Frosi et al., http://www.jcb.org/cgi/content/full/jcb.201002032/DC1

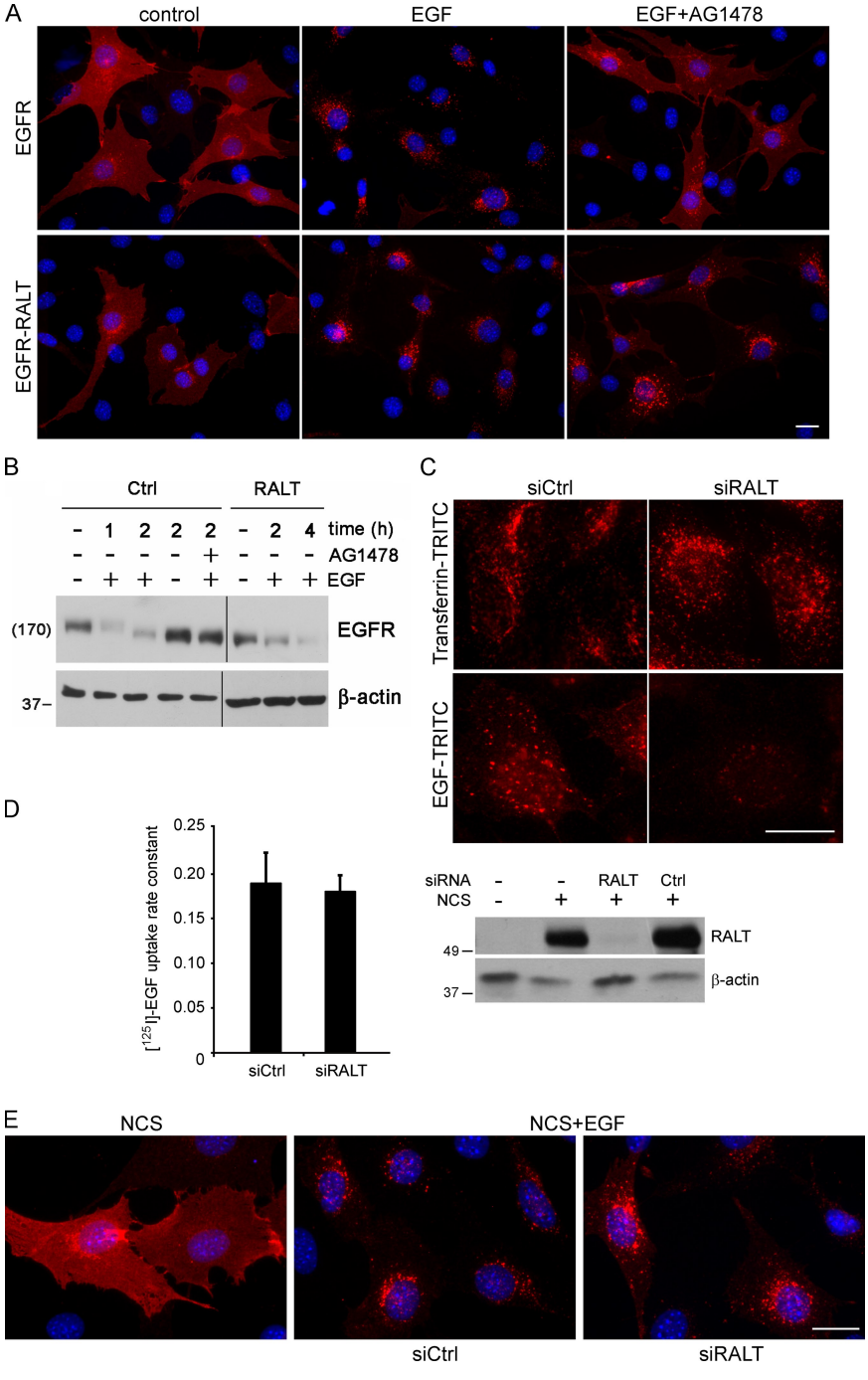

Figure S1. **RALT drives endocytosis and degradation of kinase-suppressed EGFR.** (A) Quiescent control and RALT-overexpressing NR6-EGFR cells were either left unstimulated or incubated with 20 ng/ml EGF at 37°C for 10 min (±3 µM AG1478), fixed, and stained with anti-EGFR mAb 108 (red). Hoechst 33258 was used to stain nuclei (blue). (B) Quiescent control and RALT expressing NR6-EGFR cells were stimulated with 100 ng/ml EGF for the indicated time. When indicated, 3 µM AG1478 was added to culture medium throughout the incubation time. Lysates were immunoblotted as indicated. Samples unrelated to this experiment were run in between the Ctrl and RALT series and were deleted from the panel in the process of image composition. (C) NR6-EGFR Dc214 cells were transfected with either control or RALT-specific siRNAs. Cells were made quiescent and then stimulated with 10% NCS for 3 h at 37°C to induce RALT expression. After serum wash-out, cells were incubated for 10 min at 37°C with TRITC-labeled EGF or transferrin before being processed for fluorescence detection. (D) NR6-EGFR cells were transfected with control and RALT-specific siRNA. 2 d after transfection, cells were incubated for 18 h in starvation medium and then stimulated for 3 h with 10% NCS to induce RALT expression. Rates of [^{125}I]-EGF uptake were measured as described in Fig. 1 C and reported as K_e values (left). Results were averaged from four independent binding experiments relative to two independent RNAi experiments. RALT KD was verified by immunoblot analysis, which included control nontransfected cells (right). (E) NR6-EGFR cells prepared as in D were subjected to IF to visualize EGFR endocytosis. NCS-stimulated cells were either left untreated or subjected to a 15-min incubation at 37°C with 20 ng/ml EGF. EGFR was imaged by mAb 108 (red) and nuclei were stained by Hoechst 33258 (blue). Bars: (A and E) 20 µm; (C) 10 µm.

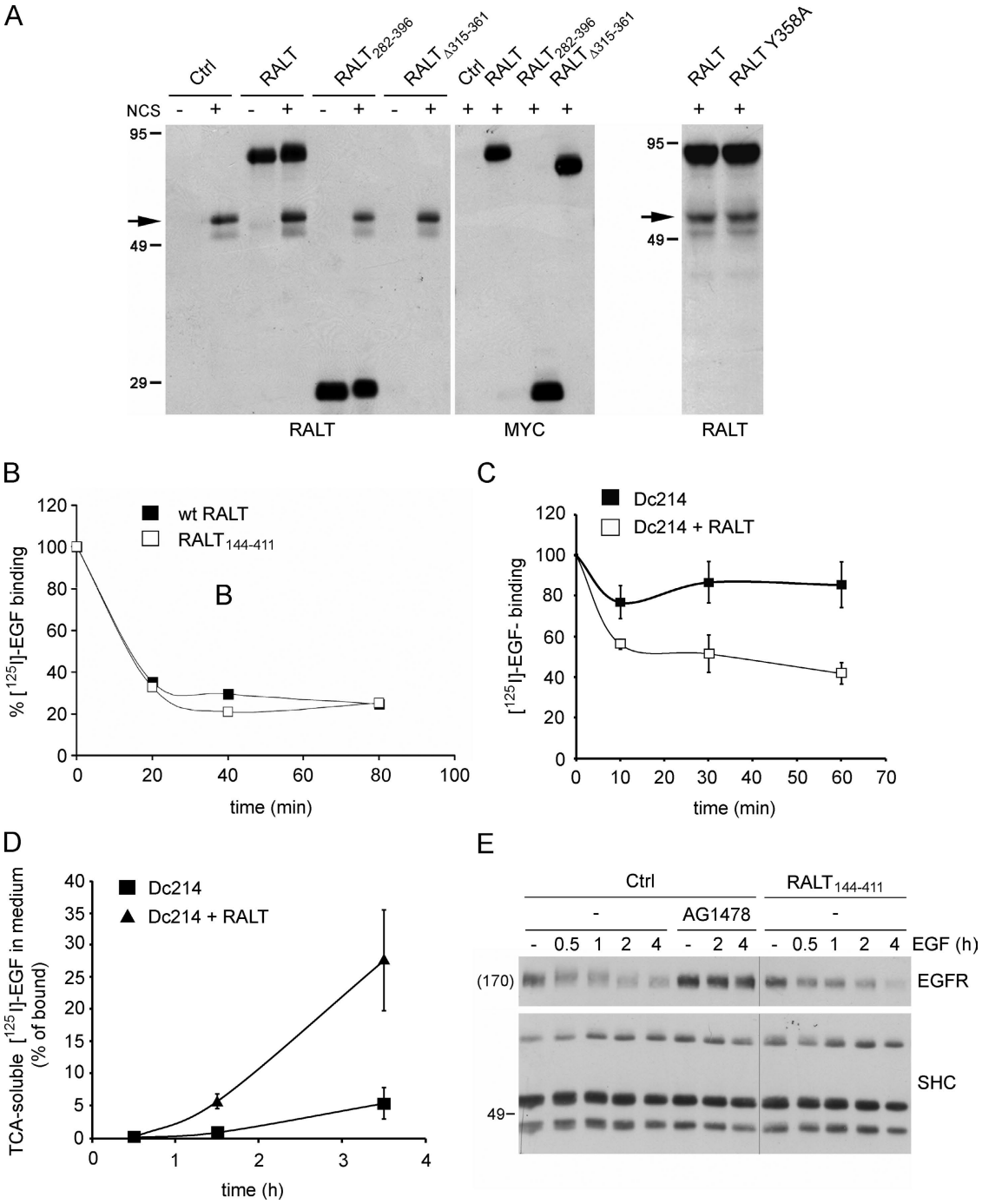

Figure S2. **The evolutionarily conserved 144–411 fragment of RALT mediates endocytosis and degradation of EGFR.** (A) NR6 cells were transduced with retrovirus stocks encoding EGFR Dc214 and the indicated MYC-RALT alleles. Quiescent cells were either left untreated or stimulated with 10% NCS for 3 h in order to induce expression of endogenous RALT protein (arrows). Lysates were immunoblotted with the indicated antibodies. (B) NR6-EGFR Dc214 cells expressing either wtRALT or $RALT_{144–411}$ were made quiescent, stimulated with 100 ng/ml EGF for the indicated time at 37°C, and processed to determine residual [^{125}I]-EGF binding at the cell surface (i.e., percentage of binding at time 0). A representative experiment of two independent assays is shown. (C) RALT-driven down-regulation of EGFR Dc214 is associated to protracted intracellular retention of EGFR Dc214. Control and MYC-RALT–expressing NR6-EGFR Dc214 cells were allowed to bind EGF (100 ng/ml) for 30 min on ice, washed with ice-cold medium, chased at 37 C° in EGF-free medium for the indicated time, and processed to determine residual [^{125}I]-EGF binding at the cell surface (i.e., percentage of binding at time 0). Results were averaged from two independent experiments. (D) [^{125}I]-EGF degradation in control and RALT-expressing NR6-EGFR Dc214 cells. The panel relates to the same set of experiments presented in Fig. 5 B and shows the quantification of TCA-soluble radioactivity in the extracellular medium. (E) Control and $RALT_{144–411}$–expressing NR6-EGFR cells were made quiescent before stimulation with 100 ng/ml EGF for the indicated time at 37°C. When indicated, 3 μM AG1478 was added to culture medium throughout the incubation time. Samples were immunoblotted as indicated. Samples unrelated to this experiment were run in between the Ctrl and RALT series and were deleted from the panel in the process of image composition. SHC proteins were used as loading control.

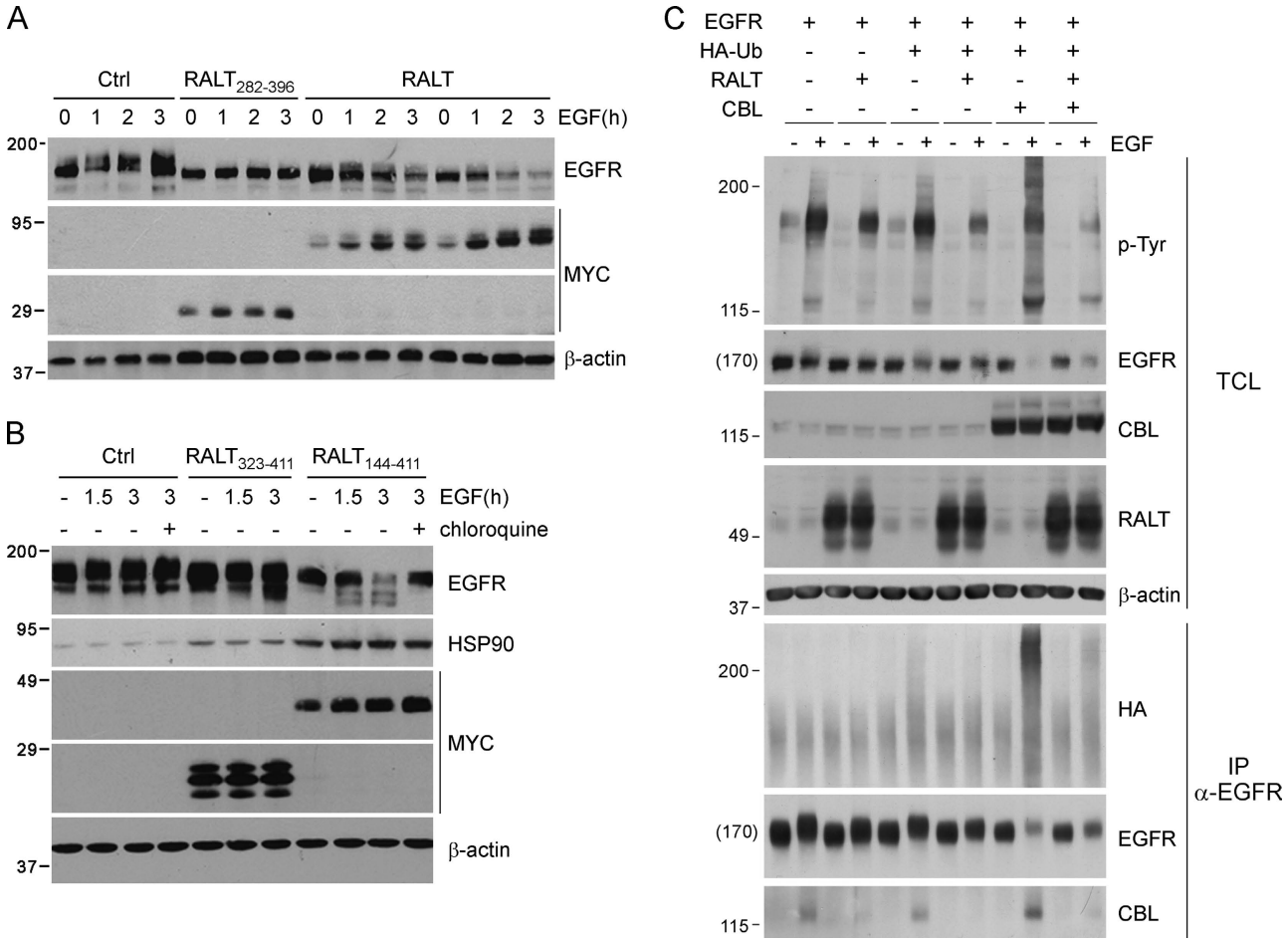

Figure S3. **RALT rescues the degradation of EGFR Y1045F independently of receptor ubiquitylation.** (A and B) CHO cells were transfected with expression vectors encoding pcDNA3-EGFR Y1045F along with either empty vector (Ctrl) or expression vectors for the indicated MYC-tagged RALT alleles. 2 d after transfection, cells were made quiescent and stimulated with 100 ng/ml EGF for the indicated time (hours). Where indicated, chloroquine was added to the culture medium throughout the EGF incubation time. Lysates were immunoblotted with the indicated antibodies. For EGFR analysis, the amount of loaded lysate was adjusted to obtain comparable sensitivity of detection among different transfectants. (C) HEK 293 cells were transfected with expression vectors for the indicated proteins. 2 d after transfection, cells were made quiescent and either left untreated or stimulated with 100 ng/ml EGF for 10 min at 37°C. Cell lysates and anti-EGFR immunoprecipitates were immunoblotted as indicated.

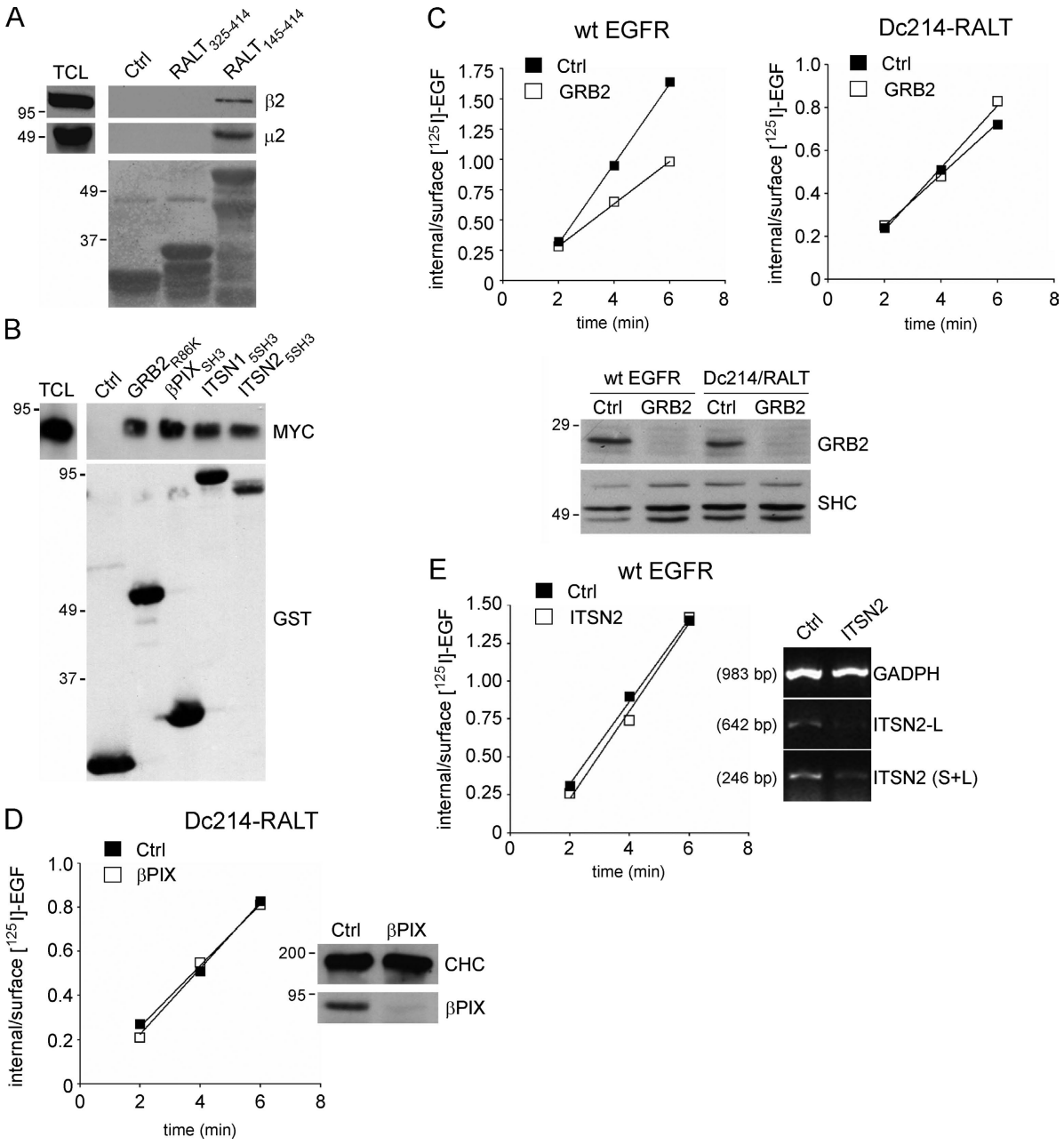

Figure S4. **Analysis of SH3-containing proteins in RALT-mediated endocytosis.** (A) Purified recombinant GST (Ctrl) and GST fusion proteins containing the indicated fragments of human RALT (fragments 325–414 and 145–414 from human RALT are colinear to fragments 323–411 and 144–411 of rat RALT, respectively) were immobilized onto agarose-glutathione beads before incubation with HEK 293 cell lysates. After washing, eluted proteins were immunoblotted with antibodies to the β2 and μ2 subunits of AP-2. GST proteins were stained with Ponceau red (bottom). (B) Purified recombinant GST (Ctrl), GST-GRB2 R86K (this mutation disables the pTyr binding activity of GRB2 SH2 domain leaving intact the binding activity of the N- and C-terminal SH3 domains), and GST fusions containing the βPIX, ITSN1 and ITSN2 SH3 domains were bound to agarose-glutathione beads before incubation with lysates prepared from HEK 293 cells expressing MYC-RALT. After washing, proteins bound to the solid phase were eluted by boiling in Laemmli buffer and immunoblotted with 9E10 anti MYC-mAb (top) or anti-GST antiserum (bottom). Total cell lysates (TCL) represent 5% of input lysate in the binding experiment. (C) NR6-EGFR and NR6-EGFR Dc214-RALT cells were transfected with either control or Grb2-specific siRNAs. Cells were made quiescent by serum deprivation and assayed for [125I]-EGF uptake (1 ng/ml at 37°C for the indicated time). An experiment representative of three independent assays is shown. Parallel 35-mm dishes were processed for immunoblot analysis to document KD efficiency; SHC proteins were used as loading control. (D) RALT-expressing NR6-EGFR Dc214 cells were transfected with control and βPIX-specific siRNAs. Cells were made quiescent and assayed for [125I]-EGF uptake (1 ng/ml at 37°C for the indicated time). An experiment representative of three independent assays is shown. Parallel 35-mm dishes were processed for immunoblot analysis to document KD efficiency; CHC was used as loading control. (E) NR6-EGFR cells were transfected with control and ITSN2-specific siRNAs. Cells were made quiescent and assayed for [125I]-EGF uptake (1 ng/ml at 37°C for the indicated time). An experiment representative of three independent assays is shown. Parallel 35-mm dishes were processed to assess KD efficiency by RT-PCR; GAPDH was used as control for RT-PCR reactions.

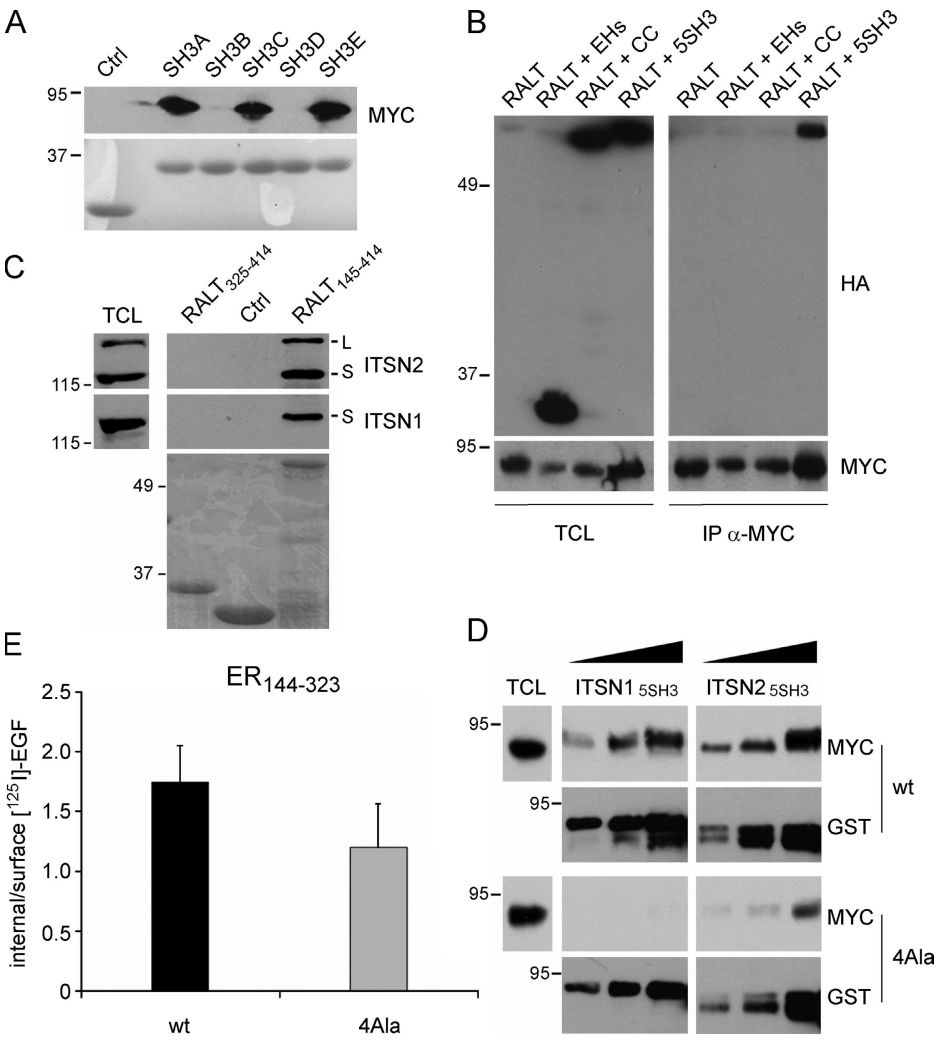

Figure S5. **Structure-function analysis of RALT–ITSN interaction.** (A) Purified recombinant GST fusion proteins containing the individual SH3 domains of *Xl*ITSN were bound to glutathione-agarose beads and incubated with lysates of HEK 293 cells expressing MYC-RALT. GST was used as control. Proteins bound to the beads were immunoblotted with anti-MYC 9E10 mAb. GST proteins were stained with Ponceau red (bottom). (B) MYC-tagged RALT was expressed in HEK 293 cells either alone or in combination with HA-tagged *Xl*ITSN fragments containing the two EPS15 homology domains (EHs), the central coiled-coil region (CC), and the five SH3 modules (5SH3). Cell lysates were subjected to immunoprecipitation with anti-MYC 9E10 mAb followed by immunoblot with anti-HA 12CA5 mAb (right). Total cell lysates (TCL) corresponding to 5% of input lysate in IPs were immunoblotted as indicated (left). (C) Purified recombinant GST (Ctrl) and GST fusion proteins containing the indicated fragments of human RALT were immobilized onto agarose-glutathione beads before incubation with HEK 293 cell lysates. After washing, eluted proteins were immunoblotted with anti-ITSN1 and anti-ITSN2 antibodies. Total cell lysates corresponding to 5% of input lysate in the binding reaction were analyzed in parallel. L and S stand for long and short isoform, respectively. GST proteins were stained by Ponceau red (bottom). (D) Increasing amounts of purified recombinant GST fusions spanning the five SH3 domains of ITSN1 and ITSN2 were immobilized onto glutathione-agarose beads. Beads were incubated for 1.5 h at 4 C° with a fixed amount of HEK 293 cell lysate expressing MYC-RALT (either wt or 4Ala). Bound proteins and 5% of input lysates were immunoblotted with the indicated antibodies. (E) $ER_{144–323}$ chimeras in either wt or 4Ala configuration were expressed stably in NR6 fibroblasts. Quiescent cells were analyzed for [^{125}I]-EGF uptake (3 ng/ml at 37°C for 5 min). Results were averaged from five independent experiments. The observed differences are statistically significant (P = 0.038).