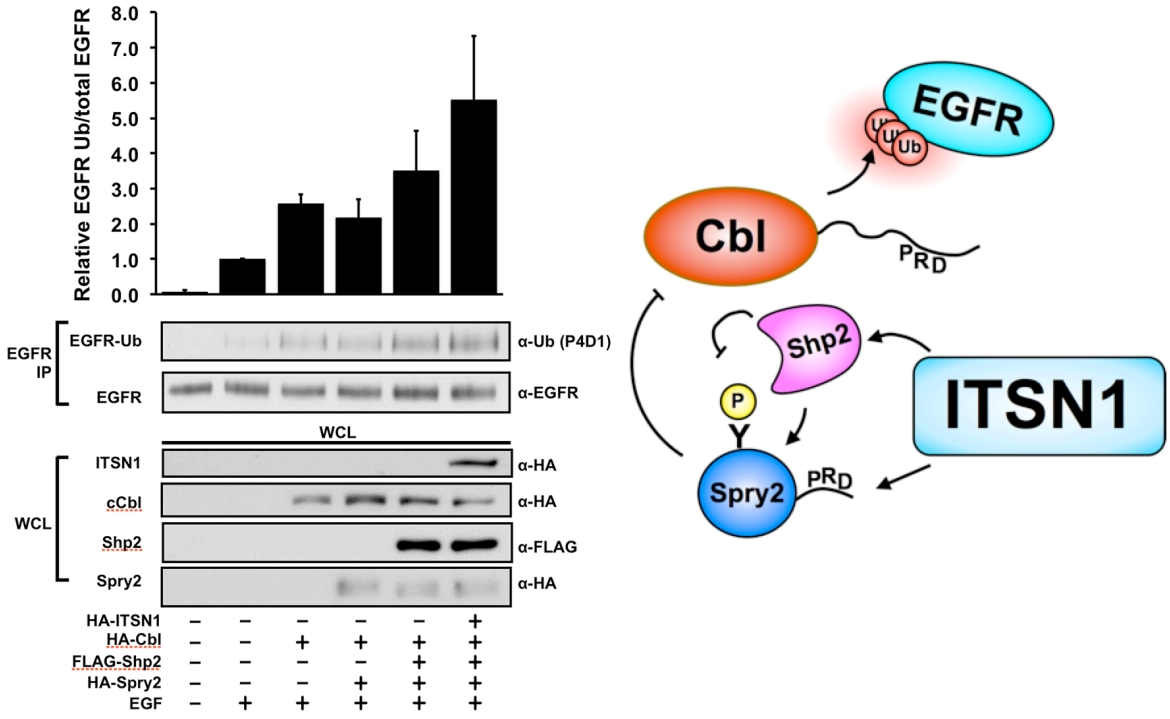

Figure 25. Shp2 and ITSN1 regulate Cbl-mediated EGFR ubiquitylation. HA-Cbl, HA-Spry2, FLAG-Shp2 and HA-ITSN1 were co-expressed in COS cells in different combinations. Cells were harvested following EGF stimulation (5 mins, 100 ng/ml) and endogenous EGFR were immunoprecipitated from cell lysates. Precipitates were fractionated on gels, transferred to PVDF membranes and probed for EGFR ubiquitylation levels. Overexpression of Cbl in COS cells enhanced EGFR ubiquitylation following EGF stimulation (Compare lanes 2&3) but addition of HA-Spry2 inhibited Cbl activity and decreased EGFR ubiquitylation levels (Compare lanes 3&4). Overexpression of FLAG-Shp2 reversed Spry2 inhibition of Cbl activity and enhanced EGFR ubiquitylation (compare lanes 4&5). Addition of ITSN1 further enhanced Shp2‘s effect on EGFR ubiquitylation (compare lanes 5&6).

& 5). Co-expression of ITSN1 with Shp2 further enhanced EGFR ubiquitylation (Figure 25, compare lanes 5 & 6).

3.6 DISCUSSION

We previously demonstrated that ITSN1 enhances EGFR ubiquitylation by blocking the inhibitory effect of Spry2 on Cbl's E3 ubiquitin ligase activity (Okur et al., 2012). However, the precise mechanism through which ITSN1 inhibited Spry2 and enhanced Cbl activity was unclear. Following EGF stimulation, Spry2 is phosphorylated at Tyr55, which is necessary for its interaction with and inhibition of Cbl (Fong et al., 2003; Hall et al., 2003; Mason et al., 2004; Rubin et al., 2003; Wong et al., 2001). Our current studies identified a novel interaction of ITSN1 with Shp2, an SH2-containing tyrosine phosphatase that targets Spry2 dephosphorylation (Hanafusa et al., 2004; Jarvis et al., 2006; Pan et al., 2010). This new finding suggests a mechanism in which EGF stimulation leads to ITSN1 recruitment of Shp2 to the Cbl-Spry2 complex leading to dephosphorylation of Spry2, decreased Cbl-Spry2 interaction, and increased Cbl activity (Figure 26). Ultimately, these changes lead to enhanced EGFR ubiquitylation. Indeed, our results demonstrate that Shp2 association with ITSN1 is induced by EGF stimulation. Furthermore, Shp2-Spry2 association is enhanced with ITSN1 overexpression leading to enhanced Spry2 dephosphorylation. The importance of Shp2 in Spry2 dephosphorylation is supported by results with the Shp2-C463S mutant which reversed the effect of ITSN1 on Spry2 dephosphorylation. Although the Shp2-C463S mutant functions in part as a substrate trapping mutant, it's ability to act as such is weak

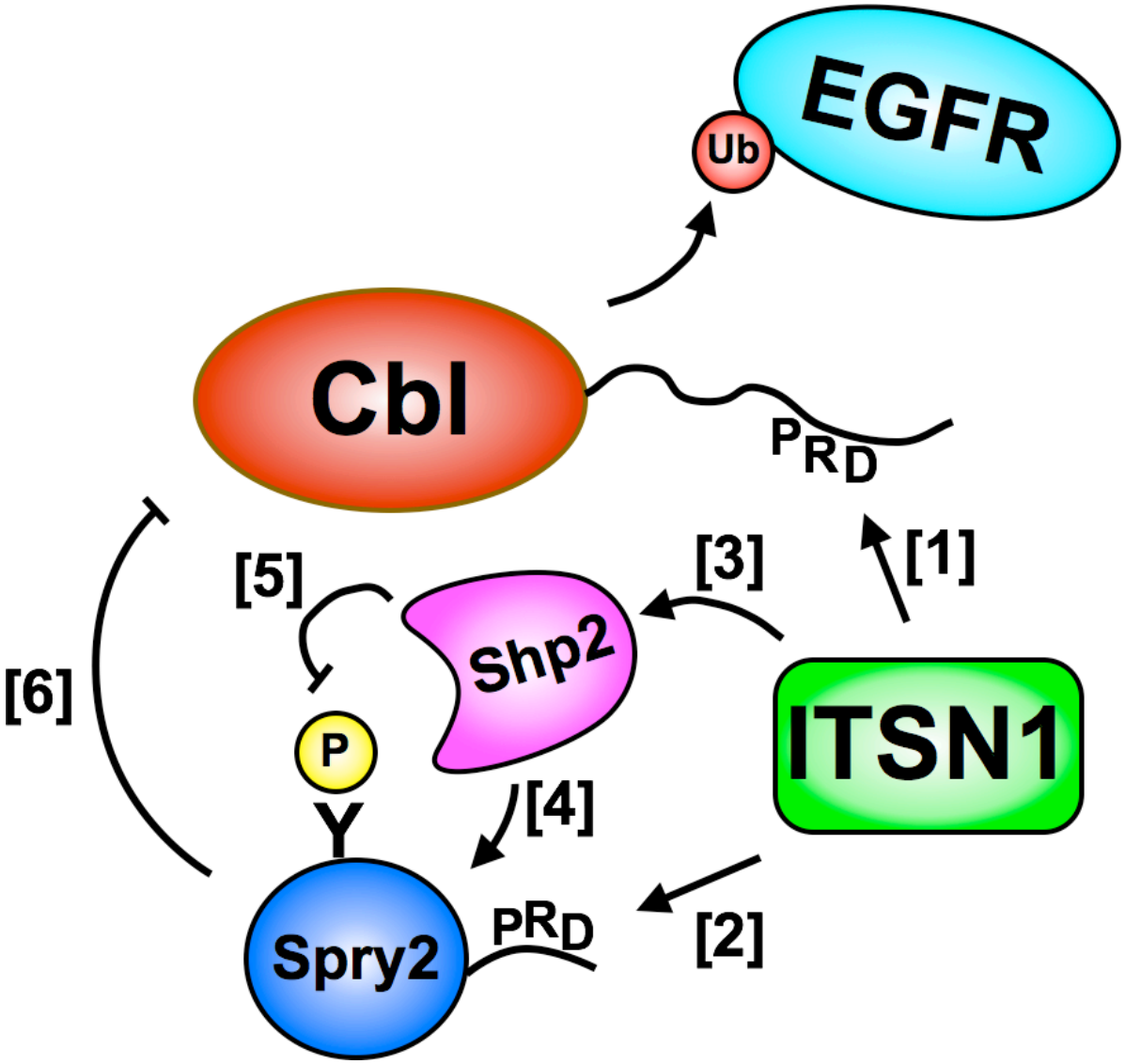

Figure 26. Role of Shp2 in ITSN1-Spry2-Cbl regulation. ITSN1's SH3 domains bind Cbl's PRD (1) and Spry2's PRD (2). Deletion of the Spry2 PRD enhances Cbl-Spry2 binding through an unknown mechanism (Okur et al., 2012) suggesting that ITSN1-binding to Spry2 (2) is inhibitory. Shp2 dephosphorylates Spry2 Tyr55 (5) leading to decreased Cbl binding (Hanafusa et al., 2004). Our results indicate that ITSN1 binds Shp2 (3) and that Shp2 binds Spry2 (4). We hypothesize that ITSN1 recruits Shp2 to the Cbl-Spry2 complex leading to decreased Spry2 tyrosine phosphorylation (5) and decreased Cbl-Spry2 binding (6). The net effect of these interaction is to modulate the activity of Cbl in regulating EGFR ubiquitylation. Note: arrows denote activation and bars denote inhibition.

(Agazie and Hayman, 2003). Therefore, we speculate that Cbl may have a higher affinity for tyrosine phosphorylated Spry2 and effectively out compete the Shp2-C463S mutant for binding Spry2. Thus, ITSN1 and Shp2 together enhanced Cbl-mediated EGFR ubiquitylation suggesting that ITSN1 recruits Shp2 to the Spry2 complex resulting in dephosphorylation of Spry2 thereby relieving the inhibition of Cbl.

Although EGF stimulation enhanced ITSN1-Shp2 interaction, the mechanism for this stimulation-dependent interaction remains unclear. We have been unable to demonstrate EGF-dependent tyrosine phosphorylation of ITSN1 (N. Martin and J.P. O'Bryan, unpublished data) similar to results from Rynditch and colleagues (Novokhatska et al., 2013). Our domain analysis experiments indicate that the coiled-coil region of ITSN1, and to a lesser extent, the SH3 region are sufficient for binding Shp2 (Figure 15B). However, our data suggest that ITSN1's coiled-coil region is the predominant means for binding Shp2. Based on coiled-coil algorithms coupled with the structure of Shp2 (PDB 2SHP) (Hof et al., 1998), there are two coiled-coil regions in Shp2 which may function as ITSN1 docking sites. These regions, encompassing aa 216-258 and 510-543, lie adjacent to one another in the overall Shp2 structure. This arrangement suggests that ITSN1 may interact with Shp2 in a bivalent manner with each of these coiled regions. Future work will be necessary to define the precise mechanism by which ITSN1 and Shp2 interact. However, given the importance of tyrosine phosphorylation of Shp2 for its activity (Grossmann et al., 2010), interaction of Shp2 with ITSN1 may also facilitate tyrosine phosphorylation and activation of Shp2 thereby enhancing Spry2 dephosphorylation.

In addition to our studies, Shp2 and Sprouty interaction has been reported by several other groups (Hanafusa et al., 2004; Jarvis et al., 2006; Pan et al., 2010; Quintanar-Audelo et al., 2011). In contrast to these studies which employed FGF, we used EGF for stimulation of cells based on our prior results (Martin et al., 2006; Okur et al., 2012). In the background of ITSN1 overexpression, increased Shp2 levels enhanced Spry2 dephosphorylation and expression of the Shp2-C463S mutant reversed this effect. These results suggest that Shp2 regulates EGF-induced Spry2 tyrosine phosphorylation. In support of this finding, Hanafusa and colleagues observed that titrating Shp2 levels resulted in a significant reduction in FGF-induced Spry2 phosphorylation (Hanafusa et al., 2004). Although Quintanar-Audelo and colleagues observed that Shp2 bound Spry2 and that this interaction was disrupted with Spry2-Y55F mutation, consistent with our results, they proposed that Spry2 might not be the preferred Sprouty family member targeted by Shp2 (Quintanar-Audelo et al., 2011). They observed that Shp2 overexpression resulted in Spry2 dephosphorylation although the effect was quite modest (6% reduction) in comparison to Shp2's effect on SPRED proteins (52% reduction) (Quintanar-Audelo et al., 2011). Although the reasons underlying these experimental differences are unclear, Pan and colleagues observed that genetic deletion of Shp2 resulted in increased Spry2 tyrosine phosphorylation consistent with our conclusion that Shp2 regulates the tyrosine phosphorylation of Spry2 (Pan et al., 2010).

The involvement of Shp2 in regulating Cbl and thus EGFR ubiquitylation is quite interesting. Our previous work demonstrated that interaction of ITSN1 with Spry2 modulates Cbl:Spry2 association in a dose-dependent manner thereby regulating EGFR

ubiquitylation. Involvement of an additional protein, Shp2, as a ITSN1 and Spry2 binding partner into this network of interactions adds a further level of complexity to the regulation of Cbl. Results with the Spry2-P304A mutant suggest that loss of ITSN1 binding to Spry2 leads to the sequestration of Shp2 from the Cbl-Spry2 complex. As a result, Spry2 tyrosine phosphorylation levels are elevated leading to enhanced Cbl interaction and inhibition of EGFR ubiquitylation. These results are consistent with our earlier work, and provide a mechanistic explanation for our observation that ITSN1 enhances the inhibitory effect of Spry2-P304 on Cbl (Okur et al., 2012). However, the question remains as to how Shp2 regulation of Cbl is related to Shp2's role in activating the ERK-MAPK pathway. As described earlier, multiple mechanisms have been described whereby Shp2 may contribute to ERK-MAPK activation by RTKs (Grossmann et al., 2010). It has been proposed that Spry2 tyrosine phosphorylation at Y55 is necessary for Spry2's antagonistic role in ERK-MAPK activation by RTKs. Thus, dephosphorylation of Spry2 by Shp2 would be consistent with blocking Spry2's inhibition of ERK-MAPK and Shp2's role in activation of this pathway. However, Spry2 has distinct, and sometimes opposite, functions downstream of different RTKs. For example, Bar-Sagi and colleagues describe a bimodal function for Spry2 in regulating EGFR signaling (Egan et al., 2002). The Cysteine-rich domain (CRD) serves to inhibit EGFR activation of ERK-MAPK but only when Spry2 is not bound to Cbl. Thus, dephosphorylation of Spry2 by Shp2 in this model would inhibit EGFR activation of ERK-MAPK through unmasking the inhibitory effect of the CRD. However, this inhibition may be tempered by Shp2's additional functions in stimulating Src family kinases and

inhibiting RasGAP. Thus, the net effect of Shp2 in this context of EGFR signaling may lead to enhanced ERK-MAPK activation.

While our results indicate that Shp2 enhances EGFR ubiquitylation, our data do not address the functional consequences of this ITSN1-Cbl-Shp2-Spry2 complex to EGFR signaling. Ubiquitylation of the EGFR is necessary for the proper trafficking and degradation of the receptor. However, signaling by the receptor is controlled by the proper spatial localization of the receptor which involves multiple pathways including ubiquitylation. Indeed, Spry2 regulate the movement of EGFR from early endosome to late endosome independent of its interaction with Cbl (Kim et al., 2007). Thus, additional studies will be needed to define the importance of this pathway to EGFR signaling.

Our findings reveal a complex network of interactions between ITSN1, Shp2, Spry2 and Cbl during the ubiquitylation process, regulation of which is critical to modulating the trafficking of RTKs in the cell. The ITSN1 gene is localized to the Down Syndrome (DS) critical region of human chromosome 21 and overexpressed in the brains of DS patients (Hunter MP, 2011) suggesting a role for this scaffold protein in DS biology (Keating et al., 2006). Given the presence of endocytic trafficking perturbations in the pathology of DS (Keating et al., 2006), elucidation of the functional role of ITSN1 in RTK ubiquitylation and trafficking will aid in understanding the defective trafficking mechanisms contributing to DS pathogenesis.

4.1 CONCLUSIONS AND FUTURE DIRECTIONS

My dissertation work has revealed a complex network of interactions between multiple proteins that regulate the receptor ubiquitylation process. However, it should be noted that each protein in this network has diverse functions and is involved in additional pathways in the cell. Therefore, the total expression levels or spatial and temporal interactions of these proteins with each other or with other proteins outside of the network might limit the availability of a member of the complex for another unique pathway and affect the physiological outcome. This phenomena usually reveals itself better if the proteins in the complexes are scaffolds or inhibitors that function by sequestering the target protein since their total expression levels and availability is crucial for the signaling pathways in which they are involved. ITSN1 is one example for this phenomenon. It is a scaffold protein with multi-modular domains with different cellular functions. One of the features of scaffold proteins is that they function at an optimum concentration in cells. If scaffolds are expressed at too low or high of a level, this can decrease or inhibit signaling due to formation of incomplete and inactive complexes leading to inefficient signaling (Levchenko et al., 2000). Indeed, Martin and colleagues observed that high amounts of ITSN1 or depletion of ITSN1 in cells both decrease EGFR internalization (Martin et al., 2006), demonstrating the importance of scaffold concentration for signal transduction pathways.

Spry2 is a protein whose distribution and availability in the cell may differentially affect pathways due to its functions as a sequestering inhibitory protein and its diverse roles in signaling pathways. Indeed, Spry2's mode of action is quite interesting regarding MAPK signaling. Spry2 represses FGF- and VEGF- induced MAPK activation

but enhances EGF-induced signaling. The molecular basis of this dual but opposing mode of action of Spry2 is not yet known. However, it is widely accepted that the positive regulatory role of Spry2 on signaling is attributed to inhibition of Cbl activity (Egan et al., 2002). Following EGF stimulation, Spry2 sequesters Cbl away from EGFR and blocks Cbl-mediated EGFR degradation, thereby resulting in sustained signaling from the activated receptors.

During my research, I discovered that ITSN1 enhances Spry2 Tyr55 dephosphorylation through recruitment of the Shp2 tyrosine phosphatase which in turn, disrupts the inhibitory effect of Spry2 on Cbl activity for EGFR ubiquitylation and signal termination. However, growth factor induced Spry2 Tyr55 phosphorylation is also crucial for Spry2 inhibition of MAPK signaling (Martinez et al., 2007) since Spry2 Y55A mutation reverses the inhibitory effect of Spry2 on growth-factor induced ERK activation. This result raises the question of whether ITSN1 enhances ERK activation by promoting the dephosphoylation of Spry2. Earlier studies in our laboratory showed that ITSN 1overexpression alone did not induce or alter ERK-MAPK activation. However, ITSN1 silencing decreased EGF induced ERK-MAPK signaling in the same cells. These results could be due to enhanced Spry2 phosphorylation in the absence of ITSN1. Indeed, Martinez and colleagues demonstrated that expression of Spry2 Pro304 mutant, which is impaired in ITSN1 binding, further inhibited ERK activation compared to WT Spry2 (Martinez et al., 2007). However, there are other potential pathways where ITSN1 might negatively or positively regulate growth factor-stimulated ERK signaling. First, ITSN1 recruits endocytic accessory factors to the vesicle and is necessary for endocytic complex formation which regulates internalization. Vieira and colleagues

(1996) reported that both EGF-induced EGFR and ERK phosphorylation are suppressed in endocytosis-defective cells. ITSN1 silencing is also reported to cause inhibition of EGFR internalization which indeed might lead to suppression of ERK activation by EGF stimulation due to defects in endocytosis (Martin et al. 2006). On the other hand, ITSN1 enhances Cbl activity resulting in enhanced EGFR ubiquitylation. Whereas the effect of ubiquitylation on internalization is still under debate, it is commonly accepted that ubiquitylation enhances receptor trafficking to lysosomal degradation, thereby terminating the signaling from receptors. Indeed, overexpression of c-Cbl nearly abolished EGF-induced MAPK activation (Waterman at al. 1999). To summarize, ITSN1 function in endocytosis might have a promoting effect on EGF-induced ERK/MAPK signaling whereas ITSN1 enhancement of Cbl-mediated EGFR degradation or ITSN1-enhanced Spry2 dephosphorylation might affect ERK/MAPK signaling negatively. It seems ITSN1 and Spry2 exerts their functions on signaling pathways differently depending on their interactions with other proteins. Thus, their cumulative effect on signaling should be considered while examining their functional role in future studies.

Although not examined in my studies, the possible effect of ITSN1 on Spry2 binding to Grb2 should also be considered due to the role of Grb2 in ERK signaling. EGF stimulation induces Spry2 interaction and co-localization with Grb2 (Hanafusa et al. 2002; Tefft et al 2002) and this binding sequesters Grb2 and impedes MAPK activation following FGF stimulation. It has been reported that Spry2-Grb2 complex formation might block FGF induced Ras-GTP loading (Gross et al., 2001) or recruitment of the Grb2–Sos complex to receptor to initiate signaling (Hanafusa et al., 2002). In

spite of these studies, the effect of Spry2 on Grb2 still remains controversial. Martinez and colleagues reported that ERK inhibition by Spry2 is independent of its interaction with Grb2 since disruption of Spry2 and Grb2 interaction does not reverse the inhibitory effect of Spry2 on growth factor-induced ERK activation (Martinez et al., 2007). However, they used Spry2 P304A to block Grb2 and Spry2 interaction and our studies showed this mutation also disrupts ITSN1 and Spry2 binding. Given that ITSN1 inhibits Spry2 phosphorylation, disruption of ITSN1 and Spry2 interaction could lead to increased Spry2 phosphorylation, which in turn, could further enhance the inhibitory effect on Spry2 on ERK activation by a different mechanism although Spry2 inhibition of Grb2 is disrupted. The fact that ITSN1 and Grb2 binds to the same Pro-rich domain on Spry2 raises other questions. Do ITSN1 and Grb2 interact with Spry2 competitively? Can they both bind to the same Spry2 protein simultaneously? Future studies addressing these questions are important because complex formation of Spry2 with these proteins competitively or simultaneously could lead to different signaling outcomes since ITSN1 acts as a Spry2 inhibitor and Spry2 acts as a Grb2 inhibitor. In addition, it could be informative to examine whether the sequestering role of Spry2 on Cbl or Grb2 is regulated from the same pool of Spry2 or different unique pools potentially due to different localization.

A similar phenomenon is seen with ITSN1-mediated Ras signaling. ITSN1 is reported to activate Ras on a subset of intracellular vesicles. However, this pool of Ras does not activate either the ERK or JNK MAPK pathways (Mohney et al., 2003). Thus, like Ras, different pools of Spry2 might be regulated by different pathways. If Spry2-mediated events are regulated from the same pool of Spry, it might be important to test

whether the availability of Spry2 for its inhibitory effect on Cbl causes depletion of Spry for its other sequestering roles and limits its availability to participate in other pathways.

In addition to ITSN1 and Grb2, another protein, CIN85, also associates with the same PXXPXR Pro-rich region of Spry2. Interaction of CIN85 with Spry2 is interesting because this protein also binds to Cbl (Soubeyran et al., 2002) and it has been identified as an ITSN1 binding partner in yeast two-hybrid system (Martin et al., 2006). CIN85 is very similar to ITSN1 with regard to function and structure. They both have SH3 and CC domains and they are both involved in the regulation of EGFR ubiquitylation and Cbl function (Martin et al., 2006; Soubeyran et al., 2002). CIN85 is reported to associate with endophilin and form a complex with Cbl to down-regulate EGFR after EGF stimulation. However, Spry2 interacts with CIN85 and acts at the Cbl/ CIN85 interface to block CIN85-mediated clustering of Cbl (Haglund et al., 2005). Given that ITSN1 disrupts Cbl and Spry2 interaction and blocks inhibitory effect of Spry2 on Cbl, these results suggest that the interaction dynamics between ITSN1, Cbl, CIN85 and Spry2 might regulate EGFR down-regulation. In addition to Shp2 recruitment to Spry2, it is possible that ITSN1 might compete with CIN85 for Spry2 binding and block the inhibitory effect of Spry2 on Cbl and CIN85. Further studies will be necessary to elucidate this possible mechanism.

In addition to its diverse interactions, spatial and temporal status of Spry2 and its availability for certain pathways at unique locations in the cell or in specific cell types could result in different signaling outcomes since Spry2 is a sequestering inhibitory protein and modified following growth factor stimulation. Interestingly, Egan and

colleagues reported that C- and N- termini of Spry2 have opposing effects on MAP kinase activation with regards to EGFR signaling (Egan et al., 2002). They demonstrated that C-terminal deletion of Spry2 potentiates ERK signaling whereas N-terminal deletion cause not only loss of signaling enhancement but also further inhibition of EGF-induced ERK activation suggesting that the inhibitory functions of Spry2 reside in the C-terminus while potentiating functions of Spry2 on signaling reside in the N-terminal domain. These findings raise a question of how dual but opposing functions of the two ends of Spry2 are regulated. Do they compete with each other to exert their effects and/or are functions of each of Spry2's ends differentially limited or activated? Given the involvement of Spry2 in the regulation of complicated mechanisms, I favor the last option since it allows Spry2 to tightly regulate the signaling processes. Spry2 is phosphorylated at Tyr55 in response to EGF stimulation but this phosphorylation is relatively transient and can be detected from 3 mins up to 30 mins or less although total Spry2 levels can still be detected at least up to 2 hours (Mason et al., 2004) suggesting that there is a temporal N-terminal activation of Spry2. Therefore I speculate the following mechanism regarding Spry2 effect on EGF-induced ERK signaling. Cbl ubiquitylation of EGFR occurs immediately around 30 seconds following stimulation, suggesting that Cbl effect on EGFR is among one of the first events following receptor activation (Yokouchi et al., 1999). Cbl is internalized and degraded at lysosomes along with the receptor after activation, causing not only Cbl depletion but also signal termination. Simultaneously, the same EGF induction also causes recruitment of Spry2 to the cell membrane where the N-terminus of Spry2 is tyrosine phosphorylated (Tyr55).

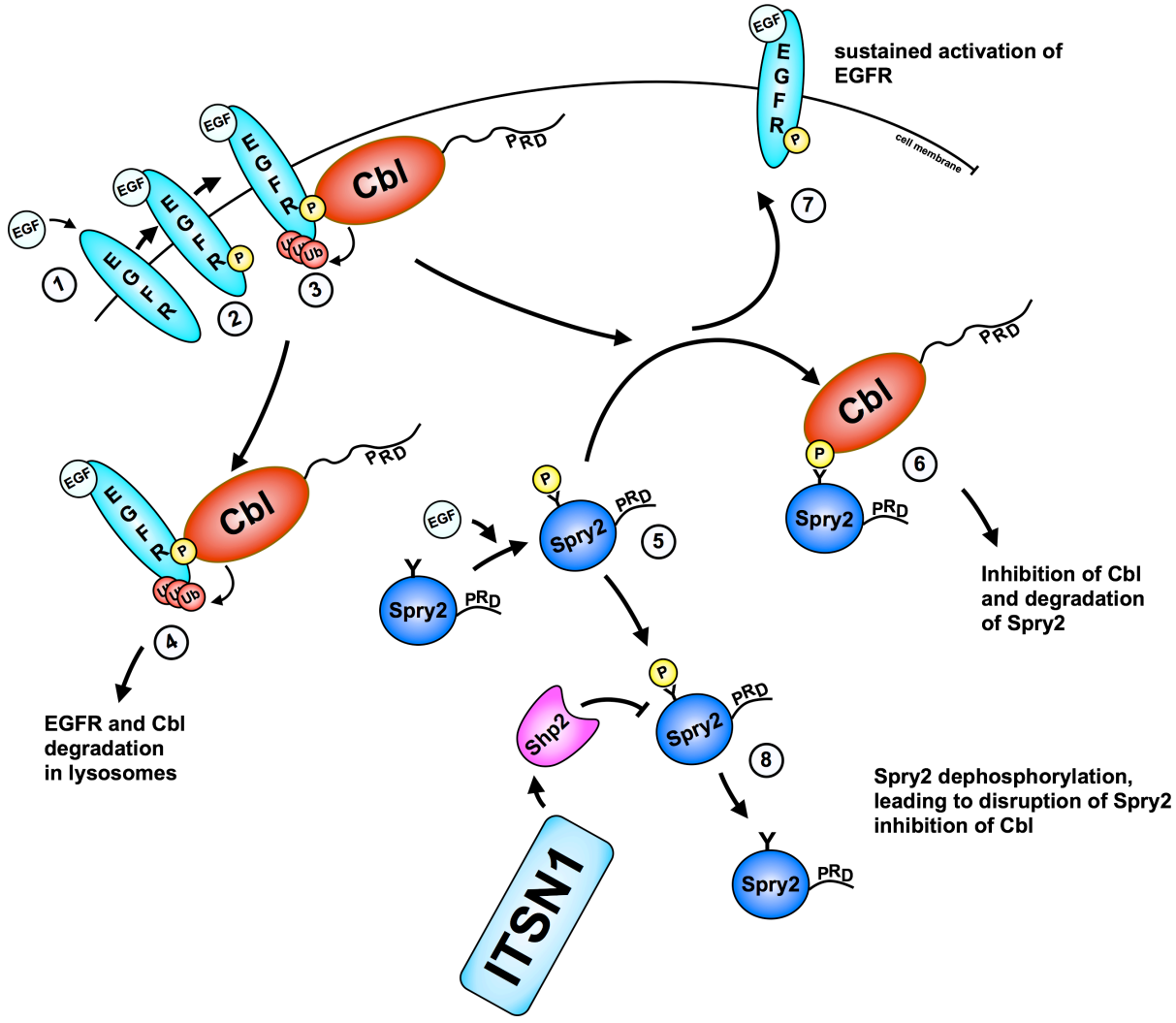

Figure 27. ITSN1 regulation of Cbl-mediated EGFR ubiquitylation. EGF binds to extracellular domain of EGFR (1). Binding of EGF leads to autophosphorylation and activation of the receptor (2). Cbl interacts with phosphorylated receptor and mediates its ubiquitylation (3), resulting in EGFR internalization and degradation at lysosome along with Cbl. (4). EGF stimulation also leads to Spry2 phosphorylation (5). Then, modified Spry2 binds to Cbl (6) and acts as a positive regulator of signaling by preventing Cbl from continuously ubiquitylating EGFR, thereby leading to sustained activation of the receptor (7). In the meantime, ITSN1 recruits Shp2 phosphatase to Spry2 and causes Spry2 dephosphorylation, thereby controlling Spry2 inhibition of Cbl to ensure fine-tuning of RTK signaling (8).

Then, modified Spry2 binds to Cbl and acts as a positive regulator of signaling by preventing Cbl from continuously ubiquitylating EGFR, thereby leading to sustained activation of the receptor. In the meantime, ITSN1 recruits Shp2 phosphatase to Spry2 and causes Spry2 dephosphorylation, thereby controlling Spry2 inhibition of Cbl to ensure fine-tuning of RTK signaling (Figure 27). In addition, binding of phosphorylated Spry2 to Cbl also causes Cbl-mediated Spry2 ubiquitylation and depletion of positively-acting Spry2 through the proteasomal degradation pathway. The potentiating role of N-terminus Spry2 is transient and limited due to depletion of phosphorylated Spry2. However, Spry2 levels continue to increase due to EGF-induced Spry2 expression (Ozaki et al., 2001). Newly synthesized Spry2 or dephospohrylated Spry2 (resulting from Shp2 recruitment through ITSN1) will probably act as a negative regulator of ERK signaling due to the inhibitory role of Spry2 C-terminal region. Therefore, Spry2 initially acts as an activator of EGFR signaling due to its N-terminus and subsequently acts as an inhibitor due to its C-terminus following its dephosphorylation or new synthesis.

My research and previous studies in the laboratory regarding EGFR ubiquitylation were mostly conducted with forced expression systems. Therefore, it will be important to test the proposed mechanism with endogenous studies. For example, the depletion of ITSN1 on Cbl-mediated EGFR ubiquitylation has not been tested. Our prediction is that ITSN1 knock-down would result in decreased EGFR ubiquitylation. In addition, the effect of ITSN1 on Spry2 phosphorylation in the background of Shp2 depletion remains to be investigated.

ITSN1 is localized to DS critical region and overexpressed in the brains of DS patients and in the mouse model for DS (Gardiner et al., 2003; Pucharcos et al., 1999). Martin and colleagues discovered that ITSN1 regulates RTK internalization and trafficking, and overexpression of ITSN1 enhances RTK degradation in lysosomes, suggesting that increased ITSN1 levels in DS may alter RTK trafficking in these patients as well. Indeed, defects in endosomal trafficking are one of the earliest hallmarks of DS. Thus, elucidation of the functional role of ITSN1 in RTK ubiquitylation and trafficking will aid in understanding of the defective trafficking mechanisms contributing to DS pathogenesis.

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6. APPENDIX

Portion of the Work demonstrated in Chapter 2 was published in a journal mentioned below;

Molecular and cellular biology : MCB

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Portion of the Work demonstrated in Chapter 3 was published in a journal mentioned below;

Receptor tyrosine kinase ubiquitylation involves the dynamic regulation of Cbl-Spry2 by intersectin 1 and the Shp2 tyrosine phosphatase.

Okur MN, Russo A., O'Bryan JP.

Mol Cell Biol. 2013.

7. VITA

NAME: MUSTAFA NAZIR OKUR

EDUCATION:

Ph.D. in Department of Biochemistry & Molecular Genetics, University of Illinois at Chicago, Chicago, USA, 2013

B.S. in Molecular Biology and Genetics, Bogazici University,
Istanbul, TURKEY, 2006

PUBLICATIONS:

1. Receptor tyrosine kinase ubiquitylation involves the dynamic regulation of Cbl-Spry2 by intersectin 1 and the Shp2 tyrosine phosphatase.
Okur MN, Russo A, O'Bryan JP.
Mol Cell Biol. 2013 October.

2.Intersectin 1 enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction.
Okur MN, Ooi J, Fong CW, Martinez N, Garcia-Dominguez C, Rojas JM, Guy G, O'Bryan JP.
Mol Cell Biol. 2012 Feb;32(4):817-25. Epub 2011 Dec 12.

3. Intersectin (ITSN) family of scaffolds function as molecular Hubs in protein interaction networks.
Wong KA, Wilson J, Russo A, Wang L, **Okur MN**, Wang X, Martin NP, Scappini E, Carnegie GK, O'Bryan JP.

4. The Role of PI3K/Akt in Insulin-Mediated Regulation of Cu Transporter ATP7A Function Required for Full Activation of Extracellular SOD in Vasculature: Novel Protective Mechanism for Endothelial Dysfunction in Type I Diabetes.
Sudhahar V, Urao N, McKinney RD, **Okur MN**, O'Bryan JP, Ushio-Fukai M, Fukai AHA Scientific sessions 2011, Nov 12-16, Orlando, FL. USA**. (in preparation)**

5. Proliferation defects and genome instability in cells lacking Cul4A.
Kopanja D, Stoyanova T, **Okur MN**, Huang E, Bagchi S, Raychaudhuri P.
Oncogene. 2009 Jul 2;28(26):2456-65. doi: 10.1038/onc.2009.86. Epub 2009 May 11.

PRESENTED ABSTRACTS:

1. Intersectin enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl-Shp2 interaction.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Cognition in Down Syndrome, 2013 March. Presented Abstract

2. Intersectin enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Pharmacology Department Retreat, 2012 September. Presented Abstract

3. Intersectin enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Post-Translational Regulation of Cell Signaling, 2012 August. Presented Abstract

4. Intersectin enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Chicago Symposium on Cell Signalling, 2012 May. Presented Abstract

5**.** Intersectin enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Cancer Research Forum, UIC, 2012 March. Presented Abstract

6. IQGAP1, a VEGFR2 Scaffold Protein, Sequesters c-Cbl and Regulates VEGF Receptor 2 Stability: Role In VEGF-induced Angiogenesis
Seok-Jo Kim, Masooma Razvi, Norifumi Urao, John P O'Bryan, **Mustafa Nazir Okur**, Ronald D McKinney, Tohru Fukai, Masuko Ushio-Fukai.
AHA Scientific sessions 2011, Nov 12-16, Orlando, FL. USA.

7**.** The Role of PI3K/Akt in Insulin-Mediated Regulation of Cu Transporter ATP7A Function Required for Full Activation of Extracellular SOD in

Vasculature: Novel Protective Mechanism for Endothelial Dysfunction in Type I Diabetes.
Sudhahar V, Urao N, McKinney RD, **Okur MN**, O'Bryan JP, Ushio-Fukai M, Fukai
AHA Scientific sessions 2011, Nov 12-16, Orlando, FL. USA.

8. Intersectin enhances Cbl ubiquitylation of epidermal growth factor receptor through regulation of Sprouty2-Cbl interaction.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
COM Research Forum, 2011 Nov. Presented Abstract

9. ITSN regulation of EGFR ubiquitylation and degradation.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Biochem. and Molecular Genetics Retreat Poster Presentation, 2011 Oct. Presented Abstract

10. ITSN regulation of EGFR ubiquitylation and degradation.
Mustafa Nazir Okur, Jolene Ooi Yu Zhu, Chee-Wai Fong, Natalia Martinez, Carlota Garcia-Dominguez, Jose M. Rojas, Graeme Guy, John P. O'Bryan
Pharmacology Retreat Poster Presentation, 2011 Sept. Presented Abstract

HONOURS, SCHOLARSHIPS, PRIZES AND AWARDS

Fall 2012	Woeltjen Student Achievement Award, 2012
Fall 2012	3rd Place, Woeltjen Presentation Award, 2012
Spring 2012	Fondation Jerome Lejeune February 2012, Session 2011b, 36.000€ over two years
Summer 2005	Scientific Scholarships: Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany - Dr. Christian Eckmann Laboratory -700 Euro
Summer 2004	Scientific Scholarships: Department of Molecular Genetics and Microbiology, Duke University Durham, NC, USA - Dr. Robin Wharton Laboratory - 6000 USD