Figure S1
Figure S3
SUPPLEMENTAL FIGURES

Fig.S1. Disruption of the CIN85-Dyn2 complex leads to sustained ERK signaling after EGF stimulation.

(A-D) Mock-treated HuH7 cells and HuH7 cells expressing FLAG-CIN85AC W->Y (A, B) or Dyn2-CBM (C, D) were serum-starved for 3 h and treated with 50 ng/ml EGF for the indicated time points. (A and C) Equal amounts of each sample were analyzed by western blot using specific antibodies against phospho-ERK (pERK) and ERK. (B and D) Quantitation of pERK levels for three independent assays. The amount of phosphorylated protein was normalized to the total protein level in each case, and data are represented as mean ± standard error. The analysis showed a significant increase and sustention of ERK activation in mutant-expressing compared with mock-treated cells (*: p ≤ 0.05).

Fig.S2. Disruption of the CIN85-Dyn2 complex has no effect on transferrin or RhEGF endocytosis.

(A, B) Internalization of Rh-Transferrin (A) and RhEGF (B) in HuH7 cells expressing the CIN85 or Dyn2 mutants as indicated (C, D). Quantitative analysis of transferrin (C) and EGF uptake (D). Values are indicated as mean ± standard error and show a significant decrease in both Rh-Transferrin and RhEGF uptake in the presence of Dyn2ΔPRD (p ≤ 0.01), whereas dominant-negative CIN85 specifically reduced RhEGF uptake (p < 0.0005). (E) Surface biotinylation of EGFR in mock-treated HuH7 cells and HuH7 cells expressing FLAG-CIN85wt or FLAG-CIN85AC W->Y after EGF stimulation. The blots are representative of three independent experiments, and data are presented as mean ± standard error. (F) Surface biotinylation of EGFR in mock-treated HuH7 cells and HuH7 cells expressing FLAG-Dyn2wt or FLAG-Dyn2-CBM after EGF stimulation. Blots are representative of three independent experiments, and data are presented as mean ± standard error. Disruption of CIN85-Dyn2 complex formation did not influence EGFR internalization.
Fig. S3. Trafficking of RhEGF through the early and late endocytic compartments in CIN85wt- and mutant-expressing cells.

(A) Trafficking of RhEGF through the GFP-Rab5-positive early endosomal compartment in CIN85wt (a-c’)- and CIN85AC W->Y (d-f’)-expressing HuH7 cells. In both conditions, no defect in trafficking into and out of the early endosomes was observed (a’-c’ and d’-f’, respectively). Bars: 10 µm. (B) Trafficking of RhEGF through the GFP-Rab7-positive late endosomal compartment in CIN85wt (a-c’)- and CIN85B W->Y (d-f’)-expressing HuH7 cells. The wt-expressing cells showed no defect in RhEGF trafficking into and out of the late endosomal compartment (a’-c’), whereas the CIN85B W->Y-expressing cells displayed a ~2-h delay in trafficking to the late endosome (d’-f’).

Fig. S4: Expression of Dyn2-CBM causes retention of RhEGF in late endosomes/lysosomes.

(A) RhEGF retention in the late endosomal/lysosomal compartment in HuH7 cells expressing Dyn2-CBM compared to mock-transfected cells on the same coverslip. RhEGF trafficked to the lamp1-positive late endosomal/lysosomal compartment (blue) in both cases (enlarged boxes 1-4), but cells expressing Dyn2-CBM (asterisk) retained RhEGF in that compartment at 3-h chase. Control cells lost the RhEGF signal due to degradation (arrows, enlarged boxes 5 and 6). (B) RhEGF trafficking in the late endosomal/lysosomal compartment in HuH7 cells expressing Dyn2-wt. The wt-expressing cells (asterisk) showed no differences in RhEGF trafficking and degradation pattern compared with mock-treated cells (compare enlarged boxes 1-3 to 7-9). Bars: 10 µm.
**Fig.S5: Re-expression of CIN85wt rescues EGFR internalization and degradation in the background of a CIN85 knockdown.**

(A) Images of intracellular RhEGF in HeLa cells treated with CIN85siRNA and re-expressing either CIN85wt (upper panel) or CIN85AC W->Y (lower panel). Cells depleted of CIN85 are marked with a pound sign; cells re-expressing CIN85wt or –ACW->Y are marked with asterisks. Note that RhEGF internalization was restored in both cases, but the siRNA-induced degradation effect was only rescued by CIN85wt re-expression. Bars: 10µm. (B) Quantitation of the amount of intracellular RhEGF from ≥3 independent experiments as described above, relative to mock-treated cells. Data are represented as mean ± standard error and show a significant delay in EGFR degradation in CIN85-depleted cells that was rescued by CIN85wt but not by the Dyn2 binding mutant (p ≤ 0.0005). (C) Representative blot of EGFR following surface biotinylaition in mock- or CIN85 siRNA-treated HeLa cells after EGF treatment. Knockdown of CIN85 did not cause a delay in EGFR internalization. (D) Representative blot for EGFR degradation assay in HeLa cells treated with non-targeting siRNA (NT), CIN85siRNA alone, or CIN85 siRNA plus re-expression of either CIN85wt or CIN85AC W->Y. (E) Quantitation of EGF-induced EGFR internalization in HeLa cells treated with CIN85 siRNA. The blot is representative of three independent experiments, and data are represented as mean ± standard error. A significant delay in EGFR degradation in CIN85 knockdown cells was rescued by CIN85wt but not by the Dyn2 binding mutant (p ≤ 0.05).

**Fig.S6: Re-expression of Dyn2-CBM delays EGFR degradation in the background of a Dyn2 knockdown.**

(A) Dyn2 levels in HeLa cells transfected with either a vector control (NC), Dyn2 shRNA (sh/-), Dyn2 shRNA plus re-expression of Dyn2wt (sh/wt), or Dyn2 shRNA plus re-expression of Dyn2-CBM (sh/CBM). (B) RhEGF uptake assay in HeLa cells transfected with sh/-, sh/wt, or sh/CBM. Transfected cells are marked with asterisks. The Dyn2 knockdown
delayed internalization and subsequent degradation of RhEGF. The CIN85-binding mutant of Dyn2 rescued the internalization defect but still delayed RhEGF degradation compared to control cells, whereas Dyn2 wt accelerated downregulation of the EGFR, resulting in a decreased RhEGF signal compared to mock-transfected cells. Bars = 10 µm. (C) Quantitation of the amount of intracellular RhEGF at various time points. For each condition, ≥40 cells from three independent experiments were analyzed, and data are represented as ratio of sh/-, sh/wt, or sh/CBM signal to the mock signal at each time point ± standard error. Knockdown of Dyn2 significantly delayed EGFR degradation, and this effect was rescued by Dyn2wt but not by the CIN85-binding mutant (*: p ≤ 0.05). (D) Quantitation of the amount of intracellular RhEGF in Dyn2-CBM–re-expressing cells relative to Dyn2wt–re-expressing cells. Data are presented as mean ± standard error and show a significant delay in Dyn2-mutant relative to – wt-expressing cells (*: p ≤ 0.0001).

**MOVIES**

**Movie 1: Tubulation of GFP-Rab7-positive late endosomes in HeLa cells co-expressing GFP-Rab7 and CIN85wt.** Movies start at chase time t=0 after 45 min of pretreatment with RhEGF at 37 °C. Images were captured every 5 s. Note that the Rab7-tubules are short and vesiculate rapidly.

**Movie 2: Tubulation of GFP-Rab7-positive late endosomes in HeLa cells co-expressing GFP-Rab7 and CIN85AC W->Y.** Movies start at chase time t=0 after 45 min of pretreatment with RhEGF at 37 °C. Images were captured every 5 s. In contrast to the CIN85wt-expressing cells, mutant cells contained longer Rab7-positive tubules that barely vesiculated during the observation time.
Movie 3: Tubulation of GFP-Rab7-positive late endosomes in HeLa cells co-expressing GFP-Rab7 and CIN85B W->Y. Movies start at chase time $t=0$ after 45 min of pretreatment with RhEGF at 37 °C. Images were captured every 5 s. Note the short Rab7 tubules that vesiculate rapidly.

Movie 4: Tubulation of GFP-Rab7-positive late endosomes in HeLa cells co-expressing GFP-Rab7 and dsRed-Dyn2wt. Movies start at chase time $t=0$ after 45 min of pretreatment with EGF at 37 °C. Images were captured every 5 s. Note that the Rab7 tubules are short and vesiculate rapidly.

Movie 5: Tubulation of GFP-Rab7-positive late endosomes in HeLa cells co-expressing GFP-Rab7 and dsRed-Dyn-CBM. Movies start at chase time $t=0$ after 45 min of pretreatment with EGF at 37 °C. Images were captured every 5 s. In contrast to the Dyn2wt-expressing cells, mutant cells displayed longer Rab7-positive tubules that did not vesiculate during the observed time.

Movie 6: Tubulation of GFP-Rab7-positive late endosomes in rat fibroblasts expressing GFP-Rab7 (MOCK). Movies start at chase time $t=0$ after 45 min of pretreatment with RhEGF at 37 °C. Images were captured every 5 s. Mock-treated cells displayed Rab7-positive vesicles and short tubules that vesiculated rapidly.

Movie 7: Tubulation of GFP-Rab7-positive late endosomes in rat fibroblasts depleted of Dyn2 and expressing GFP-Rab7. Movies start at chase time $t=0$ after 45 min of pretreatment with RhEGF at 37 °C. Images were captured every 5 s. In contrast to the control cells, Dyn2-deficient cells displayed longer Rab7-positive tubules that did not vesiculate during the observed time.