A Dyn2–CIN85 complex mediates degradative traffic of the EGFR by regulation of late endosomal budding

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The epidermal growth factor receptor (EGFR) is over-expressed in a variety of human cancers. Downstream signalling of this receptor is tightly regulated both spatially and temporally by controlling its internalization and subsequent degradation. Internalization of the EGFR requires dynamin 2 (Dyn2), a large GTPase that deforms lipid bilayers, leading to vesicle scission. The adaptor protein CIN85 (cbl-interacting protein of 85 kDa), which has been proposed to indirectly link the EGFR to the endocytic machinery at the plasma membrane, is also thought to be involved in receptor internalization. Here, we report a novel and direct interaction between Dyn2 and CIN85 that is induced by EGFR stimulation and, most surprisingly, occurs late in the endocytic process. Importantly, disruption of the CIN85–Dyn2 interaction results in accumulation of internalized EGFR in late endosomes that become aberrantly elongated into distended tubules. Consistent with the accumulation of this receptor is a sustention of downstream signalling cascades. These findings provide novel insights into a previously unknown protein complex that can regulate EGFR traffic at very late stages of the endocytic pathway.

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Introduction

The epidermal growth factor receptor (EGFR) is over-expressed in a variety of human cancers. Activation of the EGFR by EGF initiates various signalling cascades that control important cellular processes such as proliferation and growth. Therefore, activation and downstream signalling of the EGFR are tightly regulated both spatially and temporally. One mechanism used by cells to modulate downstream

signalling cascades is to control the internalization and subsequent degradation of this receptor. The formation of clathrin-coated vesicles is thought to be the major mechanism responsible for sequestering and internalizing cell surface receptors and their bound ligands (Huang *et al*, 2004), but there are also numerous reports indicating that the activated EGFR can be internalized in a clathrin-independent manner (Sigismund *et al*, 2005; Orth *et al*, 2006), probably depending on the EGF concentration.

Both of these endocytic processes are known to require dynamin 2 (Dyn2), a large GTPase that assembles into circular polymers that constrict and deform lipid bilayers, leading to vesicle scission (Hinshaw and Schmid, 1995; Pucadyil and Schmid, 2008). The function of the dynamins in the endocytic process has traditionally been viewed as providing either a mechano-chemical (Stowell *et al*, 1999; Danino *et al*, 2004) or possibly a regulatory function (Sever *et al*, 2000; Takei *et al*, 2005) in liberating nascent endocytic vesicles. Generally, dynamin has not been considered to have a function in receptor cargo sequestration and trafficking, despite the fact that it is linked to multiple endocytic cargo-binding adaptor proteins including Grb2 (Miki *et al*, 1994), Eps15 (Sengar *et al*, 1999), and AP2 (Wang *et al*, 1995).

Ubiquitination of EGFR is sufficient for proper sorting and targeting of the receptor to the lysosome for degradation (Ettenberg *et al*, 2001; Huang *et al*, 2006). Detailed studies revealed that cbl-mediated ubiquitination of the receptor occurs at the plasma membrane (Stang *et al*, 2000) and is also essential for EGFR's exit from early endosomes (Ravid *et al*, 2004). Cbl has been shown to recruit cbl-interacting protein of 85 kDa (CIN85) to the endocytic complex in an EGF-dependent and ubiquitin-ligase-independent manner. CIN85 functions as an adaptor by binding to various endocytic proteins such as endophilin, Src family kinases, and Grb2 (reviewed by Dikic, 2002). Previous studies suggested that CIN85 participates not only in the initial steps of EGFR internalization (Soubeyran *et al*, 2002; Schmidt *et al*, 2004), but also in post-endocytic receptor trafficking and degradation (de Melker *et al*, 2001; Kowanetz *et al*, 2004).

Here, we report a novel EGF-induced direct interaction between Dyn2 and CIN85. Surprisingly, this CIN85–Dyn2 interaction occurs late in the endocytic process at a Rab7-positive late endosome. Disruption of this interaction results in a defect in vesicle budding from late endosomes with a concomitant accumulation of the EGFR in this compartment.

Results

The CIN85–Dyn2 interaction is induced by EGF

To determine whether Dyn2 and CIN85 could interact, we first examined the subcellular localizations of CIN85 and Dyn2 under basal conditions (10% FBS). As shown in Figure 1A, a partial overlap between CIN85 (green)-

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and Dyn2 (red)-positive spots was observed at punctate, endosomal-like structures (arrows) and in a perinuclear-localized compartment. His-Tag- and GST-pulldown assays

from HeLa cell lysates confirmed the interaction of these two proteins (Figure 1B and C). In addition, we performed co-immunoprecipitation assays using different epithelial cell

types. As shown in Figure 1D, CIN85 and Dyn2 were isolated as a complex from all cell types examined. Of note, the endogenous CIN85 levels in the cell types examined were low, and, therefore, the signal in the input lanes was weak relative to the precipitated protein by western blot.

Dyn2 is well known for its function in the severing of nascent endocytic vesicles, whereas CIN85 is involved in EGFR trafficking. Therefore, we hypothesized that the interaction between CIN85 and Dyn2 might be induced by EGF. To test this hypothesis, we immunoprecipitated CIN85 from HuH7 cell lysates at different time points after EGF stimulation and observed an induction of the CIN85–Dyn2 interaction after 30–60 min of EGF treatment (Figure 1E and F). This observation was confirmed by immunofluorescence (IF) analysis before and after EGF stimulation (Figure 1G), which showed that EGF stimulation of Clone 9 cells clearly increased the perinuclear co-localization of Dyn2 and CIN85 (arrows). The formation of the CIN85–Dyn2 complex at a relatively late time point after EGF stimulation was unexpected because Dyn2 is thought mainly to act at the plasma membrane or early endosomes. In contrast, the data provided here suggest that the interaction between CIN85 and Dyn2 might be necessary for later stages of EGFR trafficking/degradation.

SH3A and SH3C domains of CIN85 interact with the proline-rich domain of Dyn2

CIN85 contains three N-terminal SH3 domains (Figure 2A), whereas Dyn2 contains a C-terminal proline-rich domain (PRD). Thus, an SH3–PRD interaction could mediate the formation of the CIN85–Dyn2 complex. To test this hypothesis, we performed GST-pulldown assays using HeLa cell lysates. Dyn2 interacted with full-length CIN85wt protein (Figure 1C) as well as with the N-terminal portion of CIN85 containing all three SH3 domains (SH3ABC) and with the isolated SH3A and SH3C domains (Figure 2B). However, no interaction was detected between Dyn2 and the SH3B domain, which was shown to be the predominant one of three potential cbl-interacting sites within CIN85 (Take *et al*, 2000; Kowanetz *et al*, 2003). Previously published data showed that mutation of a tryptophan residue in the putative-binding pocket of Fyn abolished binding to a defined SH3 domain ligand (Panchamoorthy *et al*, 1994). Therefore, two similar point mutations were introduced into the putative-binding pocket of each of the three CIN85 SH3 domains, either independently or simultaneously. As shown in Figure 2B, mutation of one SH3 domain (SH3A W→Y) or all SH3 domains (SH3ABC W→Y) was sufficient to completely abolish the binding of CIN85 to Dyn2. We next performed GST-pulldown assays using an SH3A(B)C W→Y mutant that

contained mutations of the SH3A and SH3C, but not of the SH3B domain. This mutant failed to interact with both Dyn2 from HeLa cell lysate (Figure 2C) and with purified His–Dyn2 (Figure 2D), indicating that the SH3A and SH3C domains of CIN85 are necessary and sufficient to mediate a direct interaction with Dyn2.

In a second approach, we identified the binding motif for CIN85 in the Dyn2–PRD. As shown in Figure 2E, the PRD of Dyn2 contains two potential CIN85-binding regions, PAAPSR and PTPQRR, both corresponding to the consensus sequence for CIN85 SH3-binding sites (PX[P/A]XXR; Kurakin *et al*, 2003). To determine whether one or both of these sites are necessary for Dyn2 binding to CIN85, we created two mutants of the Dyn2–PRD: ΔP1, with deletion of the PAAPSR motif, and a double mutant called Dyn2–CIN85-binding mutant (CBM) that also bears two point mutations in the second motif (PTPQRR→ATAQRR). GST-pulldown assays indicated that the Dyn2ΔP1 mutant showed markedly reduced binding to CIN85 compared with Dyn2wt, whereas the double mutant CBM almost completely abolished CIN85 binding (Figure 2F; the exposure times were chosen so that the CBM does not appear completely blank). This result suggested that both motifs in the Dyn2–PRD are necessary for efficient CIN85 binding. Taken together, these data provide evidence for a novel, specific, and direct interaction between CIN85 and Dyn2.

Interfering with formation of the CIN85–Dyn2 complex leads to a delay in EGFR degradation and sustained cell signalling

The EGF-induced formation of the CIN85–Dyn2 complex suggested that it might function in EGFR trafficking or degradation. To test these possibilities, we first performed biochemical EGFR degradation assays in HuH7 cells expressing either CIN85wt or the Dyn2-binding mutant of CIN85 (CIN85AC W→Y). Indeed, cells expressing the CIN85AC W→Y mutant showed a substantial delay in EGFR degradation compared with mock- or wt-transfected cells (Figure 3A and B; $P = 0.0006$). Similarly, over-expression of the Dyn2–CBM mutant, but not of Dyn2wt, showed a comparable delay of EGFR degradation (Figure 3C and D; $P = 0.02$). Relative to wt-expressing cells as the control, both binding mutants displayed an approximately two- to four-fold increase in EGFR levels at late time points (90 min), indicative of a substantial delay in EGFR downregulation.

In addition to the biochemical assays, IF-based Rhodamine-EGF (RhEGF) uptake/degradation assays were performed. Disruption of the CIN85–Dyn2 complex by over-expression of either CIN85AC W→Y or Dyn2–CBM caused a significant ($P \leq 0.003$) retention of RhEGF in a perinuclear endosomal compartment (Figure 4A and C), resulting in

Figure 1 The interaction between CIN85 and Dyn2 is induced by EGF. (A) Clone 9 cells were stained for CIN85 (green) and Dyn2 (red) under full-serum conditions. The merged image, indicated as a magnification of the boxed area, shows obvious overlap between the two proteins. Bar, 10 μ m. (B) Pulldown assay using either purified His–Dyn2 or His alone incubated with HeLa cell lysate under full-serum conditions to assess its interaction with CIN85. (C) Pulldown assay using GST–CIN85 or GST alone incubated with HeLa cell lysate to assess its interaction with Dyn2. The size of GST-tagged full-length (FL) CIN85 is indicated by an arrow. (D) Co-immunoprecipitation of CIN85 and Dyn2 from HuH7 or HeLa cell lysates under full-serum conditions. (E) Co-immunoprecipitation of CIN85 and Dyn2 from HuH7 cells stimulated with 50 ng/ml EGF for the indicated time points. (F) Quantitative analysis of the EGF-induced CIN85–Dyn2 interaction from three independent experiments performed in HuH7. The amount of co-precipitated Dyn2 was normalized to the amount of CIN85 in each sample, and the data are represented as mean $\pm$ standard error. Statistical analysis revealed a significant difference in the CIN85–Dyn2 interaction between early and late time points, P -values (*) = 0.03. (G) Co-localization of CIN85 (green) and Dyn2 (red) before and after EGF stimulation in Clone 9 cells. Arrows point to the prominent perinuclear localization of the CIN85–Dyn2 complex. Bar, 10 μ m.

Figure 2 Identification of specific domains that mediate the CIN85–Dyn2 interaction. **(A)** Schematic representation of the CIN85 domain structure. CIN85 consists of three SH3 domains (SH3A–C), a proline-rich region (PPro), and a coiled-coil domain at the C-terminus. **(B)** GST-pulldown assay using various CIN85 constructs: each of the SH3 domains separately, an N-terminal fragment containing all three SH3 domains (SH3ABC), the SH3A domain containing two W→Y mutations that inhibit binding to proline-rich regions (SH3A W→Y), or the N-terminal fragment containing two W→Y mutations in each of the three SH3 domains (SH3ABC W→Y). Arrows point to the size of the GST-fusion proteins as labelled above the blot. **(C, D)** GST-pulldown assay using purified GST-CIN85-fusion proteins incubated with HeLa cell lysate under full-serum conditions **(C)** or incubated with purified His-Dyn2 **(D)** to assess a direct interaction with Dyn2. The A(B)C W→Y construct bears two W→Y mutations in both the SH3A and SH3C domain that abolish SH3–PRD interactions. **(E)** Schematic representation of the proline-rich domain (PRD) of Dyn2, consisting of amino acids 747–866. The black boxes mark the two potential-binding sites for CIN85, and the binding motif for each site is indicated. Two Dyn2–PRD mutants were generated: ΔP1 has a deletion of the C-terminal PAAPSR motif, whereas the CBM bears the PPASR-deletion plus a PTPQRR→ATAQRR double point mutation. **(F)** GST pulldown using GST-CIN85 and purified Dyn2wt, ΔP1, or CBM.

~60% more RhEGF in the mutant-expressing cells at late time points (3 h) relative to mock-treated cells. In contrast, over-expression of CIN85wt or Dyn2wt had no effect on EGFR downregulation compared with mock-treated cells (Figure 4B and D). We predicted that delayed degradation of the EGFR in the mutant-expressing cells would lead to sustained downstream signalling. Consistent with this finding, we observed a significant increase in ERK activation in CIN85 or Dyn2 mutant-expressing cells ($P \leq 0.05$; Supplementary Figure S1), whereas Akt activation was not affected (data not shown). This observation is in agreement with the previous reports (Haugh *et al*, 1999a, b; Burke *et al*, 2001), showing that EGFR signalling is maintained from the endosome after internalization.

As the early stages of EGFR endocytosis appeared unaffected in the mutant-expressing cells, we further examined the effects of CIN85 mutants on the uptake of both EGF and transferrin (Tf). As expected, none of the CIN85 mutations affected internalization of Tf, although the dominant-

negative form (CIN85ABC) decreased the amount of internalized EGF ($P < 0.0005$), as reported previously (Soubeyran *et al*, 2002; Supplementary Figure S2A–D). This result indicates that manipulation of CIN85 selectively influences EGFR-mediated internalization. The IF data were confirmed by assaying EGFR internalization with surface biotinylation in HuH7 cells that expressed either wt or mutant proteins of the two binding partners (Supplementary Figure S2E and F). Consistent with the IF data, the exogenously expressed proteins did not influence early steps of EGFR internalization. In addition, we examined the influence of wt and mutant forms of CIN85 and Dyn2 on EGFR internalization and recycling when lower concentrations of EGF (1.5 and 5 ng/ml) were used. Significant differences between mock-treated and transfected cells were not observed (data not shown). These data further support the concept that a CIN85–Dyn2 complex forms and functions mainly at organelles involved in post-endocytic EGFR trafficking and/or signalling.

Figure 3 Disruption of the CIN85–Dyn2 complex impairs EGFR degradation. Mock-treated HuH7 cells and HuH7 cells expressing FLAG-CIN85wt, FLAG-CIN85AC W→Y (A, B), FLAG-Dyn2wt, or FLAG-Dyn2-CBM (C, D) were serum starved for 4 h in the presence of 50 µg/ml cycloheximide (CHX) and treated with 50 ng/ml EGF plus CHX for the indicated time points. Equal amounts of each sample were analysed by western blot to assess EGFR degradation (A, C). (B, D) Quantitation of ≥ 3 independent EGFR degradation assays comparing CIN85wt and CIN85AC W→Y (B) or Dyn2wt and Dyn2-CBM (D) to mock-treated cells. The amount of EGFR in each sample was normalized to actin, and data are represented as mean $\pm$ standard error. Both mutants caused a significant delay in EGFR degradation compared with wt ($P = 0.006$ and 0.02 , respectively).

The pronounced effects of a disrupted CIN85–Dyn2 complex on EGFR degradation suggest that delivery of the EGFR to the lysosome for degradation might be altered. To determine where EGFR retention might occur, we performed a morphological analysis using marker proteins for early and late endosomes. RhEGF was used to visualize EGFR trafficking, and GFP-Rab5 and GFP-Rab7 were used to label early and late endosomes, respectively. First, mock-transfected cells (control) or cells expressing CIN85AC W→Y were subjected to a pulse-chase assay followed by IF processing. In control cells, we found that the RhEGF reached the early endosomes after 15 min (Supplementary Figure S3A) and continued to traffic to the late endosomes, where it was found in Rab7-positive structures at about 45 min–1 h of chase (Figure 5A, insets b and b'). Over time, the RhEGF signal decreased and mostly disappeared after 1–2 h of chase (Figure 5A, insets c and c'). In an interesting contrast, in the CIN85 mutant-expressing cells, trafficking through the early endosomal compartment (Supplementary Figure S3A) and entry into late endosomes were not disturbed (Figure 5A, insets d–e'), but we observed a marked accumulation of RhEGF in GFP-Rab7-positive late endosomes at times when the RhEGF signal was mostly diminished in control cells (Figure 5A, compare insets f and f' with c and c'). RhEGF was eventually degraded in the context of the CIN85 mutant background, although with a delay of ~ 1 –2 h. Over-expression of CIN85wt had no effect on RhEGF entry into and exit out of the late endosome, whereas the CIN85 cbl-binding

mutant, CIN85B W→Y (see below), displayed an obvious delay in trafficking to the late endosome ($P < 0.0005$; Figure 5B; Supplementary Figure S3B). In agreement with the observed RhEGF trafficking defects in CIN85AC W→Y-expressing cells, over-expression of Dyn2-CBM, but not of Dyn2wt, caused retention of RhEGF at the late endosome at late time points (Supplementary Figure S4). Taken together, these results indicate that disruption of the CIN85–Dyn2 complex leads to retention of EGFR in the late endosome.

We next examined whether CIN85 and Dyn2 localize to the Rab7-positive late endosome, consistent with a function in late endosomal trafficking. These studies were performed in living cells co-expressing either GFP-Rab7 and dsRed-Dyn2wt or GFP-Rab7 and dsRed-CIN85wt. Interestingly, both proteins preferentially localized to dynamic Rab7 structures, especially small tubules. Figure 5C shows a clear localization of Dyn2 (insets a–b') and CIN85 (insets c–d') at the Rab7-positive late endosome. Dyn2-CBM did not colocalize with Rab7 (Figure 5D, insets a and a'), suggesting that CIN85 might recruit Dyn2 to that compartment. To test this hypothesis, we examined the effects of CIN85wt or CIN85AC W→Y on the late endosomal localization of Dyn2. As expected, CIN85AC W→Y, but not CIN85wt, prevented the association of Dyn2 with the Rab7-positive late endosome (Figure 5D, compare insets b and b' with c and c').

Moreover, we tested biochemically for interactions between the CIN85–Dyn2 complex and Rab7 as a protein component of late endosomes. Towards this end, we per-

Figure 4 Disruption of the CIN85–Dyn2 complex retains EGFR in a perinuclear compartment. (A–D) RhEGF uptake/degradation assay in HuH7 cells over-expressing either CIN85wt, CIN85AC W→Y (A, B), Dyn2wt, or Dyn2-CBM (C, D). In each case, transfected cells are marked by asterisks. Bars, 10 μ m. (B, D) Quantitation of the amount of RhEGF at the indicated time points in cells expressing CIN85wt, Dyn2wt, or binding mutants of both proteins. For each time point, ≥ 30 cells were counted, the amount of intracellular RhEGF was calculated relative to mock-treated cells, and data are represented as mean $\pm$ standard error. Both mutants induced a significant delay in RhEGF degradation ($P \leq 0.003$), whereas the wt proteins had no effect compared with mock-treated cells.

formed co-immunoprecipitation assays from lysates of HuH7 cells expressing GFP–Rab7 under basal conditions (10% FBS). In support of our findings, CIN85 and Dyn2 specifically associated with the late endosomal marker Rab7 (Figure 5E).

As the SH3 domains of CIN85 have been shown to interact with a number of proteins such as cbl, it was important to test whether the effects of the CIN85AC W→Y mutant were specifically because of impaired Dyn2 binding and not to reduced cbl binding. Therefore, we compared the capacity of the CIN85AC W→Y and CIN85B W→Y proteins to bind to cbl. We expected a reduction in cbl binding by the CIN85B W→Y mutant because that region was shown previously to be important for the CIN85–cbl interaction (Kowanetz *et al*, 2003). As expected, the CIN85 AC W→Y mutation had no effect on cbl binding, whereas the CIN85B W→Y mutation completely abolished the cbl–CIN85 interaction (Figure 6A). In addition, over-expression of CIN85B W→Y, but not of CIN85wt or CIN85AC W→Y (Figure 6B and C; Supplementary Figure S3B), in HuH7 cells caused a substantial delay in RhEGF degradation ($P < 0.0005$) because of retention

in the early endosome (Figure 6B, compare insets c' and f'), which is consistent with the proposed function for cbl in the exit of EGFR from early endosomes (Ravid *et al*, 2004). The differences in EGF accumulation based on point mutations in the different SH3 domains of CIN85 further support the specificity of the CIN85AC mutant and its decreased interaction with Dyn2.

Depletion of CIN85 or Dyn2 delays EGFR degradation

To further determine the effects of CIN85 on EGFR trafficking/degradation, we depleted CIN85 in HeLa cells using siRNA. SiRNA-induced knockdown of CIN85 (Figure 7A) delayed EGFR degradation in both biochemical and morphological assays (Figure 7B–E). Interestingly, knockdown of CIN85 modestly, but not significantly, reduced RhEGF internalization at 15 min (Figure 7B and C; Supplementary Figure S5), although surface biotinylation assays did not reveal an endocytosis defect in CIN85 siRNA-treated cells (Supplementary Figure S5C). The difference between mock- and siRNA-treated cells was more pronounced at later time points when the EGFR traffics through the endosomal path-

Figure 5 Inhibiting CIN85–Dyn2 complex formation leads to retention of EGFR in Rab7-positive late endosomes. **(A)** RhEGF trafficking assay in HuH7 cells expressing either GFP-Rab7 (green) alone (a–c') or together with CIN85AC W→Y (d–f'). Arrowheads point to the Rab7 rings that are positive for RhEGF. (a'–c') and (d'–f') show magnifications of the boxed areas in (a–c) and (d–f), respectively. Bars, 10 μ m. **(B)** Quantitation of the amount of RhEGF colocalizing with GFP-Rab7-positive endosomes at various time points in HuH7 cells expressing either GFP-Rab7 alone ('MOCK', green line) or together with CIN85wt (yellow line), CIN85AC W→Y (blue line), or CIN85B W→Y (red line). For each condition, ≥ 30 cells were counted, and data are represented as mean $\pm$ standard error. Disruption of CIN85–Dyn2 complex formation caused a significant delay in EGFR trafficking out of the late endosome ($*P < 0.0005$), whereas interfering with the CIN85–cbl interaction resulted in a significant delay in EGFR trafficking to the late endosome ($*P < 0.0005$). **(C)** Colocalization of dsRed-Dyn2 (a–b') or dsRed-CIN85 (c–d') with GFP-Rab7 in living cells. Images were captured 45–60 min post-EGF stimulation. Bars, 10 μ m. **(D)** Loss of Dyn2 localization on the Rab7-positive endosomes upon over-expression of Dyn2-CBM (a, a') or CIN85AC W→Y (b, b'), but not in the presence of CIN85wt (c, c'). Stills were taken from living cells 45–60 min post-EGF stimulation. Bars, 10 μ m. **(E)** Co-immunoprecipitation of GFP-Rab7, CIN85, and Dyn2 in HuH7 cells under full-serum conditions. The control IP was performed using non-specific IgG, and E-cadherin was used as a negative control for Rab7 interaction.

way, resulting in $\sim 80\%$ less RhEGF degradation in the siRNA compared with mock-treated cells (3 h chase; Figure 7B and C). Re-expression of CIN85wt completely rescued the internalization and trafficking/degradation defect of CIN85 siRNA-treated cells. In contrast, re-expression of CIN85AC W→Y, although able to rescue the internalization defect, failed to restore EGFR trafficking/degradation (Supplementary Figure S5A, B and D, E). Taken together, these observations support the idea that the interaction between CIN85 and Dyn2 is

required for the efficient transport of EGF/EGFR from a late endosomal compartment to lysosomes.

As an extension of the knockdown studies described above, an shRNA-knockdown/re-expression system (Nolz *et al*, 2007) was used to specifically knockdown Dyn2 (Gomez *et al*, 2005) and concomitantly re-express either Dyn2wt or Dyn2-CBM in HeLa cells. As shown in Supplementary Figure S6A, Dyn2 was efficiently knocked down ($\sim 90\%$), and Dyn2wt and Dyn2-CBM were over-

Figure 6 Specificity of the CIN85AC W→Y mutant: interfering with CIN85–cbl binding leads to retention of RhEGF in early endosomes. (A) Co-immunoprecipitation assay from HuH7 cells co-expressing HA-cbl and either FLAG-CIN85wt, FLAG-CIN85AC W→Y, or FLAG-CIN85B W→Y. Cells were treated with 50 ng/ml EGF for 15 min after serum starvation o/n. (B) RhEGF uptake/degradation assay in HuH7 cells expressing either GFP-Rab5 alone (a–c') or together with FLAG-CIN85B W→Y (d–f'). Boxes indicate the areas of higher magnification shown in (a'–c') and (d'–f'), respectively; arrows point to the RhEGF (red) in Rab5-labelled early endosomes (green). Bars, 10 μ m. (C) Quantitation of the amount of RhEGF colocalizing with GFP-Rab5-positive endosomes at various time points in HuH7 cells expressing either GFP-Rab5 alone ('MOCK', green line) or together with CIN85wt (yellow line), CIN85AC W→Y (blue line), or CIN85B W→Y (red line). For each condition, ≥ 30 cells were counted, and data are represented as mean $\pm$ standard error. Interfering with CIN85–cbl complex formation resulted in a significant delay in EGFR trafficking out of the early endosome (* $P < 0.0005$).

expressed compared with endogenous levels. RhEGF uptake/degradation assays in HeLa cells (Supplementary Figure S6B–D) showed that knockdown of Dyn2 reduced EGFR internalization by a modest $\sim 30\%$ relative to mock and caused an expected delay in EGFR degradation. Concomitant re-expression of Dyn2wt rescued both the internalization and degradation defects and, in fact, caused slightly faster degradation of EGFR compared with control cells. In contrast, re-expression of Dyn2-CBM, although able to rescue the internalization defect, markedly delayed RhEGF degradation compared with surrounding control cells. Quantitation of intracellular RhEGF levels under the various experimental conditions showed that at 3 h chase, Dyn2-depleted and CBM-re-expressing cells contained 60–70% more intracellular/undegraded RhEGF than the control cells. In contrast, the Dyn2wt-re-expressing cells had $\sim 20\%$ less RhEGF, which suggests faster degradation be-

cause of more available Dyn2 for transport to the lysosome (Supplementary Figure S6C; P -values < 0.0005). When Dyn2-CBM-re-expressing cells are compared with wt-re-expressing cells, the difference at the late time point is even more pronounced ($\sim$ two-fold increase in intracellular RhEGF in CBM compared with wt-expressing cells), although there was no difference in the initial RhEGF internalization (15 min pulse; Supplementary Figure S6D; P -values < 0.0005).

The CIN85–Dyn2 complex mediates vesiculation of late endosomes

To gain mechanistic insight into how the CIN85–Dyn2 complex might regulate trafficking events from the late endosome, we performed time-lapse microscopy of RhEGF-stimulated HeLa cells co-expressing GFP-Rab7 and CIN85wt or CIN85AC W→Y. Expression of mutant CIN85 induced a

Figure 7 Knockdown of CIN85 delays EGFR degradation. **(A)** HeLa cells were treated with CIN85 siRNA for 72 h, and the amounts of CIN85 and actin (as loading control) were determined by western blot. **(B)** RhEGF uptake/degradation assay in HeLa cells treated with CIN85 siRNA. Under each condition, cells were stained for CIN85 (top panel) to identify CIN85-depleted cells, and lamp1 as a late endosomal/lysosomal marker was co-stained in blue. Asterisks indicate the cells with reduced CIN85 levels. Bars, 10 μ m. **(C)** Quantitation of the amount of intracellular RhEGF at various time points. For each condition, ≥ 40 cells from three independent experiments were analysed, and data are represented as the mean ratio of the RhEGF in siCIN85- to mock-treated cells at each time point $\pm$ standard error. CIN85 knockdown caused a significant delay in EGFR degradation ($*P < 0.0005$). **(D)** Biochemical EGFR degradation assay in HeLa cells treated with CIN85 siRNA compared with mock-transfected cells. **(E)** Quantitative analysis of EGFR levels in four independent biochemical experiments. The amount of EGFR was normalized to actin levels in each case, the resulting values were normalized to $t = 0$ under each condition, and data are represented as mean $\pm$ standard error. Depletion of CIN85 significantly delayed EGFR degradation ($P = 0.05$).

striking difference in the morphology of GFP-Rab7-positive structures. Whereas CIN85wt-expressing cells showed more puncta and shorter ($\sim 1.5 \mu$ m) tubules (Figure 8A; Supplementary movie 1), the CIN85AC W $\rightarrow$ Y-expressing cells displayed a large number of long tubules ($\sim 3 \mu$ m) in addition to the puncta (Figure 8B; Supplementary movie 2). In the wt-expressing cells, numerous vesicles rapidly budded from these tubules, but the vesiculation process was attenuated in the mutant-expressing cells, which resulted in the accumulation of long undulating tubules (compare Supplementary movies 1 and 2) that were also positive for RhEGF. Quantification

of GFP-Rab7 tubule length revealed that the CIN85 mutant-expressing cells had a significant, four-fold increase in long tubular late endosomes compared with CIN85wt-expressing cells (Figure 8D). In contrast, cells expressing the cbl-binding mutant CIN85B W $\rightarrow$ Y did not have the elongated Rab7 tubules and, in fact, had fewer and slightly shorter Rab7 extensions compared with wt (see Supplementary movie 3; Figure 8C and E, and data not shown). In addition, RhEGF appeared to overlap only occasionally with the Rab7-vesicles, although numerous RhEGF- and Rab7-positive structures were in close proximity (arrows in Figure 8C).

Figure 8 Over-expression of CIN85AC W→Y, but not CIN85B W→Y, prevents late endosomal budding and increases GFP-Rab7 tubule length. (A–C) Movie stills of HeLa cells co-expressing either GFP-Rab7 and CIN85wt (A), CIN85AC W→Y (B), or CIN85B W→Y (C) and stimulated with RhEGF. Note that in the wt-expressing cells, Rab7 and RhEGF localized to spots and short tubules, whereas CIN85AC W→Y-expressing cells showed extremely long tubules (arrows) of both markers. Over-expression of CIN85B W→Y did not cause elongation of Rab7-positive structures and led to significantly fewer and slightly shorter tubules devoid of RhEGF (arrows). Bars, 5 μm . (D) Quantitation of the number of GFP-Rab7 tubules $\leq 1.5 \mu\text{m}$ or $\geq 3.0 \mu\text{m}$ in CIN85wt- and CIN85AC W→Y-expressing cells. GFP-Rab7 tubules were measured in 29 cells for the wt and 36 cells for the mutant. Data are represented as mean $\pm$ standard error; $P \leq 0.03$. (E) Quantitation of the number of GFP-Rab7 tubules $< 1.5 \mu\text{m}$ in CIN85wt- and CIN85B W→Y-expressing cells. GFP-Rab7 tubules were measured in 26 cells for both the wt and the mutant. Data are represented as mean $\pm$ standard error, $P = 0.009$.

In addition, experiments performed using either Dyn2wt or Dyn2-CBM provided similar results as for the CIN85wt and CIN85AC W→Y mutant, respectively (Figure 9A and B; Supplementary movies 4 and 5). Dyn2-CBM-expressing cells showed an ~ 2.5 -fold increase in tubules $\geq 1.5 \mu\text{m}$ (Figure 9D) compared with wt-expressing cells. Consistent with our observations for the CIN85AC W→Y mutant, Dyn2-CBM induced long Rab7 tubules that contained RhEGF (Figure 9A, insets b and b'). In further agreement with these observations, siRNA-mediated knockdown of Dyn2 (Figure 9C) in rat fibroblasts (RF) or in HeLa cells resulted in the elongation of Rab7-positive tubules and a concomitant ≥ 2.5 -fold increase in the number of longer tubules

($\geq 3 \mu\text{m}$) compared with the mock-treated cells (Figure 9E; Supplementary movies 6 and 7). The Rab7 tubules caused by Dyn2 depletion were also positive for RhEGF (Figure 9C, insets c and c'), consistent with the results for the mutant over-expressing cells. Dyn2 knockdown was assessed through western blot analysis of transfected cells, which revealed 95 and 75% reductions in Dyn2 levels in fibroblasts and HeLa cells, respectively (data not shown). Taken together, these data suggest that the CIN85–Dyn2 complex is necessary for the formation of carriers emanating from the late endosome, and interference with this interaction results in a delay of EGFR degradation and sustained downstream signalling.

Figure 9 Knockdown of Dyn2 or over-expression of Dyn2-CBM, but not Dyn2wt increases GFP-Rab7 tubule length. (A–C) Movie stills of HeLa cells co-expressing either GFP-Rab7 and Dyn2wt (A, insets a–b') or Dyn2-CBM (B, insets a–b') and stimulated with RhEGF. Note that in the Dyn2wt-expressing cells, Rab7 localizes to spots and short tubules, whereas Dyn2-CBM-expressing cells show very long tubules (arrows) of this late endosome marker. RhEGF is retained in the GFP-Rab7 tubules in Dyn2-CBM-expressing cells, but appears punctate in the Dyn2wt-expressing cells, corresponding with the Rab7 pattern (compare insets b and b' in A, B). Bars, 5 μm . (C) Movie stills of GFP-Rab7-positive endosomes in rat fibroblasts, either mock treated (inset a) or depleted of Dyn2 (inset a') and stimulated with RhEGF. Mock-treated control cells display more Rab7-vesicles and short tubules, whereas the Dyn2-deficient cells have an increased number of long tubules (arrows). The elongated Rab7 tubules in the siDyn2-treated cells also contain RhEGF (arrows), but the mock-treated cells display clear puncta of the ligand, corresponding to the Rab7 pattern (compare insets b and b' with c and c'). Bars, 5 μm . (D) Quantitation of the number of GFP-Rab7-vesicles $> 1.5 \mu\text{m}$ in Dyn2wt- and Dyn2-CBM-expressing cells. GFP-Rab7 tubules were measured in 43 cells for the wt and 46 cells for the mutant. Data are represented as mean $\pm$ standard error and show a significant increase in the number of elongated tubules in mutant-expressing cells compared with wt ($P = 0.02$). (E) Quantitation of the number of GFP-Rab7-vesicles $\geq 3 \mu\text{m}$ in mock- and Dyn2-siRNA-treated rat fibroblasts (RF) and HeLa cells. GFP-Rab7 tubules were measured in 10 cells for each condition in HeLa cells, in 33 cells for mock-treated RFs, and in 21 cells for Dyn2-siRNA-treated RFs. Data are represented as mean $\pm$ standard error and show a significant increase in the number of long tubules in Dyn2-depleted cells compared with mock-treated cells ($P = 0.04$).

Discussion

In this study, we describe a novel EGF-induced interaction between Dyn2 and the adaptor protein CIN85. These data provide evidence that the interaction is direct and is mediated by the first and third SH3 domains of CIN85 and two CIN85-binding motifs in the PRD of Dyn2 (Figures 1 and 2). Previous studies from multiple groups have identified a variety of proteins that interact with CIN85 (Dikic, 2002). Our finding that an essential component of the endocytic machinery,

Dyn2, is also a CIN85-interacting partner emphasizes Dyn2's important function in the downregulation of growth factor receptor tyrosine kinases such as the EGFR. Although this study is the first to determine a functional interaction between CIN85 and Dyn2, a recent proteomic screen for proteins that interact with the SH3 domains of CIN85 (Havrylov *et al*, 2009) also identified this interaction.

Surprisingly, in contrast to the conventional function of Dyn2 and its endocytic partners in the severing of nascent vesicles from the plasma membrane, CIN85 and Dyn2 do not

appear to interact when receptor internalization is initiated. Instead, the CIN85–Dyn2 complex forms ~30–60 min after the addition of EGF, which suggests that the major site of action of this complex is at late endosomes. Indeed, interference with the CIN85–Dyn2 interaction resulted in delayed EGFR degradation, sustained ERK activation, and accumulation of RhEGF in an Rab7-positive late endosomal compartment (Figures 3–5; Supplementary Figures S1 and S4). Moreover, the CIN85–Dyn2 complex localized to late endosomes in a CIN85-dependent manner and could be co-immunoprecipitated with Rab7 (Figure 5). Thus, this study reveals not only a novel interaction between two components of the EGFR trafficking machinery, CIN85 and Dyn2, but also an unexpected function for this complex in the efficient transport of the EGFR from late endosomes.

CIN85–Dyn2 complex at the late endocytic pathway

The observed effects of CIN85 mutants on EGFR trafficking at the stage of late endosomes are supported by the fact that this adaptor is known to associate with the EGFR as it is transported along the degradative pathway (Haglund *et al*, 2002; Soubeyran *et al*, 2002). Moreover, the CIN85 homologue CD2AP/CMS was reported to be associated with Rab4-positive early endosomes and is thought to have a function in the morphology of early endosomes as well as in transport processes between early and late endosomes (Cormont *et al*, 2003). Dyn2 associates with and acts on endosomal structures to help mediate the recycling of endocytosed Tf receptors to the plasma membrane (van Dam and Stoorvogel, 2002), the recycling of the cation-independent mannose 6-phosphate receptor from late endosomes to the *trans*-Golgi network (TGN; Nicoziani *et al*, 2000), and efficient transport of Shiga toxin from late endosomes to the TGN (Lauvrak *et al*, 2004).

To our knowledge, Dyn2 or CIN85 has not been previously implicated in the transport of internalized receptors from a late endosomal to lysosomal compartment. Furthermore, our data show for the first time a function for Dyn2 in the EGFR sorting and trafficking processes by way of its association with CIN85. Indeed, inhibiting the CIN85–Dyn2 interaction prevented the exit of RhEGF out of late endosomes (Figure 5; Supplementary Figure S4), a phenotype similar to that of cells expressing an Rab7 dominant-negative mutant (Ceresa and Bahr, 2006) or Rab7 knockdown (Vanlandingham and Ceresa, 2009). Moreover, the specificity of this interaction at the late endosome is supported by the fact that disruption of the CIN85–cbl interaction resulted in retention of RhEGF in Rab5-positive early endosomes (Figure 6), in agreement with the suggested function for cbl at the early endosome (Ravid *et al*, 2004).

An effect of the CIN85AC W→Y mutant on EGFR degradation and signalling because of sequestration of cbl from its site of action is unlikely for several reasons. First, one would expect a retention in the early rather than in the late endosome, as seen in the CIN85B W→Y mutant. Second, the CIN85 knockdown would also lead to defects in exit from the early endosome. Third, the Dyn2-CBM would show a phenotype different from the CIN85AC W→Y mutant. Finally, an inappropriate sequestration of cbl would not alter endocytic vesiculation in the cell, whereas this phenomenon is typical for non-functional/missing dynamin (Kreitzer *et al*, 2000; Roux *et al*, 2006). As with any study testing the function of

protein–protein interactions, one cannot completely rule out the potential for inhibiting the binding of other effectors. For example, Dyn2 can interact with the CIN85 homologue Cd2AP, although the levels of this homologous adaptor in the cells used in this study are nearly undetectable (data not shown).

Consistent with our over-expression studies, knockdown of CIN85 or Dyn2 led to defective delivery of RhEGF out of late endosomes, along with delayed EGFR degradation (Figure 7; Supplementary Figures S5 and S6). In CIN85 KD cells, there is a modest, but not significant, reduction of internalization, but this cannot account for the substantial delay in EGF arrival to the late endosome. Further, in contrast to the reports of others (Soubeyran *et al*, 2002), these findings suggest that CIN85 is not essential for EGFR endocytosis, but rather for its proper trafficking (see also Havrylov *et al*, 2010). This other study used different cell types and dominant-negative CIN85 protein expression rather than siRNA knockdown. Taken together, our results support the idea that the CIN85–Dyn2 complex serves an essential function in EGFR sorting and trafficking. In addition, our data extend the cellular sites of Dyn2 function from the plasma membrane to another membranous compartment along the endocytic pathway, namely the late endosome.

As indicated by the co-immunoprecipitation and IF data shown in Figure 5, Dyn2 appears to be present on late endosomes in a complex with CIN85 and Rab7. In agreement with this finding, we observed a direct effect of the CIN85–Dyn2 complex on the length of GFP-Rab7 tubules and, therefore, on late endosomal budding (Figures 8 and 9). Disrupting the CIN85–Dyn2 complex significantly increased the number of long Rab7 tubules in the mutant-expressing cells by 2.5–4-fold over wt, and these longer tubules also contained RhEGF. Whereas the Rab7 tubules in the wt-expressing cells vesiculated within a very short period of time (within seconds), the long tubules in mutant-expressing cells rarely fragmented over many minutes. This was not observed in cells expressing the cbl-binding mutant, CIN85B W→Y, again showing the specificity of the CIN85–Dyn2 interaction (Figure 8, and data not shown). These observations provide mechanistic insight into how disrupting the CIN85–Dyn2 complex leads to retention of the EGFR in late endosomes and results in sustained downstream signalling. In addition, our data strongly suggest that CIN85 recruits Dyn2 to the late endosome (Figure 5), where Dyn2 mediates a post-endosomal budding process that helps downregulate EGFR.

Our findings are consistent with the premise of a novel, ligand-induced link between two important components of the growth factor receptor sorting and trafficking machinery, Dyn2 and CIN85. Moreover, instead of mediating the initial internalization event at the plasma membrane, the CIN85–Dyn2 complex appears to function predominantly at later stages of EGFR trafficking, namely in the transport of receptors from Rab7-positive late endosomes. Future studies will be needed to examine the exact function of the CIN85–Dyn2 complex in receptor degradation in general and in EGFR sorting/degradation in particular. In addition, the identification of putative additional components of the CIN85–Dyn2–Rab7 complex will be necessary to gain further insight into the requirements for efficient receptor sorting and degradation at the stage of late endosomes.

Materials and methods

Cell culture and transfection

HeLa and HuH7 (human hepatocellular carcinoma) cells were incubated in MEM. Clone 9 rat hepatocytes were incubated in F12K medium. All media were supplemented with 10% FBS, 1.5 g/l sodium bicarbonate, 50 U/mg penicillin + 50 µg/ml streptomycin, 1 × non-essential amino acids (MEM only), and 1 mM sodium pyrophosphate. Cells were transfected using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions.

Protein purification and GST pulldown

GST-fusion proteins were expressed in *Escherichia coli* BL21 cells and purified using glutathione-coated beads (Amersham-Pharmacia) according to the manufacturer's instructions. His-tagged Dyn2 was purified using Ni²⁺-coated beads (Roche) according to the manufacturer's instructions.

HeLa cells were lysed in lysis buffer (25 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM DTT, 0.5% NP-40, 2 mM Na₃VO₄, 15 mM NaF, 0.1 mM EDTA, and protease inhibitors), and 500 µg lysate was added to the purified GST-fusion protein for 2 h at 4°C. The beads were washed five times in wash buffer (lysis buffer containing 300 mM NaCl), and the bound protein complexes were eluted by applying 50 µl 1 × SDS sample buffer (2% SDS, 10% β-mercaptoethanol, 5% glycerol, 50 mM Tris pH 7, 10% bromophenol blue) for 2 min at 95°C. The eluted protein complexes were separated by SDS–PAGE and analysed by western blot. Transfection and purification of EE-tagged Dyn2wt and Dyn2-CBM mutants were performed as described in Gomez *et al* (2005).

Immunoprecipitation

HeLa or HuH7 cells were plated in 100-mm Petri dishes and grown to 70–90% confluence. The cells were serum starved for 16 h before adding EGF (50 ng/ml) for the indicated time points. Cell lysates were collected in lysis buffer (10 mM Hepes pH 7.5, 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM EDTA, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, protease inhibitors), sonicated, and centrifuged for 10 min, 14 000 r.p.m. at 4°C. A total of 500–800 µg lysate was added to 5 µg antibody and incubated for 2 h at 4°C. Control samples contained non-specific IgG or beads alone. Antibody-bound complexes were precipitated by adding Protein A- or G-coated beads (Sigma) for 1 h at 4°C, washed five times, and subjected to western blot analysis.

Surface biotinylation after EGF stimulation

HuH7 cells expressing the indicated plasmids were serum starved overnight (o/n) in 0.2% FBS, and endocytosis was initiated by addition of 50 ng/ml EGF for the indicated time points. Cells were transferred to 4°C, rinsed with chilled PBS, and incubated with 0.5 mg/ml biotin (EZ-link[®] Sulfo-NHS-LC Biotin, Thermo Scientific) for 30 min. Subsequently, biotin was quenched with 50 mM NH₄Cl. Cells were rinsed with PBS, lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8), sonicated, and centrifuged for 10 min, 14 000 r.p.m. at 4°C. Equal amounts of protein were added to 50 µl Streptavidin agarose beads (Thermo Scientific), incubated o/n, washed three times in RIPA buffer, and subjected to western blot analysis together with ~10% of the input to determine the total amount of receptor in each sample.

IF and image acquisition and manipulation

IF staining was performed as described previously (Henley *et al*, 1998). Fluorescence micrographs were acquired using a Zeiss Axiovert 35 epifluorescence microscope (Carl Zeiss) equipped with a Hamamatsu Orca II camera (Hamamatsu Photonics, Hamamatsu City, Japan), and images were processed using Adobe Photoshop (Adobe). Time-lapse movies were acquired using the same instrumentation as described above, and Rab7 tubule length was measured using IPLab software (Scanalytics). Images were taken every 5 s over a total time of up to 5 min for each movie. The time-lapse images were converted to movies using IPLab software.

Rhodamine-EGF uptake, quantification, and statistical analysis

HuH7 or HeLa cells expressing the indicated plasmids or treated with CIN85 siRNA were serum starved for 16 h, pre-treated with

cycloheximide (CHX, 50 µg/ml) at 37°C for 60 min, and then cooled to 4°C. Subsequently, 100 ng/ml RhEGF was applied at 4°C in the presence of CHX to allow ligand binding to the receptor. Afterwards, cells were pulsed for 15 min at 37°C, washed, and chased in low-serum medium plus CHX for the indicated time points. To ensure visualization of internalized ligand only, coverslips were acid stripped (2 × 2 min, 1 × 1 min) and then subjected to IF processing using 0.0001% digitonin for permeabilization. For IF-based quantifications, all images were taken at the same exposure time and analysed using IPLab software. To measure RhEGF in a single cell, cells were circled and the mean IF intensity per circled area was measured. Background fluorescence was acquired in the same way and subtracted from the values obtained for cell measurements. For each condition, ≥40 cells were analysed, and data were represented as mean ± standard error. ImageJ was used for the quantification of the amount of RhEGF in the Rab5- or Rab7-positive compartment. Briefly, all RhEGF spots were counted in the red channel, the counting mask was transferred to the green channel (GFP-Rab5 or GFP-Rab7), and all green spots or circles having a marker from the RhEGF mask were counted positive for colocalization. The amount of colocalization was calculated as the ratio of the RhEGF spots in the respective compartment to the total number of RhEGF spots, and data were represented as mean ± standard error. For each condition, ≥30 cells were counted.

Statistical analysis was performed using a two-tailed, paired Student's *t*-test for each sample group. *P*-values ≤0.05 were considered statistically significant and are indicated in each figure.

EGFR degradation assay

HuH7 cells were transfected with the indicated plasmids as described above, serum starved for 4 h in the presence of 50 µg/ml CHX, and stimulated with EGF (50 ng/ml) in the presence of CHX for the indicated time points. Total cell lysates were collected in RIPA buffer (see above), and equal protein amounts of each sample were subjected to western blot analysis. Densitometry was performed using Bio-Rad Molecular Analyst software (Bio-Rad). HeLa cells were treated as described above but serum starved o/n in growth medium with 0.2% FBS.

Antibodies and reagents

The antibodies against Dyn2 and MC63 were described previously (Henley and McNiven, 1996). The CIN85 antibody used for immunoprecipitation was generated against aa 291–400 of CIN85 and kindly provided by Dr Daniel Billadeau (Mayo Clinic, Rochester). All other antibodies were purchased from the following companies: anti-CIN85 from Upstate; anti-GFP from Roche; anti-CIN85, anti-actin, and anti-GST from Sigma; EGFR, anti-FLAG, anti-Cd2AP, anti-pERK, and anti-ERK from Cell Signaling; and anti-lamp1 from Santa Cruz. GFP-Rab7 was kindly provided by Dr Richard Pagano (Mayo Clinic); GFP-Rab5 was provided by Dr Bruce Horzodovsky (Mayo Clinic). Rhodamine-EGF was purchased from Invitrogen, and human EGF and CHX were purchased from Sigma.

Plasmids and siRNA

The following constructs were kindly provided by Dr Daniel Billadeau: pCMS4-H1p-EGFP (referred to as NC herein; Nolz *et al*, 2007); pCMS4-H1p(shDyn2c)-EGFP (referred to as sh/-); pCMS4-H1p(shDyn2c)-Dyn2wt-EGFP (referred to as sh/wt); pCMS4-H1p(shDyn2c)-CBM-EGFP (referred to as sh/CBM); FLAG-YFP-CIN85ABC W→Y; FLAG-CIN85AC W→Y; FLAG-CIN85wt; FLAG-CIN85SH3ABC W→Y; FLAG-CIN85SH3A, B, and C; and GST-fusion constructs for all three isolated SH3 domains and the dominant-negative SH3ABC fragment. On the basis of these templates, we generated GST fusions of CIN85SH3A W→Y and CIN85 SH3ABC W→Y by cloning them into the *Bam*HI/*Eco*RI sites of pGEX-3X (Amersham-Pharmacia). GST-CIN85 wt was cloned into the *Bam*HI/*Eco*RI sites of pGEX-3X. GST-A(B)C W→Y was obtained by reverting the W→Y mutation in the SH3B domain in the GST-CIN85ABC W→Y construct. DsRed versions of CIN85wt, -AC W→Y, and -B W→Y were subcloned into the *Xho*I/*Hind*III sites of pDsRed-Monomer-N1 (Clontech). The dsRed versions of Dyn2wt and Dyn2-CBM were cloned into the *Xho*I/*Eco*RI sites, CIN85 siRNA was purchased from Santa Cruz, and non-targeting siRNA and siRNA against rat Dyn2 were purchased from Dharmacon Inc. Cells were transfected using RNAiMAX (Invitrogen) according to the manufacturer's instructions. Knockdown of CIN85 and Dyn2 was observed 72 h post-treatment.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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