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INTERSECTIN REGULATES EPIDERMAL GROWTH FACTOR RECEPTOR ENDOCYTOSIS, UBIQUITYLATION, AND SIGNALING

Negin P. Martin, Robert P. Mohney, Sara Dunn, Margaret Das, Erica Scappini and John P. O'Bryan*

Laboratory of Signal Transduction (N.P.M., R.P.M., M.D., E.S. J.P.O.) and Laboratory of Neurobiology (N.P.M., E.S.), National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina 27709; Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL 60612 (S.D., J.P.O.)

Running Title: ITSN binds c-Cbl and regulates EGFR trafficking and signaling

*Address correspondence to: John P. O'Bryan, Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL 60612: Tel: 312-996-6221; Fax: 312-996-1225; Email: obryanj@uic.edu;

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Abbreviations: ITSN, intersectin; YFP, yellow fluorescent protein; EGF(R), epidermal growth factor (receptor); RTK, receptor tyrosine kinase; RNAi, RNA interference; GST, glutathione-*S*-transferase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; HA, hemagglutinin; HRP, horseradish peroxidase; SH2/3, Src homology 2/3; PRD, Pro-rich domain; PTB, phosphotyrosine binding domain;

Abstract

Receptor tyrosine kinases (RTK) are critical for normal cell growth, differentiation and development but contribute to various pathological conditions when disrupted. Activation of RTKs stimulates a plethora of pathways, including the ubiquitylation and endocytosis of the receptor itself. Although endocytosis terminates RTK signaling, it has emerged as a requisite step in RTK activation of signaling pathways. Previously, we discovered that the endocytic scaffolding protein intersectin (ITSN) cooperated with EGFR in the regulation of cell growth and signaling. However, a biochemical link between ITSN and EGFR was not defined. In this study, we demonstrate that ITSN is a scaffold for the E3 ubiquitin ligase Cbl. ITSN forms a complex with Cbl *in vivo* mediated by the SH3 domains binding to the Pro-rich COOH-terminus of Cbl. This interaction stimulates the ubiquitylation and degradation of the activated EGFR. Furthermore, silencing ITSN by RNAi attenuated EGFR internalization as well as activation of the ERK-MAPK pathway thereby demonstrating the importance of ITSN in EGFR function. Given the cooperativity between ITSN and additional RTKs, these results point to an important evolutionarily conserved, regulatory role for ITSN in RTK function that is necessary for both signaling from receptors as well as the ultimate termination of receptor signaling.

RTKs control a plethora of intracellular pathways involved in cell growth, differentiation, development and apoptosis through translating extracellular stimuli into biochemical signals that alter cellular function. These receptors activate numerous signaling pathways, with the Ras-MAPK pathway as perhaps the most well-characterized of these pathways. Upon ligand binding, receptors dimerize and undergo trans-autophosphorylation on tyrosine residues thereby creating high affinity binding sites for proteins with Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Pawson and Nash, 2003). These phosphotyrosine binding proteins recruit components of specific pathways such as Shc, Grb2, Src, PI3K, PLCγ, or Cbl, thus ultimately affecting cell fate decisions regarding growth, differentiation and apoptosis.

Following activation, a receptor must be inactivated to prevent chronic stimulation of cells. Prolonged activation of RTKs resulting from receptor amplification, chromosomal translocation, or point mutations is associated with development and progression of numerous tumors (Blume-Jensen and Hunter, 2001). However, a number of feedback control mechanisms aid in attenuating activated RTKs including Ser/Thr phosphorylation of the receptor or its substrates, activation of phosphatases, and endocytosis. Ubiquitylation also plays an important role in trafficking and degradation of RTKs by lysosomes. In the case of EGFR, monoubiquitylation is sufficient for receptor internalization, although more recent studies suggest that EGFR is monoubiquitylated at multiple sites (Haglund et al., 2003). c-Cbl is an E3 ubiquitin ligase responsible for the ubiquitylation of EGFR (Schmidt and Dikic, 2005). The amino-terminal SH2-like tyrosine kinase binding (TKB) domain of Cbl binds phosphotyrosine sites within the activated receptor thereby allowing the RING finger domain of c-Cbl to catalyze the transfer of ubiquitin from an E2-ubiquitin complex to the EGFR. Ubiquitylation of EGFR results in the

recruitment of endocytic proteins containing ubiquitin-interacting motifs such as Eps15 (de Melker et al., 2004). Overexpression of c-Cbl increases the degradation of EGFR, whereas dominant-negative mutants of Cbl with impaired ligase activity (i.e. v-Cbl or 70Z-Cbl) inhibit down-regulation of EGFR (Schmidt and Dikic, 2005). In addition to the TKB and RING finger domains, Cbl also contains a Pro-rich domain (PRD) that recruits a number of SH3-containing proteins such as Grb2, Nck, Src and CIN85 (Schmidt and Dikic, 2005). Although these interacting proteins are important in Cbl regulation, deletion of the PRD of Cbl has mixed effects on Cbl's ability to regulate RTKs (Petrelli et al., 2002). Thus, the details of how these interacting proteins regulate Cbl remain unclear.

Endocytosis is also important for the activation of specific signaling pathways although the mechanistic details of this activity remain unclear [reviewed in (Sorkin and Von Zastrow, 2002)]. For example, endocytosis is involved in the activation of MAPK by RTKs and G-protein coupled receptors (Ceresa and Schmid, 2000). Furthermore, endocytic vesicles provide a platform for compartmentalized activation of particular pathways (Sorkin and Von Zastrow, 2002). These findings suggest that endocytosis is both a positive mediator of cellular signaling as well as an attenuator of those signals.

We have demonstrated that the endocytic scaffolding protein ITSN stimulates mitogenic signaling pathways (Mohney et al., 2003). Like many signal transduction proteins, ITSN is composed of multiple protein:protein interaction domains including two amino-terminal Eps15 homology (EH) domains, a central coiled-coil domain, and five tandem Src homology 3 (SH3) domains (see Figure 3C). Mammalian ITSNs also possess a longer splice product, termed ITSN-

long, with an extended carboxyl-terminus encoding an exchange factor domain specific for activating Cdc42 (Hussain et al., 2001). The presence of multiple protein:protein interaction domains in ITSN each with distinct ligand specificities suggests that ITSN acts as a scaffolding or adaptor protein that regulates various biochemical pathways. Our work and that of others supports this model (Irie and Yamaguchi, 2002; Jenna et al., 2002; Mohney et al., 2003; O'Bryan et al., 2001; Predescu et al., 2003; Zamanian and Kelly, 2003). However, the elucidation of these pathways and their relationship to endocytosis and signaling remain to be determined.

Although a link between ITSN and EGFR signaling has been described (Adams et al., 2000), the role of ITSN in EGFR function has not been defined. Here, we report that ITSN is necessary for internalization of the activated EGFR as well as activation of the ERK-MAPK pathway by the receptor. In addition, we report that ITSN associates with Cbl to coordinate the ubiquitylation and degradation of the EGFR. Finally, we demonstrate that ITSN cooperates with additional growth factors in the stimulation of transcription. These results reveal a prominent role for ITSN in the regulation of the EGFR and potentially other RTKs as well.

Materials and Methods

Cell Lines and Transfection. HEK293T, COS1, and A431 cells were maintained according to standard protocols. HEK293T cells were transfected by the calcium phosphate precipitation method as previously described (Mohney et al., 2003). COS1 and A431 were transfected with Lipofectamine (InVitrogen Life Technologies) and Fugene6 (Roche Diagnostics Corp.) as recommended by the manufacturers.

DNA Constructs. cDNA constructs encoding the various ITSN proteins were described previously (Mohney et al., 2003). Hemagglutinin (HA) epitope-tagged wild-type human c-Cbl and Cbl mutant constructs were gifts from Drs. Yosef Yarden (Weizmann Institute of Science) and Ivan Dikic (Goethe University) and are described elsewhere (Kowanetz et al., 2003; Levkowitz et al., 1999) and in Supplementary Materials. Glutathione-S-transferase- (GST-) tagged SH3 proteins consisting of the individual ITSN SH3 domains were purified as described elsewhere (Mohney et al., 2003).

***in vitro* Binding Assays.** -Lysates from HEK293T cells either non-stimulated or stimulated with EGF were prepared as described below. Binding to Cbl was determined as described previously (Mohney et al., 2003).

Generation of anti-ITSN SH3 Antibodies. ITSN antibodies were generated by immunizing rabbits with GST-SH3D derived from *Xenopus* ITSN (Mohney et al., 2003). This antibody recognizes human, mouse, rat and *Xenopus* ITSN isoforms (both short and long). However, this antibody has weak to no reactivity for ITSN 2 proteins.

Immunoprecipitation and Western Blot Analysis. Lysates were prepared as previously described (Oldham et al., 2002). For analysis of ubiquitylation, lysis buffer was supplemented with 5mM N-ethylmaleimide. Unless noted, EGF was used at 100 ng/ml for stimulation of cells. Equal amounts of protein extracts were pre-cleared then incubated with appropriate antisera for 1 h. For immunoprecipitation of HA epitope-tagged proteins, a mouse anti-HA antibody (Babco) was utilized. Rabbit anti-Cbl antisera (Santa Cruz Biotechnology, Inc.), specific for the carboxy-terminal 15 amino acids of Cbl, was used to immunoprecipitate c-Cbl proteins. Endogenous EGFR was immunoprecipated using Ab-13 antibody (NeoMarkers). Immunopurified proteins were then analyzed by SDS-PAGE. Membranes used for detection of ubiquitylated proteins were denatured in 6M Guanidine-HCl/20 mM Tris-HCl, pH 7.5/5 mM β-mercaptoethanol plus protease inhibitor at 4 ^{o}C for 30 min, washed in PBS, incubated with methanol, and dried prior to incubation with ubiquitin antibody (Covance). Blots were washed several times with TBS-T, probed withHRP-linked secondary antibody and then developed with SuperSignal® West Pico chemiluminescence reagents (Pierce).

For Western blotting of GST-pull downs, 10 µl of *in vitro* mixes were electrophoresed on NuPAGE gels, then transferred to Immobilon™-P membranes. The upper halves of the membranes were probed with PY20 antibodies then stripped and re-probed for endogenous Cbl. The lower halves of the membranes were probed with HRP-linked rabbit anti-GST antisera (Santa Cruz Biotechnology, Inc.). Signals were developed as stated above.

Internalization Assays. HEK293T cells were stably transfected with EGFR and maintained in selection media. Cells were transfected with ITSN siRNA or scrambled siRNA as a control (see Supplementary Information for sequences). Twenty hours post-transfection, cells were split into 24 well plates and serum starved overnight (1 ml DMEM per well). Forty-eight hours post-transfection, cells were incubated with I^{125}EGF (1 ng/ml) on ice and later moved to 37° C for the indicated times. Surface-bound and internalized EGFR was determined according to standard protocol (Haglund et al., 2003). Briefly, media in each well was replaced with 250 µl of block solution containing DMEM, 25mM HEPES, and 0.1%BSA and plates were placed on ice for 30 minutes. Cell surface receptors were coated with I^{125}EGF (1 ng/ml) in block solution (250 µl per well) for 1 hour. Duplicate wells received I^{125}EGF (1 ng/ml) and unlabeled EGF (100 ng/ml) for later determination of non-specific binding. Next, cells were either lysed immediately or the media in each well was replaced with 500 µL of block solution and cells were incubated at 37°C for appropriate amount of time prior to being lysed. The total radioactivity in cells that were lysed initially was used as total bound. Following incubation at 37°C and endocytosis of radioligand, cells were washed twice with PBS, acid washed twice (10 min per wash) in 50mM acetic acid and 150 mM NaCl , and lysed in 0.1%SDS and 0.5 M NaOH. Samples were all prepared in duplicate wells. Non-specific binding was subtracted for each sample at each time point. The amount of internalized receptor was determined as a ratio of internalized radioactivity compared to total cell-associated radioactivity.

Confocal Microscopy. Transfected cells were plated on glass bottom plates and serum-starved over night. On the following day, media was removed and cells were washed with cold serum-free media containing 20mM HEPES, pH 7.5 and 1mg/ml BSA placed on ice for 15-30 minutes

to stop endocytosis. Labeled EGF (Alexa Fluor555, Molecular Probes) was added to media (C_f=1 μg/ml) and cells remained on ice for an additional hour. For all time points (5, 15, 30, 60 minutes) except for "0" time point, media was replaced with warm serum-free media and cells were shifted to 37°C in order to start endocytosis. Cells were placed on ice after the indicated times, and washed 2X with cold PBS. For the 5-60 min samples, duplicate wells were washed twice with 0.2M acetic acid/0.5M NaCl for 8 minutes followed by two washes with PBS. Cells were fixed in 4% formaldehyde, permeabilized with PBS+3%BSA+0.1% TritonX-100 (block solution) either at room temperature for 1hr or overnight at 4°C. For immunostaining of tagged proteins, primary antibodies (either anti-SH3 for ITSN, monoclonal anti-HA) were added to samples for 1 hour at room temperature. Samples were washed with PBS then incubated with secondary antibody (anti rabbit/mouse-FITC, TRITC, or AlexaFlour350 conjugated) prepared in block solution and added to each sample for 1 hour at room temperature. Following three washes with PBS, Zeiss LSM 510UV was used for confocal microscopy.

Reporter Assays. HEK293T cells were transfected with reporter plasmids and luciferase activity was measured as described previously (Mohney et al., 2003). Silencing RNAs were used as described for internalization assays (see Supplementary Information for sequences).

Results

ITSN Regulates EGFR Ubiquitylation and Degradation. In addition to its role as an endocytic scaffold, ITSN stimulates specific signaling pathways in cells leading to oncogenic transformation and cooperates with the EGFR in these events (Adams et al., 2000; Mohney et al., 2003). Given ITSN's association with numerous components of the endocytic machinery and its role as a stabilizing scaffold in the process of endocytosis (Koh et al., 2004), we postulated that ITSN may cooperate with RTKs through modulating the trafficking and signaling of these receptors. To address this question, we transiently expressed YFP-tagged ITSN in COS cells and then examined the effect of ITSN on trafficking of the EGFR in individual cells. Given the cell-to-cell variation in expression of transfected proteins, we specifically chose cells that expressed low levels of the transfected construct and were also adjacent to cells not expressing YFP-ITSN. This approach allowed for comparison of EGF internalization in YFP-ITSN expressing and non-expressing cells in the same microscopic field and also decreased the likelihood of inhibitory effects due to vast overexpression of this scaffold protein (Ferrell, 2000). Overexpression of ITSN did not affect binding of Alexa555-EGF (Figure 1A) or total EGFR levels (Figure 1C and 1D). Shifting cells to 37^{o}C to promote endocytosis resulted in rapid internalization of the Alexa555-EGF into endocytic vesicles as apparent by the disappearance of surface fluorescence and formation of numerous cytoplasmic vesicles. Alexa555-EGF and ITSN co-localization on these vesicles was evident at 5 and 15 minutes following the shift to 37^{o}C. However, at 15, 30 and 60 min (not shown), we observed significantly reduced Alexa555-EGF fluorescence in YFP-ITSN cells as compared to cells without YFP-ITSN. This result suggested that ITSN expression either blocked internalization of the receptor (and ligand) or promoted more rapid trafficking and degradation of the complex by the lysosome. In order to distinguish between these possibilities,

we compared ^{125}I-EGF internalization in cells overexpressing ITSN versus vector control cells. Overexpression of ITSN had no significant effect on ^{125}I-EGF internalization (Figure 1B). However, analysis of EGFR turnover revealed that ITSN overexpression enhanced EGF-stimulated disappearance of the receptor as compared to vector-transfected cells (Figure 1C). In the absence of EGF, ITSN overexpression did not affect the steady-state levels of receptor (Figure 1D). The effect of ITSN on EGF-stimulated receptor turnover was dependent on the full-length protein as expression of the isolated EH domains did not enhance EGFR disappearance (Figure 1E). These results indicated that ITSN did not alter the steady-state levels of receptor but rather enhanced ligand-dependent disappearance of the receptor.

To determine whether this disappearance was due to enhance degradation of the receptor in the lysosome, we pre-treated cells with chloroquine (Figure 2). Inhibition of lysosomal activity resulted in equivalent Alexa555-EGF signals in cells with or without YFP-ITSN expression (Figure 2A, compare 60 min time points +/- chloroquine). In addition, chloroquine treatment reversed the effect of ITSN overexpression on ligand-induced EGFR disappearance (Figure 2B).

Given that EGFR internalization and trafficking are regulated in part through monoubiquitylation of the receptor (Haglund et al., 2003), we tested whether ITSN enhanced ubiquitylation of the receptor. Due to the low levels of EGFR in many cell types and the inherent difficulty in detecting endogenous ubiquitylation of cellular proteins, we turned to the A431 human carcinoma cell line which overexpresses the endogenous EGFR due to genomic amplification. Stimulation of these cells with EGF increased ubiquitin conjugation to the activated receptor (Figure 2C, lane 2). Overexpression of ITSN significantly enhanced EGFR ubiquitylation

following stimulation (Figure 2C, compare lane 2 to lane 4) suggesting that ITSN regulated trafficking and degradation of activated EGFR through increasing the ubiquitylation of the activated receptor.

ITSN and Cbl Interact on Cytoplasmic Vesicles. Both genetic evidence in *C. elegans* and *D. melanogaster* and biochemical studies in mammalian cells revealed that Cbl negatively regulates RTKs. Since ITSN's SH3 domains bound Pro-rich peptides *in vitro* that resembled several of the sequences in Cbl, we examined whether Cbl and ITSN were associated in cells. Endogenous Cbl and ITSN co-localized on vesicles in cells (Figure 3A) and were co-immunoprecipitated from mouse brain lysates (Figure 3B). Using truncation mutants of ITSN, we observed that the region encoding the 5 SH3 domains was sufficient for co-immunoprecipitation of endogenous Cbl (Figure 3C). Far Western analysis of cell lysates expressing various truncation mutants of Cbl (Figure 3D), revealed specificity in the interaction of the individual SH3 domains with Cbl. SH3A, C and E each bind Cbl, although SH3A and E appear to bind more avidly (Figure 3E). Similar results were obtained using these same bacterially expressed GST fusions to purify Cbl from cell lysates (Supplementary Figure 1A). Although the 1st Pro-rich sequence in Cbl (aa 491-504) represents a consensus binding site for SH3A of ITSN (Tong et al., 2000), mutation of the Pro residues in this sequence did not significantly decrease binding of ITSN (data not shown) further highlighting the multivalent nature of this interaction. Using a series of Cbl deletion constructs, we mapped the interaction of ITSN with Cbl to the carboxy-terminal PRD domain (Figure 3E and Supplementary Figure 1B). Full-length ITSN (data not shown) and the isolated SH3 region, *i.e.* SH3s A-E, specifically co-precipitated c-Cbl, 70Z-Cbl or Cbl-C (Supplementary Figure 1B). Another SH3-containing protein, CIN85, also associates with Cbl and endophilin to

downregulate EGFR following ligand binding (Soubeyran et al., 2002). Mutation (CblR829A) or truncation (CblΔ655) of Cbl to remove the CIN85 binding site did not alter interaction of ITSN's SH3A domain with Cbl (Supplementary Figure 1C) indicating that ITSN and CIN85 bind distinct sites in Cbl.

EGF stimulation increased association of Cbl and CIN85 and this increase was abrogated by mutation of the Cbl phosphorylation sites (Y700/731/774F; Cbl Y3F) (Soubeyran et al., 2002). However, association of ITSN and Cbl was unaffected by mutation of these tyrosine residues (Cbl Y3F) consistent with the lack of effect of EGF on ITSN-Cbl association (Supplementary Figure 1C and data not shown). Indeed, Cbl and ITSN were constitutively associated in HEK cells (Figure 3A) and this interaction was not altered by EGF stimulation (data not shown). Given the rapidity with which clathrin-coated pits are released from the plasma membrane, we examined whether incubation of cells on ice to inhibit endocytosis and trafficking (Stang et al., 2004) might reveal any difference in Cbl localization following EGF stimulation in the presence or absence of ITSN overexpression. In contrast to cells grown at 37^{o}C (Figure 3A), Cbl was localized in a diffuse pattern in the absence of ITSN overexpression and re-localized to vesicles upon overexpression of ITSN (Figure 4, upper panels) (Stang et al., 2004). As seen with HEK cells, stimulation with EGF at 4^{o}C did not alter the association of ITSN and Cbl (Figure 4). However, we observed an increase in the localization of the ITSN-Cbl double positive vesicles near the plasma membrane upon growth factor stimulation (Figure 4, lower panel). These results suggested that ITSN promoted the accumulation of vesicles at or near the plasma membrane that are Cbl-ITSN positive.

Next, we investigated whether this change in localization resulted in higher levels of Cbl phosphorylation or extended association with EGFR. Interestingly, ITSN had no effect on Cbl association with EGFR or on EGF-induced phosphorylation of Cbl (data not shown). These results demonstrate that ITSN did not alter Cbl association with the receptor.

ITSN Enhances Cbl-Induced Ubiquitylation and Degradation of the EGFR. Cbl-mediated ubiquitylation of the EGFR resulted in trafficking of the activated receptor to the lysosome for degradation (Schmidt and Dikic, 2005). To determine the relevance of ITSN-Cbl interaction, we examined the effect of co-expression of these two proteins on EGFR ubiquitylation and turnover. Immunocytochemical analysis revealed that ITSN, Cbl and EGFR co-localized on intracellular vesicles (Figure 5A and Supplementary Figure 2) although we were unable to immunoprecipitate ITSN with the EGFR (data not shown). These results suggest that this interaction between ITSN, Cbl and EGFR may not be sufficient to allow for co-immunopurification. Expression of either Cbl or ITSN alone increased ligand-dependent ubiquitylation of EGFR; however, co-expression of ITSN and Cbl resulted in a synergistic enhancement of EGFR ubiquitylation (Figure 5B). To determine whether ITSN and Cbl acted through the same or separate pathways, we co-expressed ITSN with a Cbl dominant-negative mutant (70Z-Cbl) that also binds ITSN (Figure 3E). As illustrated in Figure 5C, expression of 70Z-Cbl blocked the increase in EGFR ubiquitylation by ITSN. Thus, Cbl ligase activity was necessary for the effect of ITSN on EGFR ubiquitylation. Furthermore, co-expression of ITSN and Cbl decreased the half-life of the EGFR following stimulation as compared to cells expressing ITSN or Cbl alone (Supplementary Figure 3A). In contrast, expression of an ITSN mutant incapable of binding Cbl did not enhance EGFR

turnover (Figure 1E). These results indicate that ITSN enhanced EGFR degradation through Cbl-induced ubiquitylation of the activated receptor.

Silencing ITSN Inhibits EGFR Endocytosis and Signaling. Our previous work demonstrated that ITSN cooperated with the EGFR to synergistically enhance transcriptional activation and transformation of cells (Adams et al., 2000). The mechanism for this cooperativity involved the MEK-ERK MAPK pathway even though ITSN itself did not activate this pathway (Adams et al., 2000). Endocytosis of the EGFR as well as certain G-protein coupled receptors is important for stimulation of the MAPK pathway (Ceresa and Schmid, 2000). Our data indicate that ITSN overexpression enhanced EGFR trafficking and signaling. To determine whether this result reflected the function of endogenous ITSN in EGFR endocytosis and signal transduction, we used RNAi to decrease ITSN expression. Transfection of silencing RNA (siRNA) directed against ITSN resulted in a 50-75% reduction in ITSN protein and a corresponding decrease in EGFR internalization as indicated by the decrease in fluorescent EGF levels in cells following acid washing of the cells (Figures 6A and B, also Supplementary Figure 3B). This decrease in ITSN resulted in a significant decrease in both the extent and duration of ERK-MAPK activation following EGF stimulation (Figure 6C) as well as a decrease in EGF-stimulated transcription (Figure 6D). A similar effect on EGFR signaling was reported by Schmid and colleagues when endocytosis of the EGFR was blocked using a dominant-negative dynamin (Vieira et al., 1996). These results indicated that ITSN was necessary for the internalization of activated EGFR and signaling from the receptor. This study represents the first demonstration that ITSN is an integral component in the regulation of EGFR endocytosis and signaling.

ITSN Cooperates With Additional Growth Factor Receptors. Given the common regulation of many RTKs and the activation of similar pathways by different RTKs, we tested whether ITSN might function in the regulation of additional RTKs. As observed with EGF, ITSN overexpression enhanced gene expression in response to stimulation with HGF or bFGF (Figure 6E). These finding suggested that ITSN may play a general role in the regulation of a number of RTKs.

Discussion

Endocytosis plays an important role in regulating mitogenic signaling pathways (Sorkin and Von Zastrow, 2002). Indeed, internalized EGFR activates pathways regulating growth and survival including Ras, ERK-MAPK and PI3K (Sorkin and Von Zastrow, 2002). Given the differential signaling of Ras at specific cellular compartments (Chiu et al., 2002), internalized receptors may activate distinct signals as compared to plasma membrane-bound receptors. We have demonstrated that ITSN activated Ras on a subset of intracellular vesicles and that this pool of Ras did not activate the ERK- or JNK-MAPK pathways (Mohney et al., 2003). Thus, endocytosis provides a means of compartmentalized activation of signaling pathways.

Our study demonstrates that ITSN associates with c-Cbl, a negative regulator of RTKs, and enhances c-Cbl mediated ubiquitylation of the EGFR. Our data suggest that ITSN acts to prime the cell for internalization of activated EGFR through recruitment of Cbl to vesicles (Figure 4) where it promotes the ubiquitylation of the activated EGFR. This interaction is provocative given the ability of ITSN to synergize with EGFR in the activation of cellular signaling pathways and oncogenic transformation of cells (Adams et al., 2000). However, our results leave unanswered the question of whether the ITSN-Cbl interaction is necessary for the synergistic activation of signaling between ITSN and EGFR. Although ITSN cooperates with Cbl in enhancing EGFR turnover following stimulation, it is not clear whether this enhancement of EGFR trafficking to the lysosome enhances signaling from the receptor prior to its degradation. Nevertheless, this study represents the first demonstration that ITSN, through interaction with Cbl, functions in the covalent attachment of ubiquitin to the EGFR. While the cooperativity of ITSN with multiple

growth factors to stimulate transcription suggests a potential role for ITSN-Cbl in mediating this effect (Figure 6E), additional studies will be needed to address this possibility.

ITSN regulates protein trafficking in part through assembly of the clathrin-coated vesicles [reviewed in (O'Bryan et al., 2001)]. However, our data indicate an additional role for ITSN in regulating receptor ubiquitylation. Thus, the decrease in EGFR internalization upon silencing ITSN may be due to a decrease in c-Cbl-directed ubiquitylation of EGFR, a decrease in functional endocytic complexes or both. We favor the last possibility. ITSN has at least two roles in regulating EGFR trafficking. First, ITSN is necessary for assembly of endocytic complexes by recruiting endocytic accessory factors to the vesicle (Koh et al., 2004). The interaction of ITSN with epsin, endophilin and arfaptin (data not shown), each of which possess domains that induce membrane curvature (Itoh et al., 2001; Kalthoff et al., 2001; Peter et al., 2004), suggests that ITSN is important for the invagination of the coated-pit or in determining the size or shape of endocytic vesicles. Indeed, mutation of *Drosophila* ITSN, Dap-160, results in fewer endocytic vesicles, abnormally large vesicles and an accumulation of endocytic intermediates (Koh et al., 2004). Second, ITSN acts through c-Cbl to regulate EGFR ubiquitylation which is necessary for sorting of the receptor to the lysosome for degradation, although the mechanism by which ITSN regulates Cbl is not clear. Deletion analysis of Cbl revealed that truncation of sequences carboxyl to amino acid 440 does not impair Cbl's ability to stimulate EGFR ubiquitylation and internalization (Levkowitz et al., 1999; Petrelli et al., 2002). However, point mutations in the PRD of Cbl (CblR829A) (Kowanetz et al., 2003) or the presence of amino acids 441-480 (Petrelli et al., 2002) attenuate Cbl activity suggesting that the carboxyl-terminus of Cbl may negatively regulate its activity. We propose the following model for ITSN in regulating RTK

endocytosis (Figure 7). ITSN binding to Cbl may relieve negative constraints on the ligase, such as those imposed by the Cbl-interacting proteins Sprouty2, Sts1/2, or Cool/Pix (Schmidt and Dikic, 2005). These interactions then allow Cbl to more efficiently ubiquitylate the activated RTKs resulting in enhanced trafficking and degradation. We are currently investigating the effects of ITSN on Cbl's interaction with the aforementioned proteins. Additionally, ITSN facilitates the recruitment of additional components necessary for clathrin-coated pit formation (e.g., AP-2, epsin, dynamin) thereby allowing for coordinate assembly and regulation of the internalization process.

ITSN shares similar activities in regard to Cbl binding and regulation of EGFR ubiquitylation with the SH3 protein CIN85, a regulator of Cbl function (Schmidt and Dikic, 2005). However, several observations suggest that these two proteins are not entirely redundant in function. Cbl possesses distinct binding sites for ITSN and CIN85 (see Supplementary Figure 1) suggesting that both proteins may be necessary for Cbl regulation. Although ITSN and CIN85 each contain a coiled-coil domain and multiple SH3 domains, ITSN has two more SH3 domains than CIN85 thus allowing ITSN to associate with additional partners. Furthermore, ITSN possesses two N-terminal EH domains thereby allowing for novel interactions through this domain (O'Bryan et al., 2001). Interestingly, we have identified CIN85 as an ITSN binding partner in yeast two-hybrid suggesting that the interaction of these two proteins may be important for the dynamic regulation of Cbl function.

The importance of our results is underscored by the conservation of both ITSN and Cbl in higher eukaryotes (O'Bryan et al., 2001; Schmidt and Dikic, 2005). Although *Drosophila* (D-Cbl) and

C. elegans (Sli-1) have been widely reported to lack the conserved SH3-binding sites found in mammalian c-Cbl and Cbl-b, these proteins do indeed possess several Pro-rich motifs, although fewer in number (Robertson et al., 2000). Both D-Cbl and Sli-1 possess a PPLPPR sequence that is nearly identical to an ITSN binding site in c-Cbl (PPVPPR). Indeed, D-Cbl interacts with Dap-160 (Robertson et al., 2000) suggesting an important role for this association. Although ITSN binds this Pro-rich sequence *in vitro* (data not shown), mutation of this site does not impair the interaction of ITSN with Cbl consistent with the multivalent interaction of Cbl with ITSN's SH3 domains.

While previous studies suggested that ITSN inhibited receptor endocytosis (Predescu et al., 2003; Pucharcos et al., 2000; Sengar et al., 1999), our data indicate that ITSN is a positive mediator of receptor endocytosis and trafficking. Silencing ITSN attenuated internalization (Figures 6A&B and Supplementary Figure 3B), and overexpression of ITSN, while not affecting internalization (Figure 1B), enhanced receptor trafficking and degradation (Figures 1 and 2). The difference in our results and previous studies may be due to two factors. First, previous studies only measured endocytosis at late times following ligand stimulation (>25-60 min) and comparison with our data indicates similar results. However, based on the earlier time points in our endocytosis assays (Figure 1A) coupled with the receptor half-life experiments (Figure 1C) and RNAi results (Figure 6 and Supplementary Figure 3B), we conclude that ITSN overexpression stimulates rather than inhibits receptor trafficking. Second, we observed that transfection of 5 fold more ITSN into cells as used in the analyses in Figure 1 inhibited ^{125}I-EGF internalization (data not shown). This result is likely due to vast overexpression of ITSN resulting in the binding of limiting amounts of endocytic components thus titrating these

components into non-productive complexes and inhibiting endocytosis. This biphasic response is a common property of scaffolds (Ferrell, 2000). Thus, the combination of these two factors may account for the observed differences.

While ITSN aids in the modulation of cargo, *i.e.* EGFR, it may also modulate the ubiquitylation of additional proteins within the endocytic complex including epsin, Eps15 and Hrs, all of which are monoubiquitylated (Oldham et al., 2002; Polo et al., 2002). Given the importance of the ubiquitin proteasome system in regulation of both pre- and postsynaptic plasticity (Ehlers, 2003; Speese et al., 2003) coupled with the observed defects in synapse morphology and synaptic transmission in ITSN mutant flies (Koh et al., 2004), ITSN likely plays an integral role in the reorganization of synapses through facilitating the targeted ubiquitylation and degradation of important components in this process.

The predicted role for ITSN in receptor endocytosis and nervous system function is further highlighted by its link to Down Syndrome (DS). ITSN is localized to Chr21 in the DS Critical Region (DSCR) and is overexpressed in the brains of DS patients and in a mouse model for DS (Gardiner, 2003; Pucharcos et al., 1999). This increased ITSN expression may enhance JNK activation and promote increased apoptosis in the brain thereby contributing to the neurodegeneration seen in DS (Mohney et al., 2003). This study suggests that the increased ITSN in DS may alter RTK trafficking. Indeed, increased NGF internalization was observed in synaptosomes from the Ts65Dn mouse model for DS (Cooper et al., 2001). Thus, ITSN overexpression in DS may result in both increased activation of the JNK pathway as well as enhanced RTK trafficking. The combined stimulation of these two pathways coupled with

increased activation of other ITSN-regulated pathways is likely a contributing factor for the sequelae of DS.

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Footnote

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Figure Legends

Figure 1. ITSN enhances EGFR trafficking and degradation. (A) Transiently transfected COS1 cells (YFP-ITSN, green) were stimulated with EGF-Alexa555 (red) for the indicated times. For the 5 and 15 min time points, an enlarged area indicating overlap of endogenous EGFR and YFP-ITSN is shown in the inset. The arrows point to YFP-ITSN expressing cells that have decreased levels of internalized EGF-Alexa555 as compared to cells in which YFP-ITSN is not expressed. The results shown are representative images of 100 captured frames from five independent experiments. Scale bar, 20 μm. (B) ITSN overexpression does not alter EGFR internalization. Uptake of ^{125}I-EGF was quantitated in cells transfected with vector (vec) or ITSN (ITSN). The graph represents the average of two independent experiments performed in duplicate +/-SEM. (C) EGFR turnover is enhanced by ITSN. Endogenous EGFR levels were measured in cycloheximide (10 μM) treated COS1 cells transfected with empty vector or ITSN. Following stimulation with EGF for the indicated times, EGFR levels were monitored by Western blot analysis of cell lysate. Calreticulin was used as a control for loading. (D) Same as (C) above except cells were unstimulated and lysed at the indicated times in hrs. ITSN expression did not alter EGFR turnover in the absence of stimulation. (E) Same as (C) above except cells were transfected with either empty vector, ITSN, or an ITSN truncation mutant lacking the coiled-coil and SH3 domains (EH). Changes in EGFR levels were determined by densitometry using NIH-Image and normalized to calreticulin levels. The results represent the average of two independent experiments.

Figure 2. ITSN-induced loss of EGFR is reversible by chloroquine treatment. (A) The internalization assay, as described in Figure 1, was carried out on cells treated with (+CHQ, 2

hours, 500 μM).) or without (-CHQ) chloroquine. Inhibition of the lysosome (+CHQ) results in equivalent Alexa555-EGF fluorescence in cells with or without ITSN overexpression. Scale bar, 20 μm. (B) ITSN-stimulated turnover of EGFR levels is reversed by inhibition of the lysosome. Cells were treated as in 1C except chloroquine was added where indicated. Cell lysates were probed with antibodies to EGFR (top panel) or calreticulin as a normalization control (bottom panel). Data are representative of three independent experiments yielding the same result. The fraction of EGFR remaining following stimulation is indicated below the blot. Each value is relative to the unstimulated counterpart. (C) ITSN enhances EGF-stimulated ubiquitylation of the EGFR. A431 cells stably expressing ITSN or control cells transfected with empty vector were stimulated with (+) or without (-) EGF for 10 min. Western blot of EGFR immunoprecipitates probed with an anti-ubiquitin antibody (top panel) or an anti-EGFR antibody (bottom panel). Vector and ITSN samples were analyzed simultaneously on the same gel. Irrelevant samples were cropped from the figure for clarity. Data are representatives of at least three independent experiments yielding the same result.

Figure 3. ITSN co-localizes and co-immunoprecipitates with c-Cbl. (A) Endogenous ITSN (green) and c-Cbl (red) co-localize on intracellular vesicles (yellow overlap) in HEK293T cells grown at 37 ^{o}C. Scale bar, 20 μm. (B) Endogenous ITSN was immunoprecipitated from mouse brain tissue using antibodies against ITSN or pre-immune serum as a control. Immunoprecipitates were probed for ITSN (top panel) and c-Cbl (bottom panel) as described in Material and Methods. The two ITSN immunoreactive bands represent the short (S) and long (L) isoforms which are both present in brain. (C) ITSN expression constructs (top panel). All proteins possess an NH_2-terminal HA epitope tag. Association of endogenous c-Cbl with ITSN

(bottom panel). Full-length or truncation mutants of ITSN were immunoprecipitated with anti-HA antibodies from extracts of HEK293T cells followed by Western blot analysis for endogenous c-Cbl. Expression of the various ITSN proteins is shown in the bottom panel. The results are representative of three independent experiments. Similar results were also obtained with HA-tagged *Xenopus laevis* ITSN (data not shown). (D) Cbl expression constructs. All proteins possess an NH_2-terminal HA epitope tag. CblY3F is mutated at Tyr 700, 731 and 774. The Cbl domains are as follows: TKB, SH2-like tyrosine kinase binding domain; RF, RING finger E3 ligase domain; PRD, Pro-rich domain; UBA, ubiquitin-associated domain. (E) Cbl proteins were immunoprecipitated with HA antibody and then analyzed for binding to either GST or GST fusions with the individual SH3 domains of ITSN by Far Western analysis. The positions of Cbl proteins are indicated by arrows. The GST probe used is indicated in each box. All the Cbl proteins were expressed equally well (data not shown). These results are representative of three independent experiments.

Figure 4. EGF stimulation promotes re-localization of ITSN-bound c-Cbl proximal to the membrane. COS1 cells transiently expressing HA-tagged Cbl and/or YFP-ITSN were serum starved overnight and incubated on ice for 30 minutes prior to stimulation with EGF at 4^oC (15 min, bottom panels). Following stimulation cells were fixed and stained as described in Material and Methods. Cbl is diffusely localized throughout the cytosol in the absence of EGF stimulation (top left) and re-localizes to the membrane following 15 minutes of EGF stimulation (bottom left). Overexpression of YFP-ITSN re-localizes Cbl to vesicles (right panels) and these ITSN-Cbl vesicles accumulate near the plasma membrane following EGF treatment (bottom right), Scale bar, 20 μM.

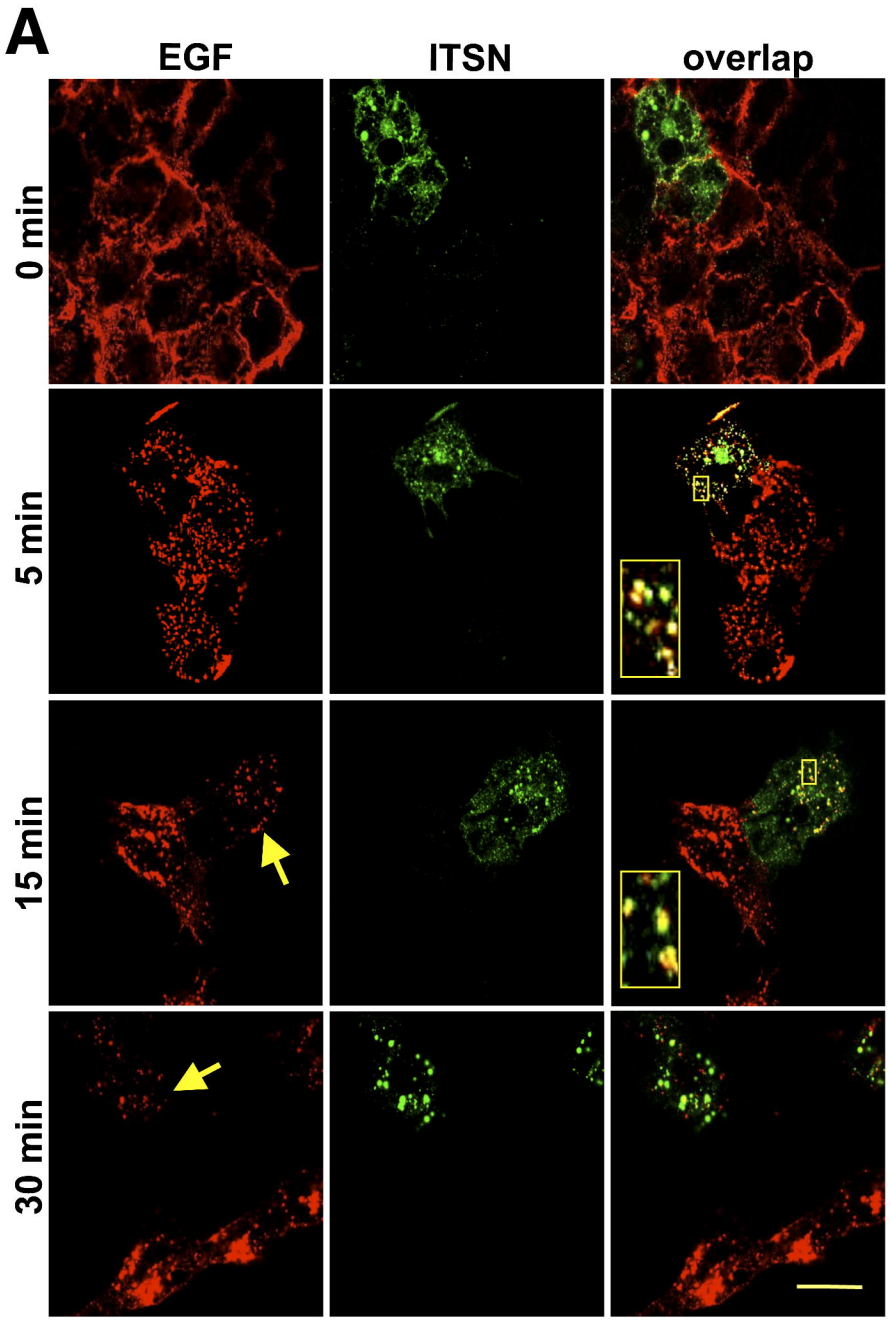
Figure 5. ITSN stimulates EGFR ubiquitylation through Cbl. (A) ITSN, c-Cbl and EGFR are found in a complex in cells. A431 expressing YFP-ITSN (green) and HA-c-Cbl (red) were stimulated with EGF for 10 min, fixed, and stained with antibodies to c-Cbl (α-Cbl) and EGFR (α-EGFR). Co-localization of c-Cbl, ITSN and EGFR is shown in the middle right panel. The inset has been enlarged (bottom right panel) to illustrate the overlap of all three proteins on enlarged vesicles. These vesicles were not due to overexpression of ITSN or Cbl as they were also present in non-transfected cells (data not shown). Scale bar, 10 μm. Additional images are provided in Supplementary Figure 2. (B) ITSN enhances ubiquitylation of EGFR. A431 cells expressing HA-ITSN, HA-c-Cbl, or both proteins were stimulated with EGF (10 min). EGFR was immunoprecipitated and analyzed for ubiquitin conjugation (top panel). Expression levels of EGFR, ITSN, and Cbl in cell lysastes are shown in the lower three panels. (C) The E3 ligase activity of Cbl is necessary for ITSN-induced EGFR ubiquitylation. EGFR ubiquitylation was measured as in (B) in A431 cells transfected with HA-ITSN, HA-Cbl, and/or dominant-negative HA-70Z-Cbl and stimulated with EGF. Expression of ITSN, Cbl, and 70Z-Cbl is shown in the bottom panels. Calreticulin was used as a loading control in (B) and (C).

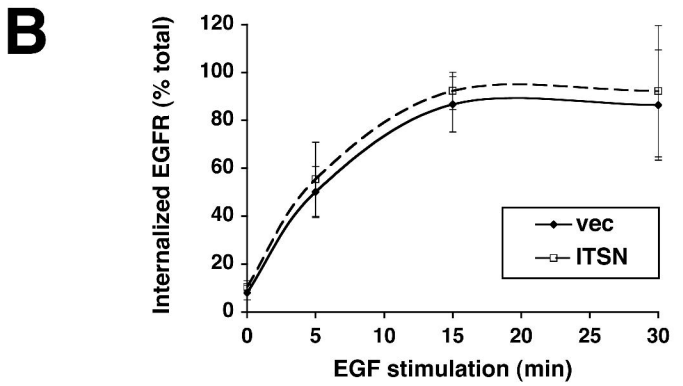
Figure 6. Silencing of ITSN decreases EGFR trafficking and signaling. (A) HEK 293T cells stably transfected with EGFR were transiently transfected with either scrambled or ITSN silencing RNAs. CFP (inset) was co-transfected with the siRNA to identify transfected cells. Cells were stimulated for varying times (min) with EGF-Alexa555 (red), acid washed (5-60 min samples) then fixed. Pictures are representative of 100 images from three independent experiments. Scale bar, 20 μm. (B) Quantitation of EGFR internalization. Level of internalized

^{125}I-EGF was monitored as described in Material and Methods. The graph represents the average of two independent experiments performed in duplicate. Similar results were obtained using a second ITSN siRNA targeting a different region of the mRNA (see Supplementary Figure 3B). The differences in ^{125}I-EGF at the 5 and 15 min time points are statistically significant in a Students t-test ($p<0.032$ and $p<0.0011$, respectively). (C and D) Silencing ITSN decreased EGF stimulation of ERK-MAPK (C) and EGF activation of ELK-1-dependent transcription (D). (C) HEK293T cells were transiently transfected with ITSN siRNAs or control scrambled siRNA. Cells were serum starved overnight, stimulated with EGF for the indicated period of time (min) and then lysates probed for ITSN, phospho-ERK and total ERK as a control for loading. (D) HEK293T cells were transiently transfected with siRNAs to ITSN or control siRNA to GFP along with the Gal-Elk reporter constructs. Relative ELK activation was determined as described previously (Mohney et al., 2003). ITSN expression is shown in bottom panel. The results presented are the mean +/-SEM from at least three independent experiments performed in duplicate. (E) ITSN potentiates ELK activation following growth factor stimulation. Cells were transfected with an expression construct encoding HA-ITSN (ITSN) or empty vector along with the Gal-Elk reporter plasmids as previously described (Mohney et al., 2003). Relative ELK activation in samples stimulated with EGF (100 ng/ml), HGF (100 ng/ml), and bFGF (10 ng/ml) were determined as described previously (Mohney et al., 2003). The results presented are the mean +/-SEM from at least three independent experiments performed in duplicate.

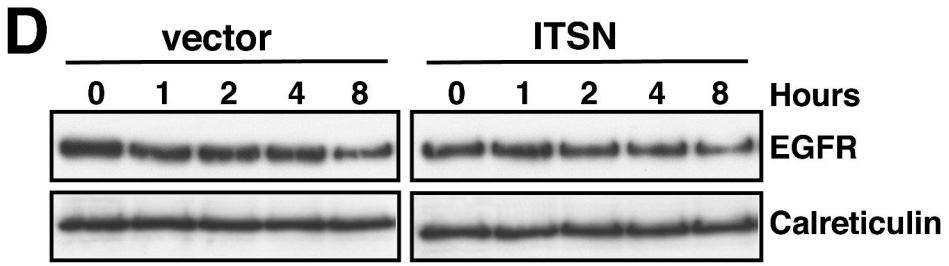
Figure 7. Model for ITSN regulation of EGFR endocytosis and trafficking. ITSN interacts with Cbl to stimulate EGFR ubiquitylation. This increase in Cbl function may arise through enhancing the interaction of Cbl activators (e.g. CIN85) or blocking the interaction with Cbl

inhibitors (e.g. Spry2, Sts1/2 or COOL/PIX). In addition, ITSN aids in assembling clathrin-coated vesicles through association with endocytic accessory proteins (e.g. Dynamin, Eps15, etc) and proteins that induce membrane curvature (e.g. Epsin, Endophilin, Arfaptin2). Together, these two functions of ITSN facilitate the endocytosis, trafficking and degradation of the activated EGFR and possibly other RTKs. Arrows denote positive interaction; bars denote inhibitory interactions.


A
EGF
ITSN
overlap
0 min
5 min
15 min
30 min


B
Internalized EGFR (% total)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
EGF stimulation (min)
vec
ITSN


C
vector
ITSN
0 1 5 15 30 60
0 1 5 15 30 60
EGF (min)
EGFR
Calreticulin


D
vector
ITSN
0 1 2 4 8
0 1 2 4 8
Hours
EGFR
Calreticulin

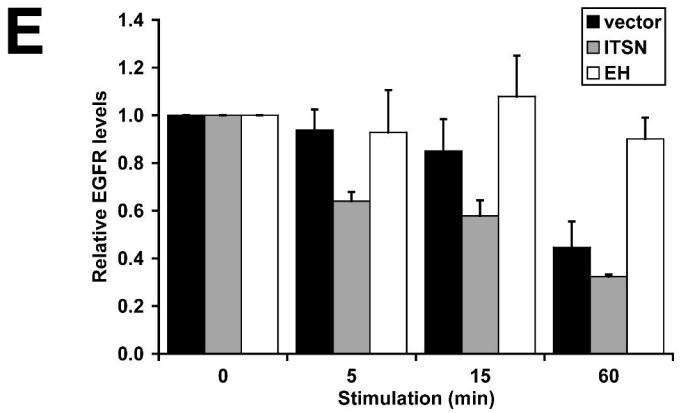

E
Relative EGFR levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
15
60
Stimulation (min)
vector
ITSN
EH

Figure 1

Figure 2

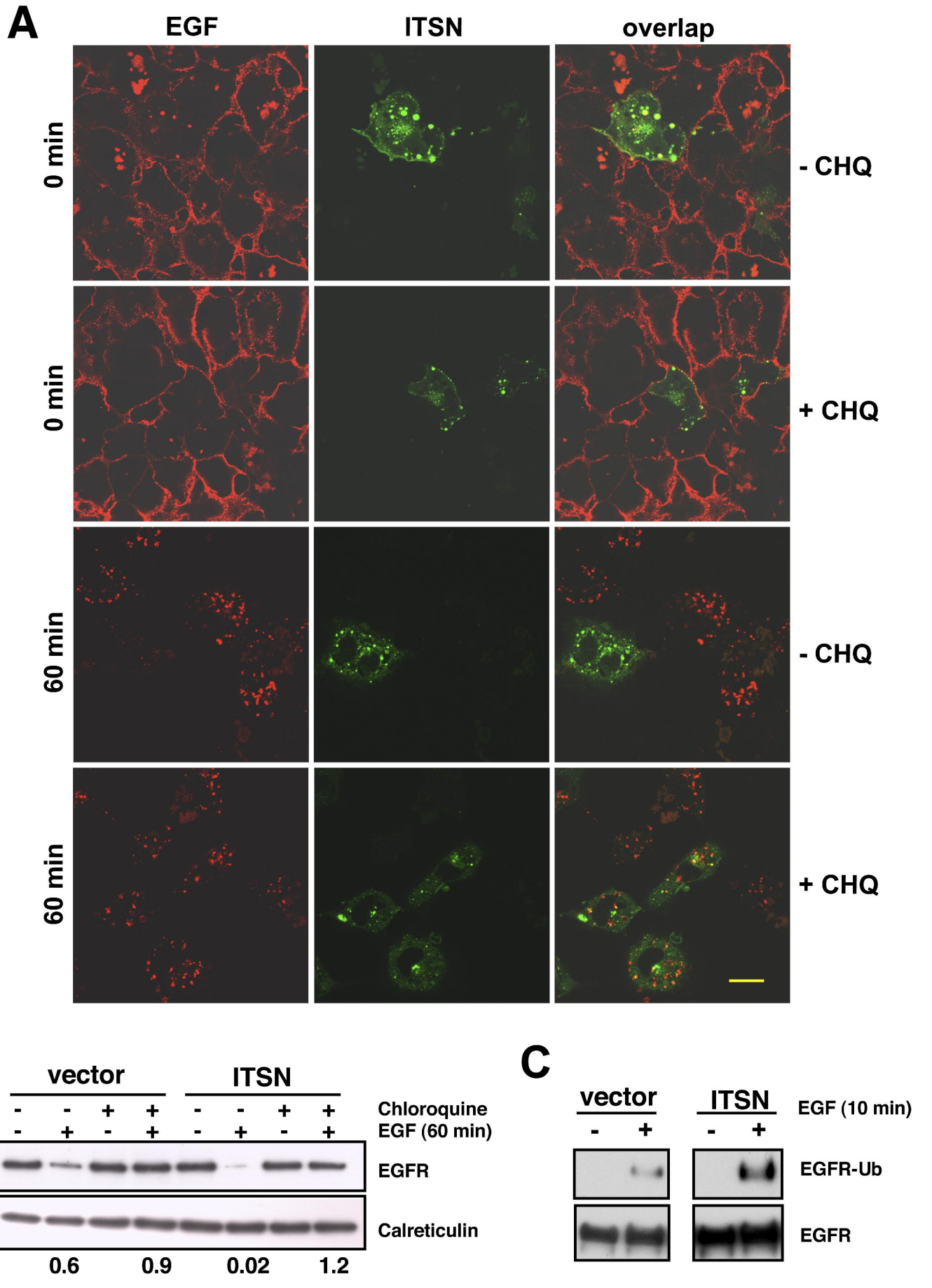

A
EGF
ITSN
overlap
0 min
- CHQ
0 min
+ CHQ
60 min
- CHQ
60 min
+ CHQ
B
vector
ITSN
Chloroquine
EGF (60 min)
EGFR
Calreticulin
0.6
0.9
0.02
1.2
C
vector
ITSN
EGF (10 min)
EGFR-Ub
EGFR

Figure 3

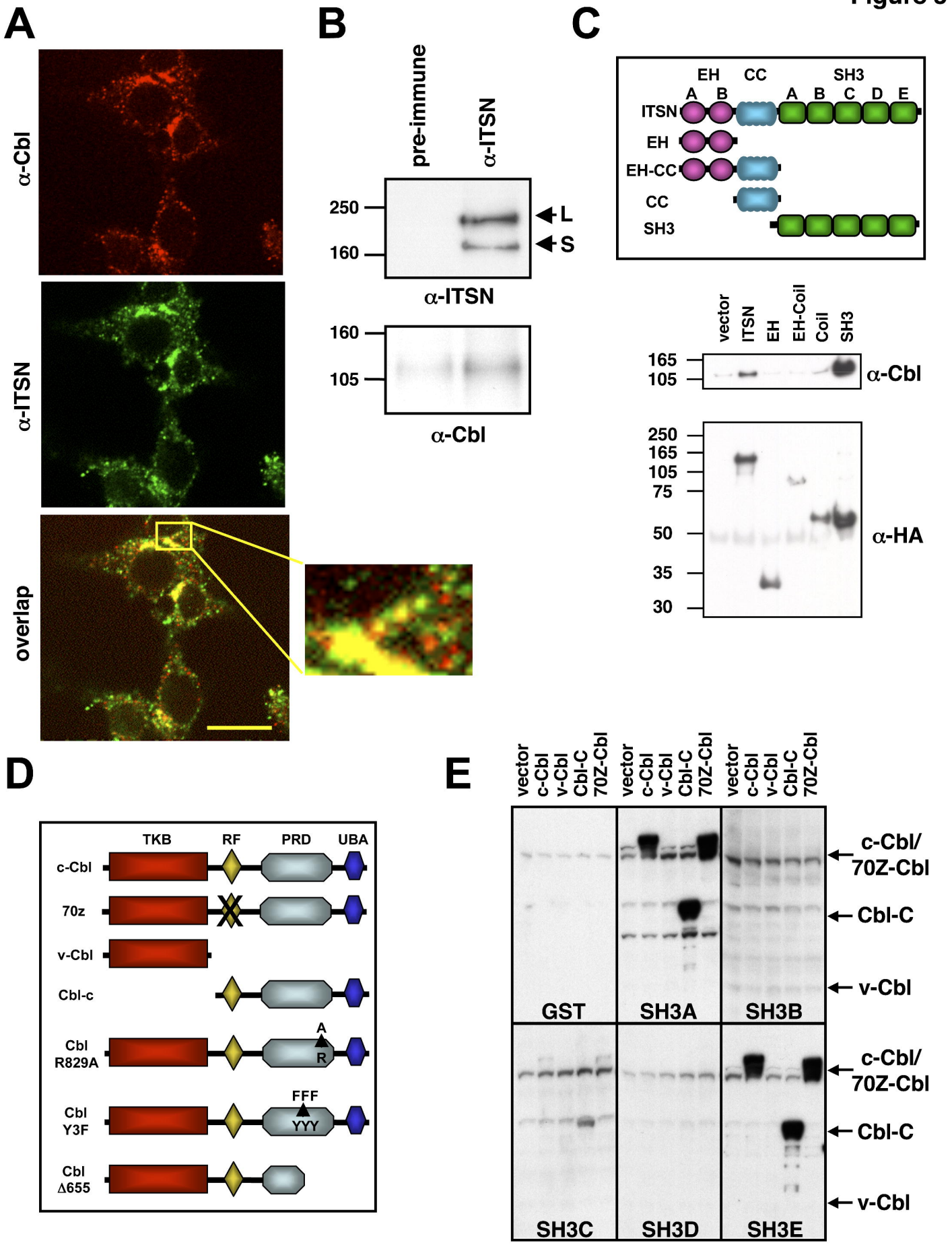
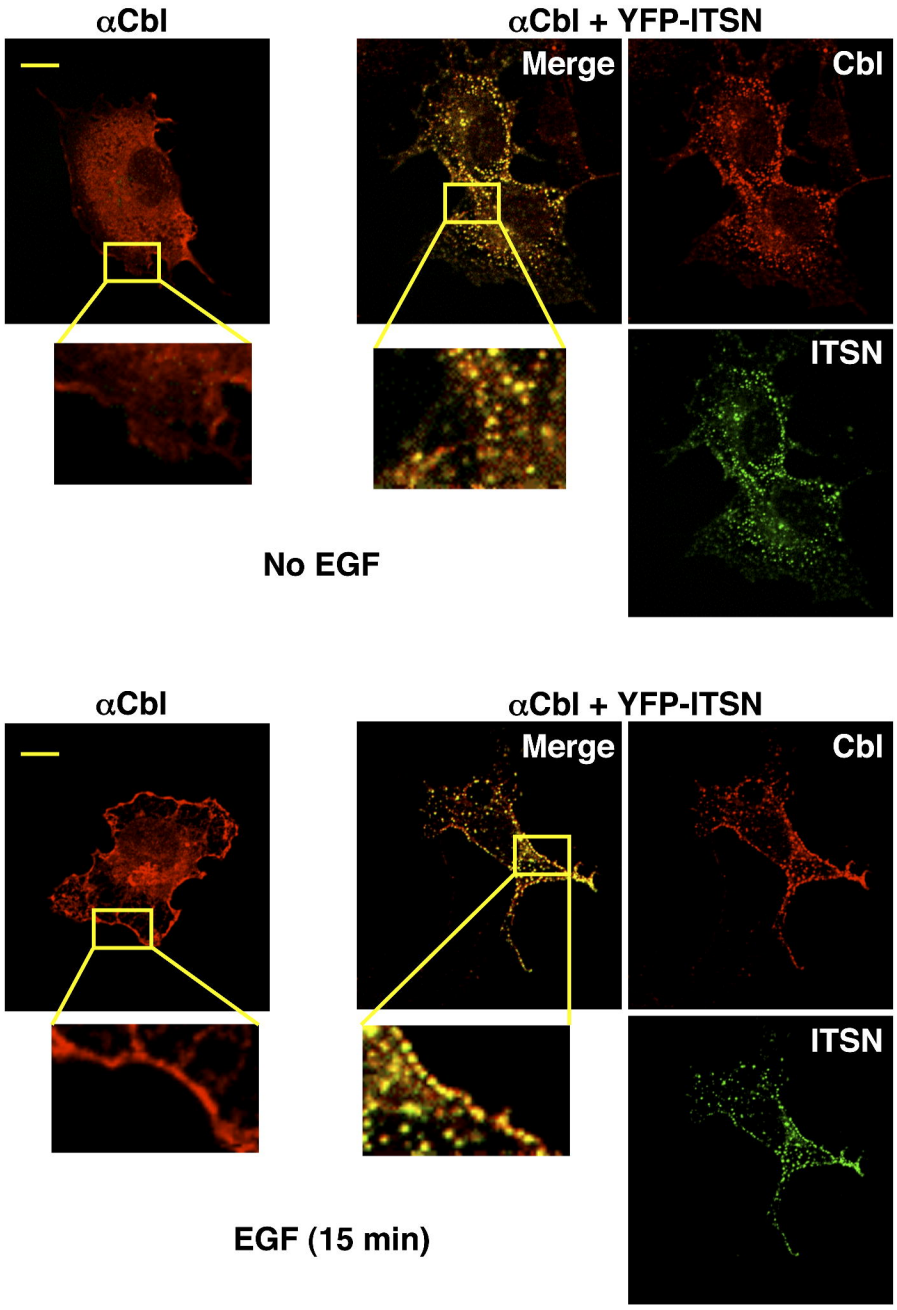
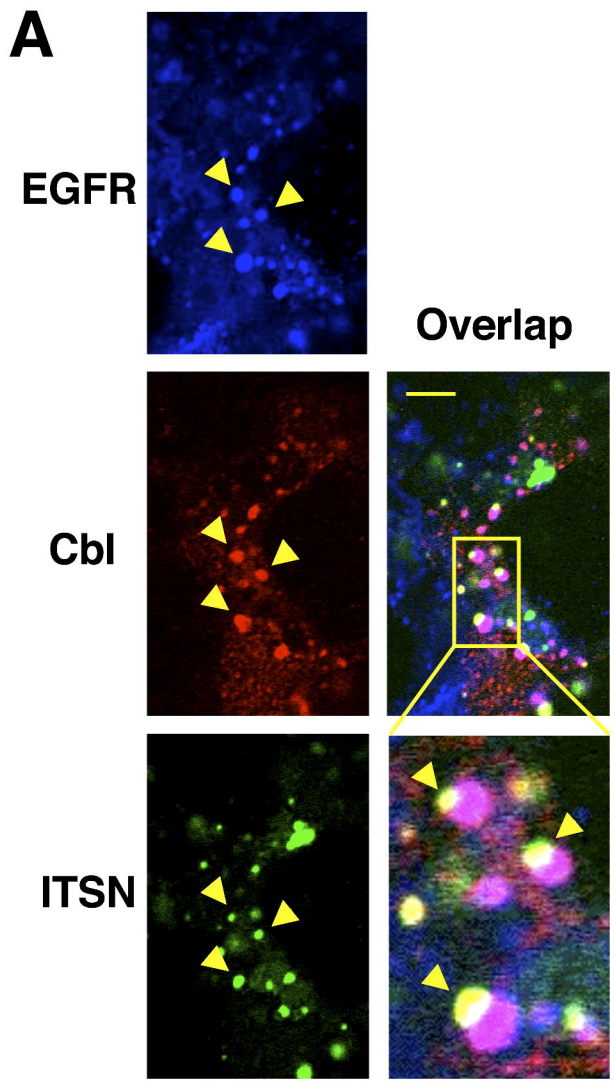
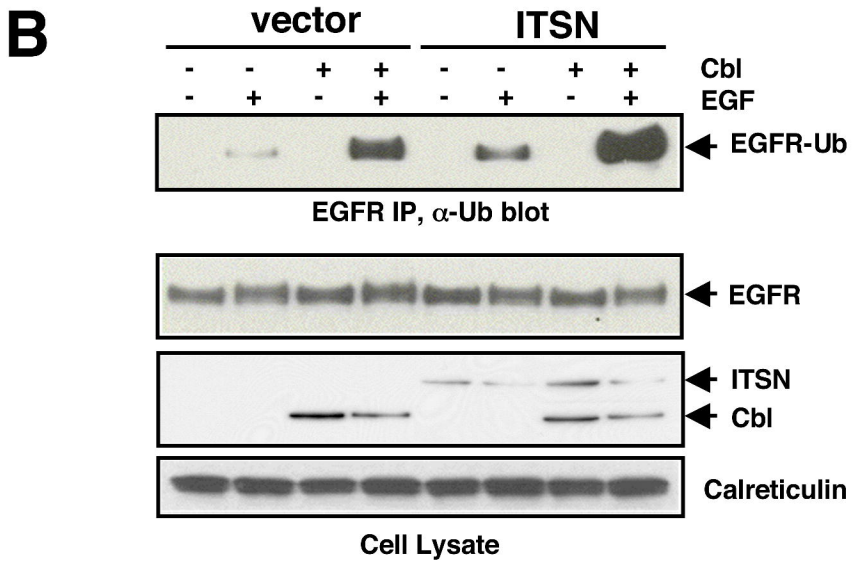

A
α-Cbl
α-ITSN
overlap
B
pre-immune
α-ITSN
250
160
L
S
α-ITSN
160
105
α-Cbl
C
EH
CC
SH3
A
B
A
B
C
D
E
ITSN
EH
EH-CC
CC
SH3
vector
ITSN
EH
EH-Coil
Coil
SH3
165
105
α-Cbl
250
165
105
75
50
35
30
α-HA
D
TKB
RF
PRD
UBA
c-Cbl
70z
v-Cbl
Cbl-c
Cbl R829A
A
R
Cbl Y3F
FFF
YYY
Cbl Δ655
E
vector
c-Cbl
v-Cbl
Cbl-C
70Z-Cbl
c-Cbl/ 70Z-Cbl
Cbl-C
v-Cbl
GST
SH3A
SH3B
SH3C
SH3D
SH3E

Figure 4


αCbl
αCbl + YFP-ITSN
Merge
Cbl
ITSN
No EGF
αCbl
αCbl + YFP-ITSN
Merge
Cbl
ITSN
EGF (15 min)


A
EGFR
Overlap
Cbl
ITSN


B
vector
ITSN
Cbl
EGF
EGFR-Ub
EGFR IP, α-Ub blot
EGFR
ITSN
Cbl
Calreticulin
Cell Lysate

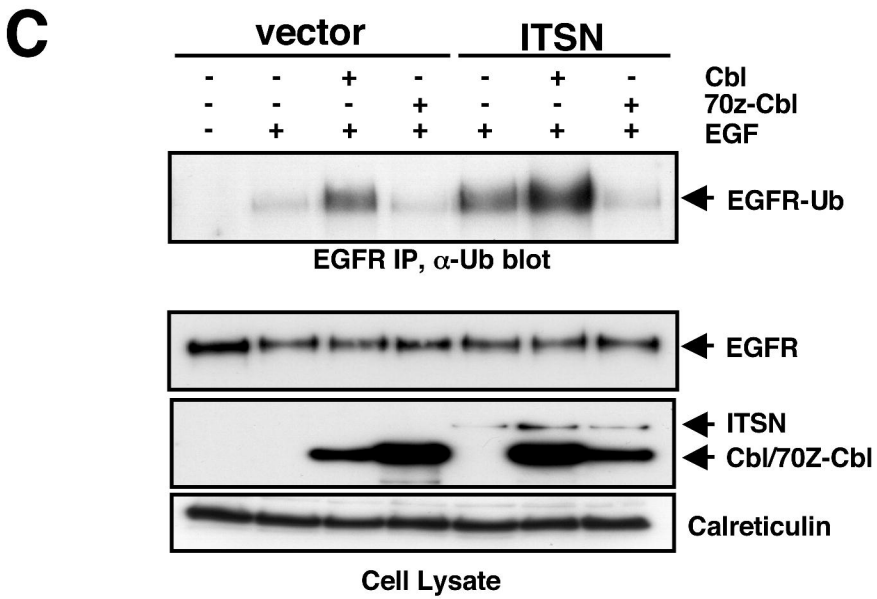

C
vector
ITSN
Cbl
70z-Cbl
EGF
EGFR-Ub
EGFR IP, α-Ub blot
EGFR
ITSN
Cbl/70Z-Cbl
Calreticulin
Cell Lysate

Figure 5

Figure 6

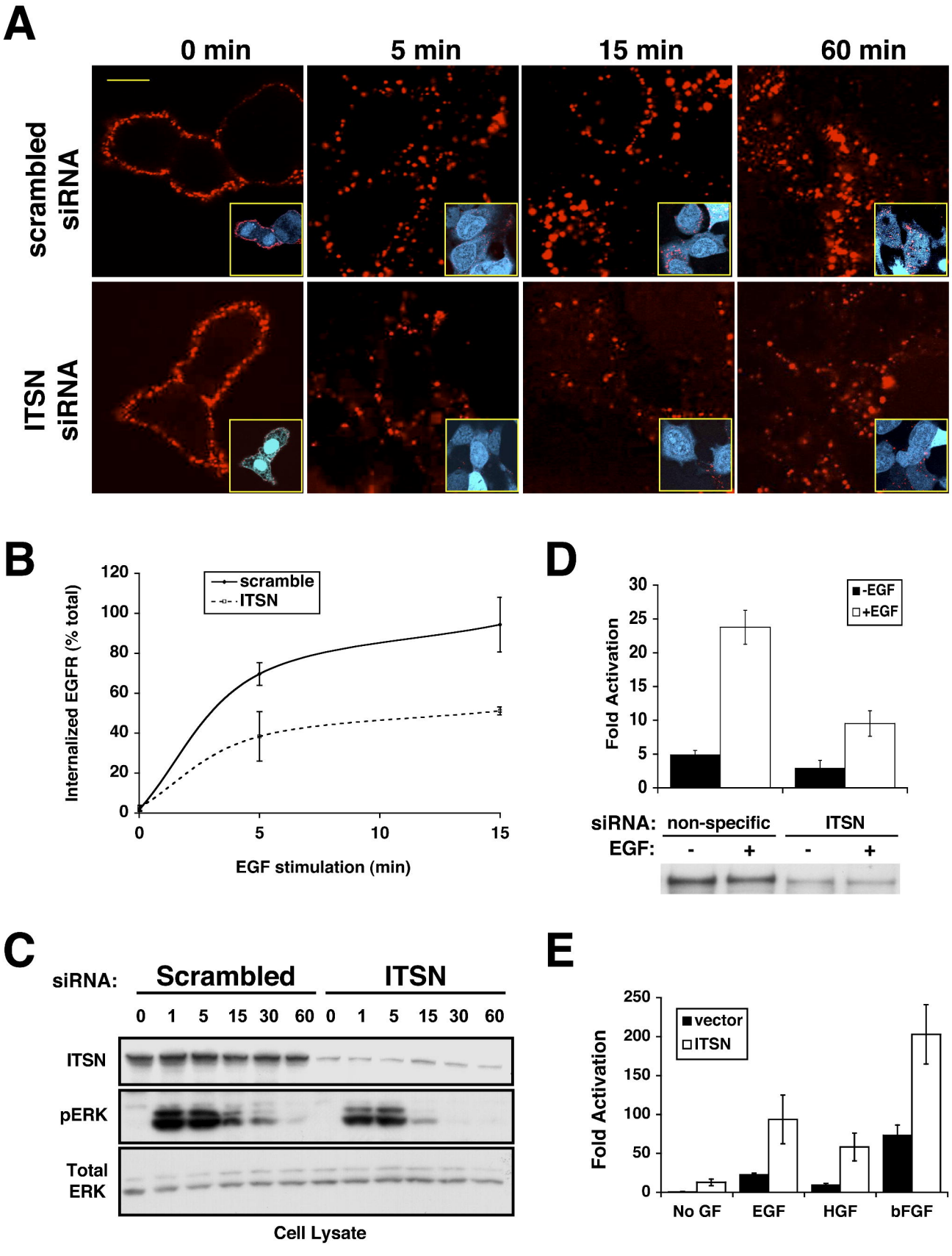
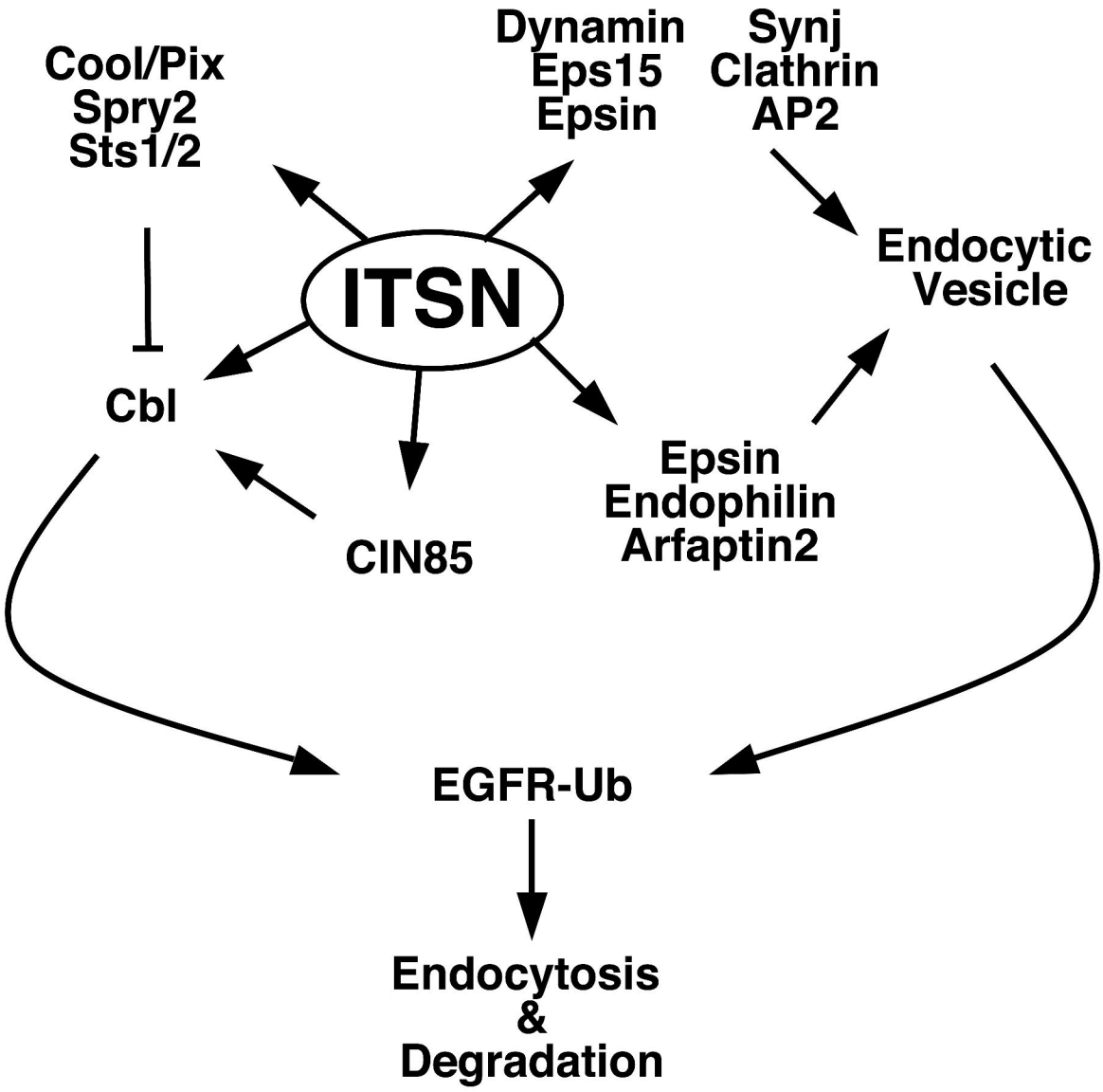

A
0 min
5 min
15 min
60 min
scrambled siRNA
ITSN siRNA
B
Internalized EGFR (% total)
scramble
ITSN
EGF stimulation (min)
D
Fold Activation
-EGF
+EGF
siRNA:
non-specific
ITSN
EGF:
C
siRNA:
Scrambled
ITSN
0 1 5 15 30 60 0 1 5 15 30 60
ITSN
pERK
Total ERK
Cell Lysate
E
Fold Activation
vector
ITSN
No GF
EGF
HGF
bFGF

Figure 7


Cool/Pix
Spry2
Sts1/2
Dynamin
Eps15
Epsin
Synj
Clathrin
AP2
ITSN
Endocytic
Vesicle
Cbl
CIN85
Epsin
Endophilin
Arfaptin2
EGFR-Ub
Endocytosis
&
Degradation