Figures and figure supplements

A nanobody-based molecular toolkit provides new mechanistic insight into clathrin-coat initiation

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Figure 1. Anti-EPS15 Nb identification. (A) Schematic diagrams of the overall architecture and structural features of H. sapiens (Hs) EPS15 and EPS15R proteins. The relative location of various GST-fusion proteins used is indicated. DPF: Asp-Pro-Phe SLiM repeat containing domain, UIM: ubiquitin-

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Tools and resources

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interacting motif. (B) Dendrogram of amino-acid sequence relationship between the seven V\textsubscript{H} anti-EPS15 Nbs selected by biopanning and ELISA screening. Some intrafamily variability may have arisen from RT-PCR and/or amplification PCR-based DNA polymerase errors. (C) Amino acid alignment. Framework regions (FR) 1–4 and interposed, color-coded CDR1-3 stretches are indicated. The pair of cysteine residues involved in disulfide formation is indicated (dashed red line), while the three residues mutated in the conditionally-destabilized (Tang et al., 2016) desNb E\textsubscript{142} protein are highlighted (blue). (D) Pull-down assay of soluble anti-EPS15 Nb in \textit{E. coli} periplasmic lysates using 50 \mu g GST, GST-EPS15 (1-109, 1-217) or (1-314). Analysis of supernatant (S) and pellet (P) fractions after incubation of Sepharose-bead-immobilized GST fusion with \textit{E. coli} periplasmic extract containing the indicated Nb. Coomassie-stained gels shown, with the position of the molecular mass standards (in kDa) indicated. Bound Nb recovered in the pellet fraction is indicated (arrowheads). (E) Binding of Nb E\textsubscript{142} to GST-EPS15 (1-134) and (121-314) lacking the EH1 domain as in D. (F) Combined ribbon and molecular surface representation of a computationally-threaded structure of Nb E\textsubscript{142} modeled by Phyre2 server (Kelley et al., 2015). The locations of the CDR1-3 on the folded V\textsubscript{H} domain model are indicated with coloring as in C, while the NPF SLiM in CDR3 is shown in stick representation and single letter amino acid code.

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Figure 2. EH domain function-blocking Nb_E_142. (A) Pull-down binding assay of Nb_E_142 and an NPF→APA E_142 mutant expressed in E. coli periplasmic extracts to either GST or increasing amounts of GST-EPS15 (1-314). Typical Coomassie-stained gel shown with the position of the molecular Figure 2 continued on next page
mass standards (in kDa) indicated on the left. (B) Schematic depiction of EPS15 and EPS15R interaction partners grouped by peptide SLiM or protein interaction type. (C) Biochemical pull-down assay of NPF-SLiM-containing partners within HeLa whole cell lysate to either immobilized GST or increasing amounts of GST-EPS15 (1-314) EH1-3 domain. After fractionation by SDS-PAGE, gels were either stained with Coomassie blue or transferred to nitrocellulose and immunoblotted with the indicated antibodies. (D) Inhibition of NPF-based partner protein interactions with 200 μg immobilized GST-EPS15 (1-314) by equimolar (45 μg) or 5-fold (225 μg) molar excess of added Nb E_142. (E) Combined ribbon and molecular surface representation of the M. musculus Eps15 EH1 domain (PDB ID: 1QJT) with location of W577 highlighted. Residues within the interaction surface (yellow oval) that undergo substantial chemical shift upon NPF ligand binding (Whitehead et al., 1999) are indicated. (F) Elimination of Nb binding by EH1 domain interaction site disrupting W577A mutation. Coomassie-stained gel of a pull-down assay using 50 μg of the indicated GST-fusion proteins with purified Nb E_142 protein. The location of the BSA carrier protein in the supernantant fractions is indicated, and bound Nb recovered in pellet fractions is indicated (arrowheads).

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Figure 3. Direct interaction of Nb E_142 with the EPS15/R but not intersectin 1 EH domains. (A) Analysis of supernatant (S) and pellet (P) fractions after incubation of 50 μg Sepharose-bead-immobilized GST, GST-EPS15 (1-314) or GST-EPS15R (1-365) with periplasmic extracts containing either Nb E_3, Nb E_142 or Nb E_142 (NPF-APA). Figure 3 continued on next page.
Figure 3 continued

 Nb E_142 or Nb E_142 (NPF→APA) as indicated. Coomassie-stained gels shown, with the position of the molecular mass standards (in kDa) indicated on the left, and bound Nb (arrowheads) shown. (B) Analysis of supernatant (S) and pellet (P) fractions after incubation of 50 µg Sepharose-bead-immobilized GST, GST-EPS15 (1–109/EH1) or GST-EPS15 (1–217/EH1-2) and GST-intersectin 1 l (ITSN1l) (1–312/EH1-2) with periplasmic extracts containing either Nb E_3, Nb E_142 as indicated. Coomassie-stained gel shown, with the position of the molecular mass standards (in kDa) indicated on the left, and bound Nb (arrowheads) shown. (C) Analysis of supernatant (S) and pellet (P) fractions after incubation of 0.5–20 µM GST-EPS15 (1–109/EH1) with 2 µM purified Nb E_142 in the presence of 100 µg/ml BSA as indicated. Coomassie-stained gel shown, with the position of the molecular mass standards (in kDa) indicated on the left. (D) Plots of the mean of three determinations that differed by less than 10% shown for GST-EPS15 (1-109) (squares) or GST-EPS15 (1-314) (circles) titrations. Estimation of $K_D$ from the quantitative analysis of the supernatant fractions yields ~3 µM for a single EH domain (1-109) and ~2 µM for the triple EH domain array (1-314). (E) Analysis of supernatant and pellet fractions after immunoprecipitation of purified GST-EPS15 (1-314) (arrowheads) or native Eps15 in K-562 cell lysate or rat brain cytosol. After fractionation by SDS-PAGE, gels were either stained with Coomassie blue or transferred to nitrocellulose and immunoblotted with the indicated antibodies. Non-specific rabbit anti-EPS15 cross-reactive material in the pellet fraction from the GST-EPS15 (1-314) incubation is identified (bracket).

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Figure 3—figure supplement 1. Quantitative titration of Nb E₁₄₂ with immobilized GST-EPS15 (1-314). SDS-PAGE analysis of supernatant (S) and pellet (P) fractions of a representative pull-down assay after incubation of 10 μM Sepharose-bead-immobilized GST or 0.25–10 μM GST-EPS15 (1–314/EH1-3) with 2 μM purified Nb E₁₄₂ in the presence of 100 μg/ml carrier BSA, as indicated. Coomassie-stained gel shown, with the position of the molecular mass standards (in kDa) indicated on the left.
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Figure 4. Intracellular expression of mNG-Nb E_142 in HeLa cells. (A–E) Selected but representative basal confocal optical sections of HeLa SS6 cells, fixed after transient expression of either control mNG (A and A'; green) or mNG-Nb E_142 (B–G), and stained with anti-EPS15 antibodies (red) and

Figure 4 continued on next page
Hoechst DNA dye (blue). The mNG-expressing transfected cells are outlined (green) and glass-attached ruptured cell cortices (arrows) and Nb E_142 colocalizing weakly with endogenous EPS15 (arrowheads) in intact cells are indicated. Color-separated enlargements (C–E) of the region boxed in B are shown with nuclei (asterisks) in intact cells identified. The presence of mNG-Nb E_142 within EPS15-positive, morphologically aberrant clathrin-coated structures is evident on the adherent membrane fragments from sheared-off non-viable cells (Heuser, 1989). Scale bar; 10 μm; 1.0 μm in C. (F) SDS-PAGE and immunoblot analysis of whole HeLa cell lysates after transfection with plasmids encoding mNG, mNG-Nb E_3 or mNG-Nb E_142 as indicated and either stained with Coomassie blue or probed with the indicated antibodies. Molecular mass standards (in kDa) are shown on the left. (G–G') Selected but representative confocal basal optical section of HeLa SS6 cells expressing mNG-Nb E_142 and analyzed as in B. mNG-expressing transfected cells are outlined. Scale bar; 10 μm.

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Figure 4—figure supplement 1. Efficient intracellular labeling of endogenous EPS15 in AP-2-positive clathrin-coated structures with a polyvalent tandem mNG-Nb E₁₄₂ x 3 fusion protein. (A) Schematic depiction of the relative domain architecture of mNG, mNG-Nb E₁₄₂, and mNG-Nb E₁₄₂ x 3 intrabody constructs. aa: amino acid. (B) Biochemical validation of expression of intact intrabody fusions in HeLa SS6 cells. Whole-cell lysates

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from cells transiently transfected with encoding plasmids as indicated, subjected to SDS-PAGE analysis and either stained with Coomassie blue or transferred to nitrocellulose and immunoblotted with the indicated antibodies. Molecular mass standards (in kDa) are shown on the left, while non-specific, cross-reactive bands observed with the anti-mNG antibody are identified (asterisks). (C–F) Selected but representative basal confocal optical section of HeLa SS6 cells, fixed after transient expression of tandem mNG-Nb E_142 x 3, and stained with the anti-AP-2 α subunit mAb AP.6 (red) and Hoechst DNA dye (blue). The mNG-expressing transfected cells are outlined (green). Scale bar, 10 μm. Color-separated enlargements of the regions boxed in C and C’ are shown below (D–F). (G) Line-scan analysis of fluorescent signal colocalization of the AP-2 adaptor (red) and ectopic mNG-Nb E_142 x 3 (green) along the white line indicated in panel D. a.u.; arbitrary units. (H–I) Selected but representative comparative basal optical sections of HeLa SS6 cells transiently transfected with either the single mNG-Nb E_142 (H–H’) or control mNG (I–I’, green) encoding plasmids.

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Figure 5. Massive endosome displacement of endogenous EPS15 with GFP-FYVE<sub>2</sub>-Nb E<sub>142</sub>. (A) Schematic depiction of the domain arrangement of the GFP-FYVE<sub>2</sub>-based fusion proteins analyzed. (B–D') Selected but representative confocal basal optical sections of HeLa SS6 cells transfected with Figure 5 continued on next page.
either negative control GFP-FYVE_2 (B, B'), GFP-FYVE_2-Nb E_142 (C, C') or destabilized GFP-FYVE_2-desNb E_142 (D, D'). Fixed cells were stained with anti-EPS15 antibodies (red) and DNA dye (blue). Scale bar; 10 μm. (E) SDS-PAGE and immunoblot analysis of whole HeLa SS6 cell lysates either mock transfected or transiently transfected with GFP-FYVE_2-based plasmids as indicated. Gels were either stained with Coomassie blue or transferred to nitrocellulose and probed with the indicated antibodies. Molecular mass standards (in kDa) are shown on the left. (F–G) Basal confocal section of transiently transfected HeLa SS6 cells expressing either GFP-FYVE_2-Nb E_142 (F) or GFP-FYVE_2-desNb E_142 (G) and stained for AP-2 α subunit (red) or EPS15 (blue). Moderate (arrows) and high-level expression (arrowheads) and EPS15 with AP-2 sequestration are indicated. DOI: https://doi.org/10.7554/eLife.41768.008
Figure 5—figure supplement 1. Endocytic transferrin uptake in GFP-FYVE₂-Nb E₁₄₂ expressing HeLa SS6 cells. (A) Schematic representation of the transient transfection and transferrin internalization protocol. (B–F) Maximal intensity projection of a deconvoluted confocal z-stack of fixed HeLa SS6 cells showing transferrin uptake and localization. (G) Transferrin-only channel. (H) GFP-FYVE₂-Nb E₁₄₂ channel. (I) AP-2 channel. (J) Summed fluorescence intensity bar graph showing the distribution of fluorescence intensity across different volume category bins. HeLa SS6 and HeLa SS6 + GFP-FYVE₂-Nb E₁₄₂ are compared.
cells previously transfected with GFP-FYVE$_2$-Nb E$_{142}$ (green) and pulsed at 37\(^\circ\)C with 25 \(\mu\)g/ml Alexa Fluor 568-labeled transferrin (red) for 5 min after starvation in serum-free medium for 60 min. After permeabilization, cells were stained with an anti-AP-2 \(\alpha\)-subunit antibody (blue). A selected but representative stack is shown with color-separated enlargements (C–F) of the region boxed in B shown below. In the presence of GFP-FYVE$_2$-Nb E$_{142}$, the grossly enlarged endosomes are apparent, yet transferrin endocytosis still ensues. In some cells, the distended endosomes contain the GFP-fusion protein (and EPS15/R), transferrin and strongly mislocalized AP-2 (arrow) while in other cells, transferrin is located in the lumen of expanded endosomes with only weak AP-2 attachment (arrowheads). Scale bar; 10 \(\mu\)m. (G–I) Quantitation of short-term transferrin uptake. Representative three-dimensional volume snapshots (G) of the same HeLa SS6 cell monolayer in B transiently transfected with GFP-FYVE$_2$-Nb E$_{142}$ illustrating the Alexa-labeled transferrin uptake channel (gray scale, (G)), segmented (transferrin pseudocolored green, (H) according to the expression pattern of the GFP-FYVE$_2$-Nb E$_{142}$ (green, (I)) decoy Nb. (J) Numerical comparison of summed transferrin fluorescence in GFP-FYVE$_2$-Nb E$_{142}$-negative and -positive segmented populations between various volume bins, analyzed over similar total cellular volumes (5849 endosome objects in 47,366 \(\mu\)m$^3$ for the untransfected and 5841 endosomes in 65,305 \(\mu\)m$^3$ for the Nb E$_{142}$-transfected cells). Bars represent the standard error of the mean (SEM).

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Figure 6. Mitochondrial capture of EPS15 with Akap1-Nb E_142-iRFP. (A–B') Single confocal sections of basal (A and B) or medial (A' and B') optical planes of fixed HeLa SS6 cells previously transfected with either control Akap1-FRB-iRFP or Akap1-Nb E_142-iRFP.
Figure 6 continued
FRB-iRFP (A–A') or Akap1-Nb E142-iRFP (B–B') (pseudocolored red) and stained with an antibody directed against EPS15 (green) and Hoechst DNA dye (blue). The iRFP-expressing transfected cells are outlined (red) and, in medial views, EPS15 assembled in clathrin-coated structures at the plasma membrane is indicated (arrows). (C–D')
Representative single basal confocal sections of fixed HeLa SS6 cells previously transfected with either control Akap1-FRB-iRFP (C–C') or Akap1-Nb E142-iRFP (D–D') (pseudocolored blue) and stained with anti-AP-2 α-subunit (red) and anti-EPS15 (green) antibodies. Relative colocalization of AP-2 with EPS15 in absence of Akap1-FRB/Nb E_142-iRFP signal (C' and D') is shown. Scale bar, 10 μm.
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Figure 6—figure supplement 1. Transferrin internalization in Akap1-Nb E142-expressing HeLa cells. (A) Schematic representation of the transient transfection and transferrin internalization protocol. (B–C') Representative maximal-intensity projections of HeLa SS6 cells transfected with either Akap1-FRB-iRFP (B–B') or Akap1-Nb E142-iRFP (C–C') (pseudocolored blue) and then pulsed at 37°C with 25 μg/ml Alexa Fluor 568-labeled transferrin (red) for 5 min after starvation in serum-free medium for 60 min. Following permeabilization, cells were stained with an anti-EPS15 antibody (green). Scale bar; 10 μm.

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Figure 7. TALEN-mediated generation of FCHO1, FCHO2 and EPS15R triple-null HeLa cells. (A) Schematic depiction of the location and organization of the EPS15L1 gene and TALEN design strategy. The assembled TALEN component repeat variable diresidues (RVDs) are shown, color-coded on the Figure 7 continued on next page
Figure 7 continued

right. The diagnostic ApaLI restriction site (boxed in pale yellow) is indicated. (B) Agarose gel electrophoresis of EPS15L1 exon 5 PCR products with and without ApaL1 digestion as indicated. Compared with the parental HeLa SS6 and clone #64/1.E cell PCR products, the abnormally large cleavage-resistant indels in the three TALEN clones is indicated. (C) SDS-PAGE and immunoblot analysis of TALEN clones. Whole cell lysates subjected to SDS-PAGE were either stained with Coomassie blue or immunoblotted with the indicated antibodies. Molecular mass standards (in kDa) are shown on the left. (D–F) Representative confocal optical section of the steady-state distribution and morphology of clathrin-coated structures at the surface of parental HeLa SS6 (D), HeLa clone #64/1.E (E) and HeLa clone #40 (F) cells stained with antibodies directed against EPS15 (red), the AP-2 adaptor α subunit (green), and Hoechst DNA dye (blue) as indicated. Scale bar; 10 μm.

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Figure 7—figure supplement 1. Defective endocytic transferrin internalization in genome-edited HeLa cells. (A) Schematic representation of the transient transfection and transferrin internalization protocol. (B–D') Representative maximal intensity projections of confocal z-sacks from confluent HeLa SS6, HeLa #64/1.E, and HeLa clone #40.
monolayers of control HeLa SS6 (B–B’), HeLa #64/1.E (C–C’), or HeLa clone #40 FCHO1/2- and EPS15R-null (D–D’) cells pulsed with 25 μg/ml Alexa Fluor 568-transferrin at 37°C for 5 min after starvation in serum-free medium for 60 min. Fixed cells were stained with antibodies against EPS15 (green) and late endosome/lysosomal compartment marker LAMP-1 (blue). The color-separated transferrin (B’–D’) signal is shown on the right. Note the abnormal clathrin-coated structure morphology and distribution in the TALEN-edited cells correlates with reduced transferrin internalization into endosomes and diffuse accumulation of the transferrin receptor at the cell surface (arrows). Scale bar; 10 μm.
Figure 8. Clathrin-coated structure abnormalities in HeLa clone #40 cells lacking FCHO1/2 and EPS15/R. (A–L) Representative confocal basal optical sections of fixed HeLa clone #40 cells expressing either control Akap1-FRB-iRFP (A and G) or Akap1-Nb E_142-iRFP (B-F, H-L; transfected cells outlined)

Figure 8 continued on next page.
Figure 8 continued

in blue) and stained with antibodies to EPS15 (green) and the AP-2 α subunit (red) or HRB (red), as indicated. Color-separated enlargements of the regions boxed in B and H are shown below, with colocalization of displaced endogenous EPS15 with Nb E_142 (arrowheads) in transfected cells and AP-2 with residual EPS15 or HRB at the plasma membrane (arrows) indicated. Scale bar; 10 μm.

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Figure 9. Defective clathrin-mediated endocytosis in Nb E_142-producing clone #40 cells. (A) Schematic representation of the transient transfection and transferrin internalization protocol. (B-G') Transiently transfected HeLa clone #40 cells expressing either negative control Akap1-FRB-iRFP (B-D) or Akap1-Nb E_142-iRFP (E–G') were incubated in starvation medium for 60 min before a 2 min pulse of 25 µg/ml Alexa Fluor 568-transferrin (red, white in Figure 9 continued on next page.)
Figure 9 continued

(E’-G’) at 37°C. The cells were then placed on ice and immediately washed three times with acid stripping buffer to remove surface bound transferrin. Then 37°C medium was readded and the cells incubated for a further 2 min at 37°C before fixation on ice. Permeabilized cells were stained with antibodies against EPS15 (green), and confocal optical sections from a basal (B and E) and medial (C and F) plane or a maximal intensity projection of alternative fields (D and G) are shown with the iRFP fusion protein pseudocolored (blue). Isolated grayscale images of the transferrin (red) channel in the Akap1-Nb E_142-iRFP-transfected preparations are shown on the right (E’-G’) to better visualize the defective clathrin-mediated endocytosis. Scale bar, 10 μm.

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Figure 9—figure supplement 1. Quantitation of short-pulse transferrin uptake in Akap1-Nb E142-iRFP transfected HeLa clone #40 cells. (A–C) Representative example of the image segmentation process of a deconvolved maximal-intensity projection image from a field of fixed, transiently-transfected HeLa clone #40 cells following the two-stage (A) transferrin uptake assay. Both the two-color (transferrin, red) and Akap1-Nb E142-iRFP (upon mitochondria and pseudocolored blue) image (B), with transfected cells highlighted in gray, and corresponding binary grayscale images (C) are shown. Numbered segmented regions are differentially color outlined. (D) Quantitation of number of transferrin-positive structures from five independent maximal-intensity projections, binned by area in the transfected HeLa clone #40 cell population. A total of 5133 transferrin-positive structures in the segmented untransfected and 3896 in the NbE_142-transfected HeLa cells were analyzed over similar total cellular areas (26,452 μm² for the untransfected and 29,061 μm² for the Nb E_142-transfected cells).

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Figure 10. PtdIns(4,5)P$_2$ synthesis in clone #40 cells expressing mitochondria-targeted Nb E$_{142}$. (A–B) Single basal optical sections of HeLa clone #40 cells co-transfected with control Akap1-FRB-iRFP (A) or Akap1-Nb E$_{142}$-iRFP (B) (blue) together with PLC$_{δ1}$ PH-GFP (green; transfected cells outlined). Fixed cells were probed with anti-EPS15 antibodies (red) as indicated. Scale bar, 10 μm.
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Figure 11. Reconstitution of clathrin-coated structure morphology in clone #40 cells with the FCHO1 disordered interdomain linker. (A–B') Representative confocal basal optical sections of HeLa clone #40 cells transiently transfected with both GFP-FCHO1 (1–609)- and Akap1-Nb E_142-iRFP.
Figure 11 continued

iRFP-encoding plasmids before fixation. Permeabilized cells were stained with antibodies directed against either the AP-2 α subunit (red in A) or EPS15 (red in B). Because of diminished GFP expression in these co-transfected cells GFP was enhanced with the GFP-Booster Nb (green; transfected cells outlined), while the iRFP is pseudocolored (blue). Individual cells transfected with the GFP-FCHO1 (1–609) plasmid alone, lacking the mitochondrial iRFP signal, are indicated (yellow arrows), where the morphology of the clathrin-coated structures is rectified by the ectopic FCHO1 interdomain linker. Color-separated enlargements of the regions boxed in A and B are shown on the right. (C–D’) Basal optical sections of HeLa clone #40 cells transfected with either the GFP-FCHO1 (1–609) (C) or Akap1-Nb E_142-iRFP (D) plasmids alone before fixation and permeabilization. The GFP-FCHO1 (1–609)-expressing cells (green; transfected cells outlined) were stained with antibodies directed against the AP-2 α subunit (blue) and EPS15 (red), while the Akap1-Nb E_142-iRFP-expressing cells were stained with anti-AP-2 α-subunit antibodies (red) and Hoechst DNA dye (blue) as indicated. Color-separated enlargements of the regions boxed in C and D are shown on the right. Scale bar; 10 μm.

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Figure 11—figure supplement 1. Quantitation of clathrin-coated structure restructuring in GFP-FCHO1 (1–609) transfected HeLa clone #40 cells. (A–B) Measured distribution of AP-2-positive clathrin-coated structures, binned into the indicated size classes, in populations of HeLa clone #40 cells transiently co-transfected with Akap1-Nb E142-iRFP and GFP-FCHO1 (1–609). Data separated into untransfected clone #40 cells (A) and dual Akap1-Nb E142-iRFP- and GFP-FCHO1 (1–609)-expressing cells (B). Combined dotplot and boxplot with whisker representation (Postma and Goedhart, 2019), where box notching represent the 95% confidence intervals. Similar total cellular areas (105,891.1 \(\mu m^2\) for the untransfected and 110,788.9 \(\mu m^2\) for the Nb E_142-transfected cells) analyzed. (C) Compared with the HeLa clone #40 cells (red), the altered size distribution of the AP-2-positive puncta in the dual Akap1-Nb E142-iRFP- and GFP-FCHO1 (1–609)-expressing HeLa clone #40 cells is analogous to the shifted distribution of AP-2-positive structures in clone #40 cells expressing only the GFP-FCHO1 (1–609) protein, when normalized to cell area.

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Figure 12. Ubiquitin-dependent degradation of EPS15 utilizing a repurposed E3 ligase. (A–B') Representative confocal basal optical sections of HeLa SS6 cells transfected with either GFE3 or G-Nb E_142-E3 plasmids before fixation and staining with anti-EPS15 antibodies (red), and Hoechst DNA dye.
Figure 12 continued

Cells expressing GFP-tagged E3 ligase fusions are outlined (green), with the separated EPS15 signal (A’, B’) shown. (C–D’’). Fixed HeLa SS6 cells, transiently XIAP E3 ligase transfected as in A and B above, were stained with antibodies against EPS15 (red) and the AP-2 α subunit (blue) as indicated. Color-separated enlargements (C’–D’’) of the regions boxed in C and D are shown below. Scale bar; 10 μm.

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