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ABSTRACT **Ubiquitination of the epidermal growth factor receptor (EGFR) by cbl and its cognate adaptor cbl-interacting protein of 85 kDa (CIN85) is known to play an essential role in directing this receptor to the lysosome for degradation. The mechanisms by which this ubiquitin modification is regulated are not fully defined, nor is it clear where this process occurs. In this study we show that EGFR activation leads to a pronounced src-mediated tyrosine phosphorylation of CIN85 that subsequently influences EGFR ubiquitination. Of importance, phospho-CIN85 interacts with the Rab5-positive endosome, where it mediates the sequestration of the ubiquitinated receptor into multivesicular bodies (MVBs) for subsequent degradation. These findings provide novel insights into how src- kinase–based regulation of a cbl adaptor regulates the fate of the EGFR.**

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INTRODUCTION

The epidermal growth factor (EGF) receptor (EGFR) controls important cellular processes such as cellular adhesion, migration, and proliferation. Accordingly, overexpression of this receptor tyrosine kinase (RTK) is associated with the development of a variety of human cancers (Roskoski, 2004; Sebastian *et al.*, 2006). Therefore tight regulation of EGFR levels, activation, and downstream signaling cascades is essential. A mechanism used by cells to maintain normal receptor levels is deliberate control of endocytic internalization, recycling, and degradation of the receptor both spatially and temporally (Zwang and Yarden, 2009).

It has been demonstrated that the cbl-mediated ubiquitination of the EGFR is essential and sufficient for proper sorting and targeting to the lysosome for degradation (Ettenberg *et al.*, 2001; Huang *et al.*, 2006) but is dispensable for EGFR internalization (Huang *et al.*, 2006; Pennock and Wang, 2008). Although partial ubiquitination of the receptor is believed to occur at the plasma membrane (Stang *et al.*, 2000; de Melker *et al.*, 2001), this posttranslational modification is not required for internalization (Huang *et al.*, 2007) nor does it appear to require internalization (de Melker *et al.*, 2001). Thus ubiquitination may actually perform its receptor-targeting function downstream of the initial endocytic event during progression from early endosomes to late endosomes/multivesicular bodies (MVBs; Ravid *et al.*, 2004). This cbl-dependent process is augmented by the action of a structural adaptor protein, cbl-interacting protein of 85 kDa (CIN85), which tethers cbl to the endocytic machinery in an EGF-dependent manner (Dikic, 2002). A number of studies have suggested that CIN85 participates in both the initial steps of EGFR internalization (Soubeyran *et al.*, 2002; Schmidt *et al.*, 2004) and also in receptor trafficking and degradation (de Melker *et al.*, 2001; Kowanetz *et al.*, 2004; Schroeder *et al.*, 2010). Of interest, it has recently been shown that knockdown of CIN85 results in a decrease in EGFR ubiquitination (Ronning *et al.*, 2011), although the underlying mechanism has not been investigated. Of greater importance, it also remains unclear whether the CIN85–cbl interaction is essential for receptor ubiquitination and appropriate downstream trafficking. Indeed, several key steps in this important

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Abbreviations used: CIN85, cbl-interacting protein of 85 kDa; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ILV, intraluminal vesicle; pTyr, phospho-Tyr; RhEGF, rhodamine-conjugated EGF; SA, streptavidin.

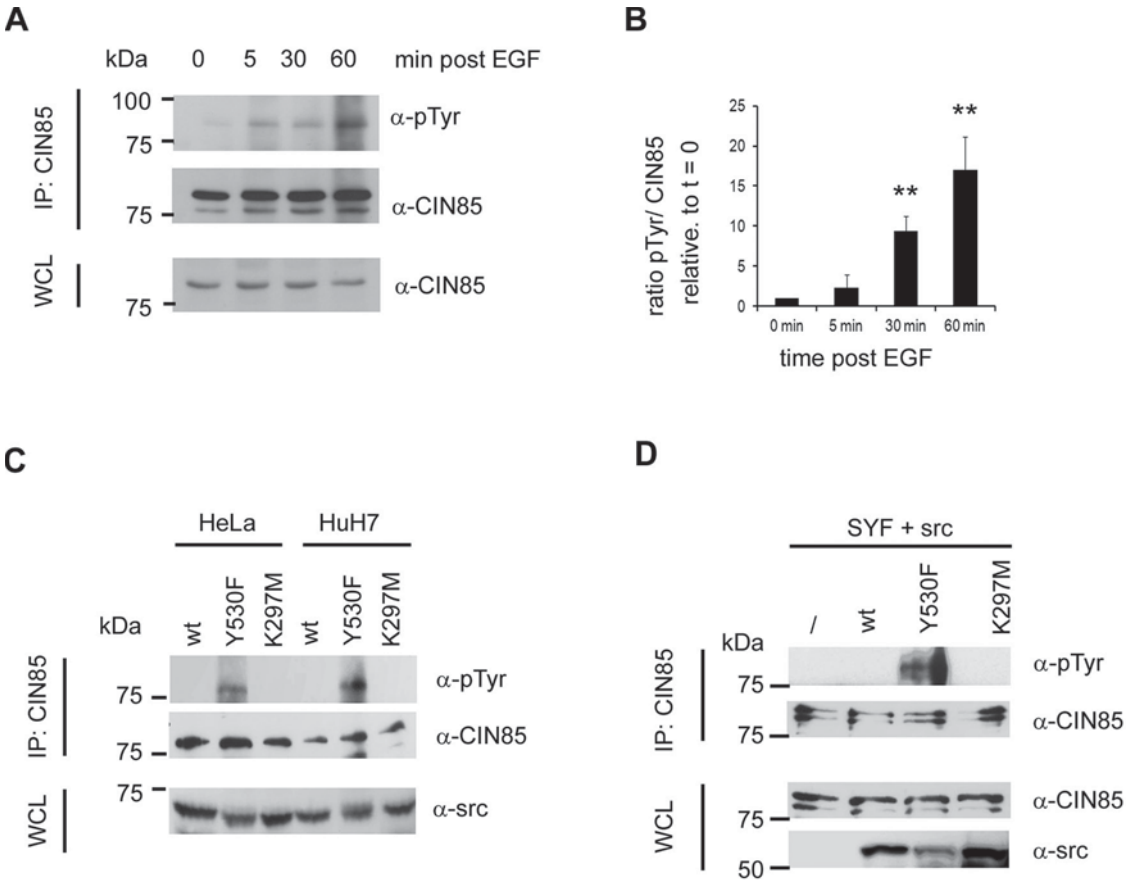

FIGURE 1: CIN85 is phosphorylated upon EGF stimulation in a src-dependent manner. (A) Representative blot showing EGF-induced CIN85 phosphorylation. CIN85 was immunoprecipitated from HuH7 cells stimulated with EGF for the indicated time points before Western blot analysis with an anti-phosphotyrosine antibody. (B) A substantial increase in tyrosine phosphorylation was observed by 30–60 min poststimulation and quantitated from three independent experiments as described in A. The pTyr signal was normalized to the amount of total precipitated CIN85 in each lane, and the data are represented as mean ± SE. (C) Representative blot showing induction of CIN85 phosphorylation by overexpression of active src (srcY530F) but not kinase-dead src (srcK297M) in HeLa and HuH7 cells under full serum conditions. (D) Representative blot showing rescue of CIN85 phosphorylation in SYF cells by reexpression of active src (srcY530F) but not kinase-dead src (srcK297M).

process remain nebulous and need to be defined further. For example, how does activation of the EGFR induce formation of the CIN85–cbl complex, what downstream signaling events stimulate this interaction, and where in the endocytic pathway does this occur? Finally, what are the consequences of preventing this stimulated interaction on EGFR traffic to the lysosome?

In this study, we report a novel EGF-induced, Tyr-based phosphorylation of CIN85 that is mediated by src kinase 30 min poststimulation, suggesting that this event occurs at a later endocytic compartment. Interference with the src-regulated phosphorylation of CIN85 results in decreased association of this adaptor with both Rab5 at the early endosomes and the cbl ligase. Most important, preventing CIN85 phosphorylation significantly reduces ubiquitination of the EGFR and leads to its accumulation in early endosomes. These findings reveal a novel src–CIN85-centric signaling pathway that regulates EGFR trafficking and subsequently its degradation.

RESULTS

CIN85 is a src substrate that is phosphorylated upon EGF stimulation

CIN85 plays an important role in EGFR down-regulation (Haglund *et al.*, 2002; Soubeyran *et al.*, 2002; Kowanetz *et al.*, 2003, 2004), although it is unclear whether this is a regulated process. Given that CIN85 contains four potential Tyr phosphorylation sites, we tested whether CIN85 might be Tyr phosphorylated upon EGF stimulation. To this end, we stimulated HuH7 cells with 50 ng/ml EGF, and we immunoprecipitated endogenous CIN85 at different time points and immunoblotted for phospho-Tyr. As shown in Figure 1A, CIN85 is indeed Tyr phosphorylated in a stepwise manner after EGF stimulation, not immediately, but at later time points (peak around 30–60 min post EGF; Figure 1, A and B; $^{**}p < 0.05$).

Because src is activated upon EGF stimulation (Oude Weernink *et al.*, 1994) and is a known binding partner of the CIN85 homologue CD2AP (Kirsch *et al.*, 1999), we tested whether this kinase could mediate CIN85 phosphorylation. We pursued this by expressing either constitutively active src (srcY530F) or kinase-dead src (src K297M) in HeLa and HuH7 cells and then monitoring CIN85 phosphorylation independent of EGF stimulation. Although wt src did not result in detectable CIN85 phosphorylation, overexpression of constitutively active src induced strong CIN85 phosphorylation, whereas the kinase-dead form was unable to do so (Figure 1C). This suggests that active src alone is sufficient to promote CIN85 phosphorylation and can mimic EGF-induced CIN85 phosphorylation. To confirm these results, we used SYF cells (src/yes/fyn triple-knockout

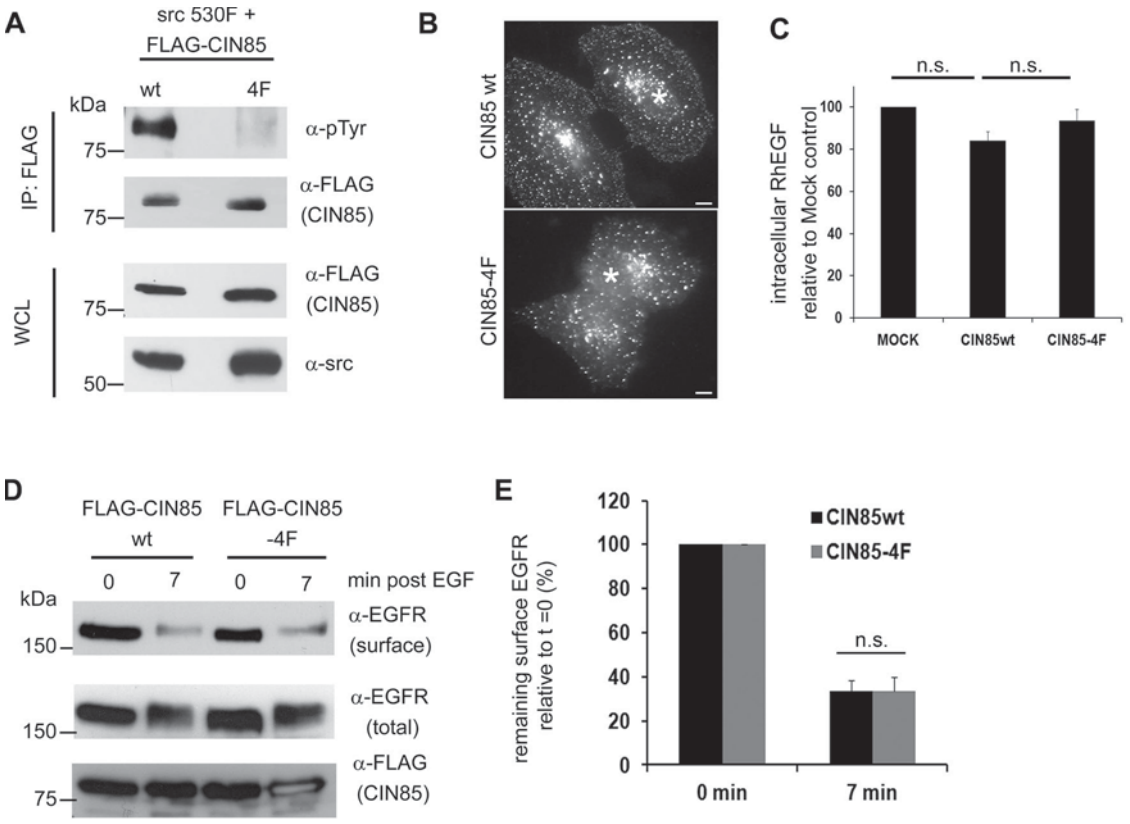

FIGURE 2: A phospho-defective CIN85 mutant, CIN85-4F, does not interfere with EGFR endocytosis. (A) Mutation of four key tyrosines in CIN85 (CIN85-4F) prevents phosphorylation of CIN85. FLAG-tagged CIN85wt or CIN85-4F was precipitated from HeLa cells coexpressing active src (srcY530F), and the CIN85 phosphorylation status was assessed by Western blot analysis. (B) Overexpression of CIN85-4F does not affect EGFR endocytosis. Representative images of RhEGF internalization in HuH7 cells expressing either CIN85wt (asterisk, top) or -4F (asterisk, bottom). Internalization was allowed for 15 min, and cells were acid stripped to visualize only internalized ligand. (C) Quantitation of three independent experiments as described in B. The data are represented as mean ± SE. (D) Representative blot of a surface biotinylation assay used to examine the effects of CIN85wt and -4F on EGFR endocytosis. HuH7 cells expressing FLAG-CIN85wt or -4F were stimulated for the indicated time points; surface proteins were biotinylated and precipitated using streptavidin (SA)-coated beads. The amount of total and surface EGFR was assessed by Western blot analysis. (E) Quantitation of four independent experiments as described in D. The data are represented as mean ± SE.

mouse embryonic fibroblasts) for rescue experiments, predicting that without src there would be no CIN85 phosphorylation and that with reexpression of active src CIN85 phosphorylation should be rescued. Indeed, CIN85 was not phosphorylated in control SYF cells, whereas its phosphorylation was restored by reexpressing active src but not kinase-dead src (Figure 1D). Of interest, reexpression of wt src alone in these cells did not result in detectable CIN85 phosphorylation, suggesting that activation of this kinase is needed. To test whether EGF-induced src activation might induce CIN85 phosphorylation in SYF cells, we expressed wt src kinase in these cells and assessed CIN85 phosphorylation with or without EGF addition. As shown in Supplemental Figure S1A, CIN85 was phosphorylated in a src- and EGF-dependent manner, revealing an EGFR–src cascade that leads to CIN85 phosphorylation. We further confirmed the src dependence of CIN85 phosphorylation by a pharmacological approach using the src inhibitor SU6656. Compared to control cells treated with dimethyl sulfoxide (DMSO), the SU6656 cells exhibited significantly reduced CIN85 phosphorylation (Supplemental Figure S1B).

Taken together, those data suggest that CIN85 is Tyr phosphorylated upon EGF stimulation in a src-dependent manner.

Inhibition of CIN85 phosphorylation does not affect EGFR endocytosis

To determine the functional relevance of CIN85 phosphorylation, we generated a phospho-defective CIN85 mutant and then examined its effects on EGFR endocytosis and trafficking. CIN85 has four potential Tyr phosphorylation sites—Y10, Y109, Y271, and Y278—all of which were converted to phenylalanine by site-directed mutagenesis to produce a mutant we termed CIN85-4F (CIN85-Y10/109/271/278F). To ensure that this CIN85 mutant is not Tyr phosphorylated, we coexpressed active src with either FLAG-CIN85wt or -4F and assessed the Tyr-phosphorylation status of the CIN85wt and mutant. As shown in Figure 2A, CIN85wt is Tyr phosphorylated in the presence of active src, whereas the CIN85-4F mutant is not. It is important to note that the defective phosphorylation of the CIN85-4F mutant is not due to impaired src binding, since the mutant bound equally as well to src as the wt protein (Supplemental Figure S1C).

To determine the functional consequences of the CIN85-4F phospho-mutant, we first examined the effects of CIN85wt and -4F on EGFR endocytosis both by immunofluorescence staining (IF) and biochemically. For the IF analysis, HuH7 cells expressing either

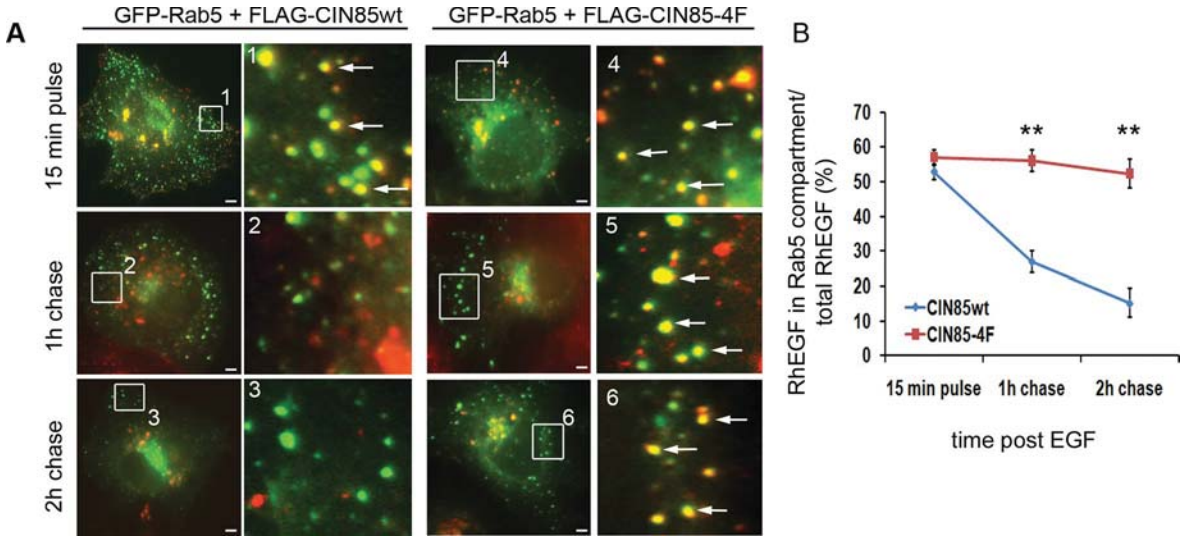

FIGURE 3: Inhibition of CIN85 phosphorylation causes a marked retention of EGFR at the early endosome. (A) RhEGF trafficking assay in HuH7 expressing GFP-Rab5 (green) together with either FLAG-CIN8wt or FLAG-CIN85-4F. CIN85wt and mutant proteins were expressed at comparable levels. Arrowheads point to the Rab5 endosomes positive for RhEGF, which are decreased substantially in wt-expressing cells as the ligand traffics through the endocytic pathway. In contrast, cells expressing the CIN85-4F mutant exhibit a marked retention of RhEGF in the Rab5 endosome. Insets 1–6 show magnifications of the boxed areas in the overview images. Bars, 10 µm. (B) Quantitation of the amount of RhEGF colocalizing with GFP-Rab5–positive endosomes at various time points in HuH7 cells expressing GFP-Rab5 together with either CIN85wt (blue line) or CIN85-4F (red line). Almost no transport of ligand from the Rab5 endosome is observed even after a 2-h chase. For each condition ≥30 cells were counted, and the data are represented as mean ± SE.

FLAG-CIN85wt or the -4F mutant were incubated with rhodamine–EGF (RhEGF) for 20 min at 37°C, and the amount of internalized RhEGF was quantitated. As shown in Figure 2, B and C, expression of either CIN85wt or CIN85-4F did not result in any significant inhibition of EGFR endocytosis. These data were confirmed by surface biotinylation assays showing that EGFR was internalized at similar levels in both CIN85wt- and mutant-expressing cells after short pulses of EGF (Figure 2, D and E). Taken together, these data show that preventing CIN85 phosphorylation does not affect EGFR endocytosis, and they are consistent with our previous observations that this adaptor participates in the later steps of endocytic trafficking.

Inhibition of CIN85 phosphorylation impairs EGFR exit from the early endosome and delays EGFR degradation

Given that EGFR internalization is not influenced by overexpression of the phospho-defective CIN85-4F mutant, we examined whether CIN85 phosphorylation might regulate EGFR endocytic trafficking. To this end, we monitored EGFR transport by assessing the colocalization of RhEGF with early and late endosomal markers in CIN85wt- and mutant-expressing HuH7 cells. Early versus late endosomes were visualized using green fluorescent protein (GFP)–Rab5 or GFP-Rab7, respectively. As shown in Figure 3, A and B, in cells expressing CIN85wt, RhEGF accumulates in early endosomes within 15 min (Figure 3A, inset 1), exits after 45–60 min (Figure 3A, insets 2 and 3), and is degraded by 2 h. In contrast, CIN85-4F–expressing cells do not exhibit alterations in the transit of the EGFR to the early endosome (Figure 3A, inset 4), but instead show a significant delay in EGFR trafficking out of the early endosome. This leads to retention of RhEGF in the early endosomal compartment even after the 2-h chase period (Figure 3A, insets 5 and 6, B; $^{**}p < 0.0005$). Consistent with these data, we observed a significant delay in EGFR transit to late endosomes when CIN85 Tyr phosphorylation was disrupted. In CIN85wt-expressing cells, RhEGF localizes to late endosomes after a 1-h chase (Figure 4A, inset 2) and has trafficked through this endocytic step to a degradative compartment by 2 h (Figure 4A, inset 3). As CIN85-4F–expressing cells accumulate RhEGF in the early endosomes, this leads to a significant reduction in the transport of the labeled ligand to the Rab7-positive late endosomal compartment (Figure 4, A, insets 5 and 6, and B; $^{**}p < 0.0005$). In addition, accumulation causes a delay in RhEGF degradation visualized by retention of the RhEGF signal even after a 2-h chase (Figure 4A, compare insets 3 and 6).

Because our data suggest that the CIN85–cbl complex mainly acts at the early endosomes and not necessarily at the initial endocytic pit, we visualized the components of this complex at the place of action. It was previously shown that cbl indeed localizes to early endocytic, EGFR-positive structures and remains associated with the receptor throughout the endocytic route (Levkowitz *et al.*, 1998; de Melker *et al.*, 2001; Visser Smit *et al.*, 2009). It was also reported that cbl preferentially localizes to the Hrs-positive subdomains of early endosomes (Umebayashi *et al.*, 2008). Therefore we chose Hrs as an early endosomal marker for our cbl colocalization studies. As shown in Supplemental Figure S2a, cbl localized to Hrs-positive endosomes 15–30 min after EGF.

Because it is difficult to localize CIN85 to endosomal structures in fixed cells, most likely due to the transient nature of this association, live-cell imaging was used to assess the localization of CIN85 on early endosomes. Subsequently, HeLa cells were cotransfected with mCherry-CIN85 and active, GFP-tagged Rab5 (GFP-Rab5Q79L) as performed previously (Schroeder *et al.*, 2010). Before imaging, cells were stimulated with 50 ng/ml EGF for 20 min, and the images were acquired 25–30 min after EGF addition. As shown in Supplemental Figure S2B, CIN85 localized to Rab5-positive early endosomes under these experimental conditions. To support these morphological studies, we performed coimmunoprecipitation assays. To

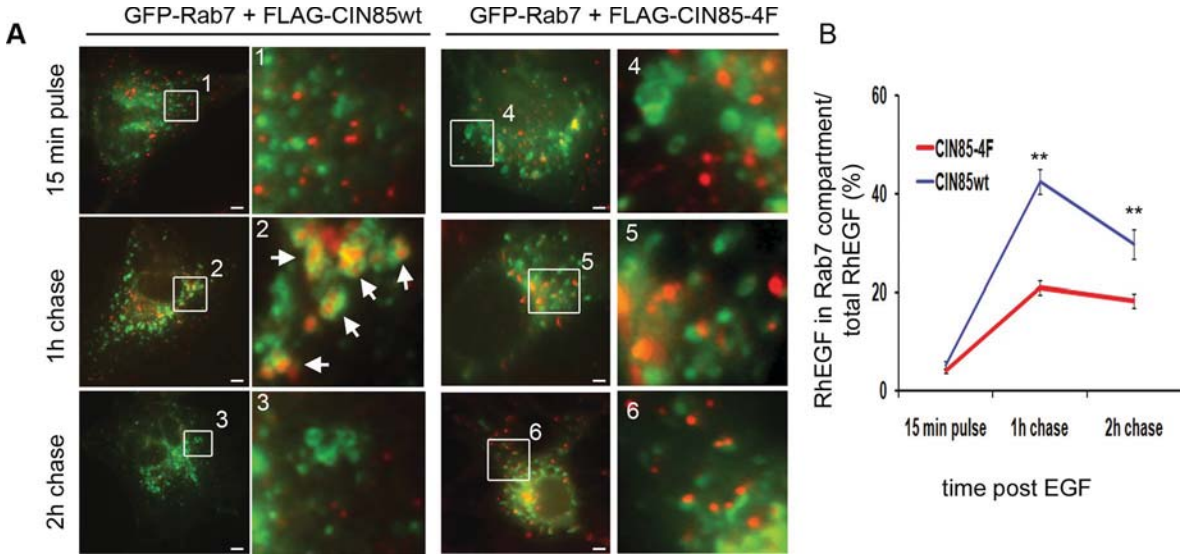

FIGURE 4: Inhibition of CIN85 phosphorylation delays delivery of EGFR to late endosomes. (A) RhEGF trafficking assay in HuH7 expressing GFP-Rab7 (green) as a marker for the late endosome together with either FLAG-CIN85wt or FLAG-CIN85-4F. CIN85wt and mutant proteins were expressed at comparable levels. Arrowheads point to the Rab7 endosomes positive for RhEGF, which are markedly increased by 60 min after chase in wt-expressing cells but minimized in the CIN85 mutant–expressing cells. Insets 1–6 show magnifications of the boxed areas in the overview images. Bars, 10 μm. (B) Quantitation of the amount of RhEGF colocalizing with GFP-Rab7–positive endosomes at various time points in HuH7 cells expressing GFP-Rab7 together with either CIN85wt (blue line) or CIN85-4F (red line). Disruption of the CIN85–cbl complex caused a significant delay in EGFR trafficking to the late endosomes. For each condition ≥30 cells were counted, and the data are represented as mean ± SE.

this end, we transfected HeLa cells with GFP-Rab5 and examined the interaction with endogenous CIN85 after EGF stimulation. Of interest, CIN85 interacts with Rab5 in an EGF-dependent manner (Supplemental Figure S2C; $p < 0.05$). Furthermore, we find that this interaction is dependent on the phosphorylation status of CIN85, as GFP-Rab5 and FLAG-CIN85wt, but not the CIN85-4F mutant protein, are seen to associate in response to EGF stimulation (Supplemental Figure S2D; $p < 0.0005$). All of these findings are consistent with the concept of a functional src-activated CIN85–cbl complex that regulates the traffic of EGFR at the Rab5 endosome.

To confirm the IF-based results, we performed EGFR degradation assays in HuH7 cells expressing either CIN85wt or the phospho-defective CIN85-4F mutant. Again, we observed a clear delay of EGFR degradation in the mutant compared with the wt-expressing cells (Figure 5, A and B; $p < 0.05$). EGFR protein levels were decreased by ~90% within 90 min in CIN85wt-expressing cells, whereas cells expressing the CIN85-4F mutant retained nearly 50% of their EGFR at the same time point. Because inhibition of the src-dependent phosphorylation of CIN85 delayed EGFR degradation, we also determined the involvement of src in that process. To this end, we performed biochemical EGFR degradation assays in SYF cells with or without reexpression of wt src, expecting that reintroducing src would somehow restore normal rates of receptor degradation in these cells. Indeed, reexpression of src wt into SYF cells accelerated EGFR degradation threefold to fourfold compared with control cells (Figure 5, C and D; $p < 0.05$). Taken together, these findings suggest that the phosphorylation of CIN85 is necessary for proper postendocytic EGFR trafficking, and impairment of CIN85 Tyr phosphorylation causes a delay in EGFR degradation due to retention of the receptor in early endosomes.

CIN85 phosphorylation is essential for EGFR ubiquitination and appropriate receptor sorting into MVBs

CIN85 was originally identified as a cbl-binding protein by Take *et al*. (2000), and a recent study demonstrated that a disruption of this interaction results in an impairment of EGFR trafficking from the early endosome (Schroeder *et al*., 2010). Because expression of the phospho-defective CIN85-4F used in this study induces the same block in EGFR traffic (Figure 3), we postulated that src-dependent phosphorylation of CIN85 might regulate the cbl interaction. To this end, we coexpressed hemagglutinin (HA)-cbl with either FLAG-CIN85wt or -4F and stimulated it with 50 ng/ml EGF for 30 min, followed by a cbl immunoprecipitation and quantitation of associated CIN85 by Western blot. Of importance, the CIN85-4F mutant showed substantially less binding (~80% reduction; $p < 0.0005$) to cbl than did the wt protein (Figure 6A). We observed a similar reduction in binding by assessing the interaction of endogenous cbl with CIN85wt or -4F (Supplemental Figure S1D; $p < 0.0005$). Given that cbl is the major E3 ligase responsible for ubiquitination of the EGFR, we next tested whether impairment of CIN85 phosphorylation might affect EGFR ubiquitination. Subsequently, HA-ubiquitin (HA-Ub) was coexpressed with either CIN85wt or the -4F mutant, and the ubiquitination status of EGFR was assessed after stimulation with 50 ng/ml EGF for 30 min. Of interest, expression of the CIN85-4F mutant caused a significant (~70%; $p < 0.05$) reduction in EGFR ubiquitination compared with wt-expressing cells (Figure 6B), suggesting that a phospho-dependent interaction between CIN85 and cbl is essential for appropriate ubiquitination of the receptor.

Appropriate ubiquitination and trafficking of the EGFR regulate sorting of the receptor into intraluminal vesicles (ILVs) of MVBs, an important step in EGFR degradation and down-regulation. Because both of these processes are significantly impaired in cells expressing the CIN85-4F phosphorylation-defective mutant, it seemed likely that

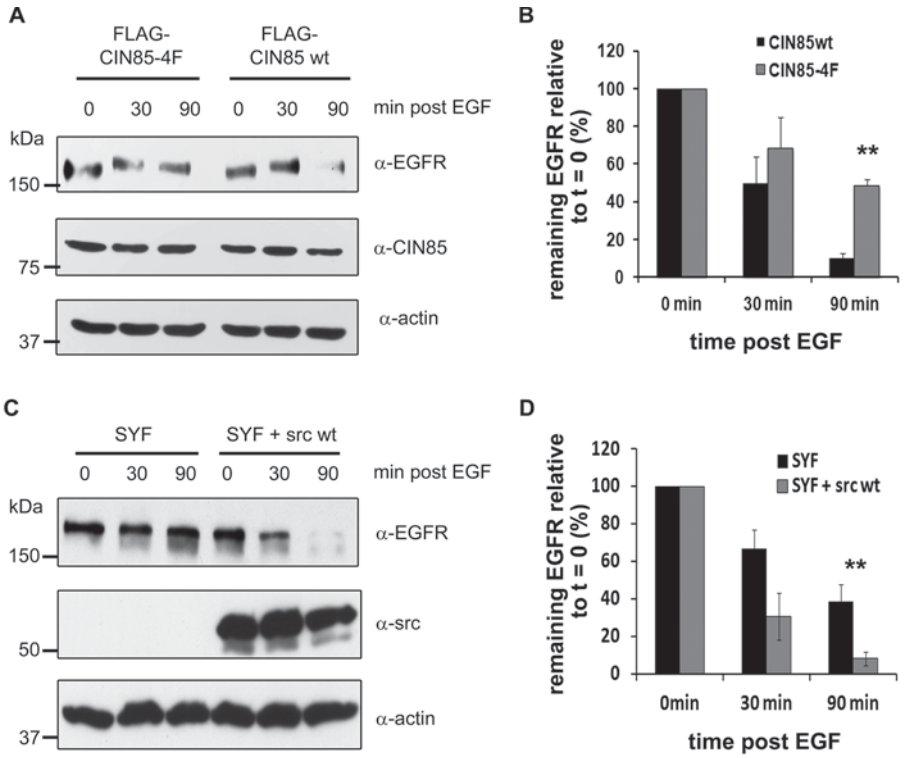

FIGURE 5: Phosphorylation of CIN85 is required for efficient degradation of EGFR. HuH7 cells expressing FLAG-CIN85wt or FLAG-CIN85-4F (A, B) or SYF cells with or without reexpression of src wt (C, D) were serum starved for 4 h in the presence of 50 µg/ml CHX and treated with 50 ng/ml EGF plus CHX for the indicated time points. Equal amounts of each sample were analyzed by Western blot (A and C) and show a strong persistence of EGFR levels in cells expressing the mutant CIN85 (A) and an acceleration of EGFR degradation in SYF cells reexpressing src wt (C). (B, D) Quantitation of ≥4 independent EGFR degradation assays comparing CIN85wt- and CIN85-4F–expressing cells demonstrating an 8- to 10-fold increase in the retention of intact EGFR in the mutant-expressing cells. SYF cells with src wt show three- to fourfold faster receptor degradation compared with control cells as assessed by quantitation of three independent experiments. The amount of EGFR in each sample was normalized to actin, and the data are represented as mean ± SE.

CIN85 phosphorylation could play a role in sorting of the receptor into ILVs of MVBs. To test the efficacy of MVB sorting in the CIN85 mutant–expressing cells, we used an approach described by Lobert *et al.* (2010) and compared EGFR sorting into MVBs in cells coexpressing GFP-tagged Rab5-Q79L with either CIN85wt or -4F mutant or control cells expressing GFP-Rab5-Q79L alone. Overexpression of active Rab5 is well known to induce enlargement of early endosomes (Stenmark *et al.*, 1994), which facilitates resolution of the MVB lumen from the vesicle limiting membrane. Graphically, inhibition of CIN85 phosphorylation greatly affected MVB sorting of the EGFR (Figure 6, C and D). In control (Figure 6C, inset 1) and CIN85wt-expressing cells (Figure 6C, inset 2), the majority of EGFR was sorted into the lumen of the early endosomes, whereas in CIN85-4F–expressing cells EGFR was missorted to the limiting membrane (Figure 6C, inset 3). Quantification revealed that MVB sorting of EGFR in cells expressing the phospho-defective CIN85 mutant was reduced by >50% compared with control cells (Figure 6D; $^{**}p < 0.0001$). These data indicate that CIN85 phosphorylation is necessary for proper sorting of EGFR into MVBs for subsequent trafficking to late endosomes and lysosomes for degradation.

The role of CIN85 in early endosomal sorting and trafficking of the EGFR was further supported by experiments using 3T9 cells depleted of endogenous CIN85. These cells were transfected with a CIN85-short hairpin RNA (shRNA)–encoding lentivirus to ensure a stable and nearly complete knockdown of CIN85 (Supplemental Figure S3A), allowing us to test EGFR endocytosis and trafficking in the context of CIN85 deficiency. As reported previously (Schroeder *et al.*, 2010), EGFR endocytosis was only mildly affected in these cells compared with cells transfected with a nontargeting control shRNA (Supplemental Figure S3, B and C). Furthermore, we observed a missorting of the receptor to the limiting membrane of enlarged endosomes (Supplemental Figure S3, D and E), as well as a delay in transport of the receptor to late endosomes (Supplemental Figure S3, F and G) and a concomitant reduction in EGFR degradation (Supplemental Figure S3, H and I). Taken together, the knockdown data confirm that CIN85 is important in conducting early to late endosomal EGFR trafficking.

DISCUSSION

In this study, we provide novel insights into the molecular mechanisms by which the endocytosed EGF receptor is directed toward the degradative pathway upon ligand stimulation. Specifically, we identify a novel EGF-induced, src-mediated tyrosine phosphorylation of the endocytic adaptor protein CIN85 (Figures 1 and 2 and Supplemental Figure S1, A–C). This phosphorylation event mediates the interaction of CIN85 with the E3 ubiquitin ligase, cbl, and is required for appropriate EGFR ubiquitination, sorting, and degradation (Figures 3–6 and Supplemental Figure S1D).

CIN85 function: from receptor internalization to degradation

Previously it was shown that the activated EGFR binds to cbl, which, in turn, recruits CIN85 to the endocytic pit. This trimeric EGFR–cbl–CIN85 complex was assumed to traffic through the endocytic pathway for subsequent degradation. Thus CIN85 action has been implicated mainly in early stages of endocytic internalization (Haglund *et al.*, 2002; Soubeyran *et al.*, 2002).

In contrast, we observed that CIN85 appears to act largely in postendocytic sorting and that the src-mediated phosphorylation of this adaptor, which peaks at ~30–60 min after EGF stimulation, is particularly important for later steps of endocytic receptor traffic. Indeed, blocking CIN85 Tyr phosphorylation has no obvious effect on EGFR endocytosis but instead affects the efficient sorting and degradation of the EGFR (Figures 2–6). These observations are in agreement with a previous study reporting the participation of CIN85 in late endosomal trafficking via an interaction with dynamin 2 and Rab7 (Schroeder *et al.*, 2010). It also confirms recent observations from our lab and by others showing that RNA interference–mediated knockdown of CIN85 does not affect EGFR endocytosis (Supplemental Figure S3A; Schroeder *et al.*, 2010; Ronning *et al.*, 2011). Consistent with this premise, expression of a phosphorylation-deficient mutant of CIN85 (CIN85-4F) resulted in retention of

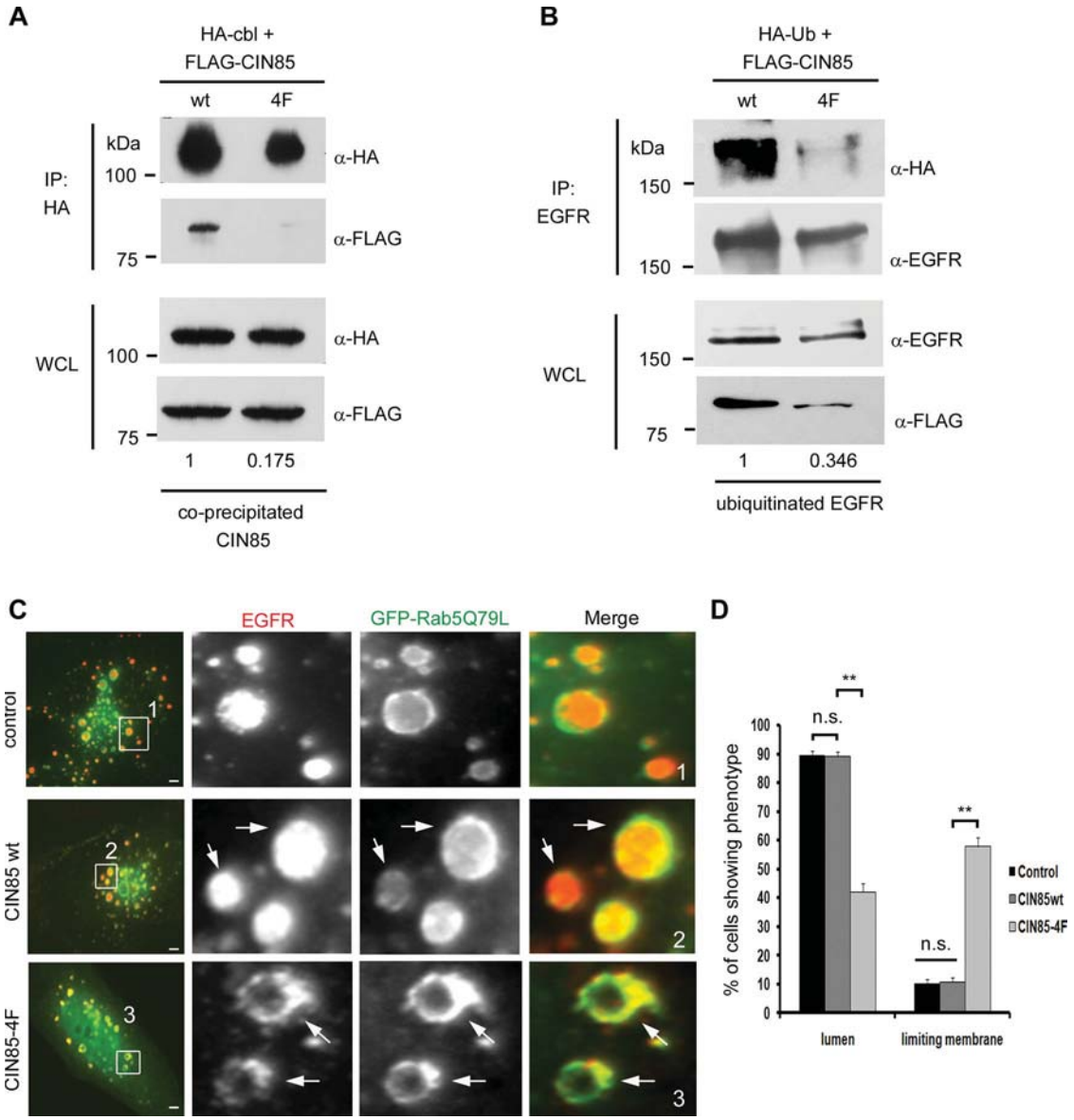

FIGURE 6: Inhibition of CIN85 phosphorylation results in reduced EGFR ubiquitination and missorting of the receptor to the limiting endosomal membrane. (A) Representative blot of a coimmunoprecipitation assay from HeLa cells coexpressing HA-cbl and either FLAG-CIN85wt or FLAG-CIN85-4F that were treated with 50 ng/ml EGF for 30 min. The levels of coprecipitated CIN85 were normalized to that of precipitated cbl, and the average amount of cbl-bound CIN85-4F relative to the wt from three independent experiments is shown. (B) Representative blot of an EGFR ubiquitination assay in HeLa coexpressing HA-Ub and either FLAG-CIN85wt or FLAG-CIN85-4F. Cells were treated with 50 ng/ml EGF for 30 min. The amount of HA-Ub was normalized to the amount of precipitated EGFR, and the average amount of ubiquitinated EGFR in CIN85-4F–expressing cells relative to wt-expressing cells from three independent experiments is shown. (C) IF images of EGFR sorting into MVBs in HeLa cells expressing either GFP-Rab5Q79L alone (control) or together with FLAG-CIN85wt or FLAG-CIN85-4F. Cells were treated with 50 ng/ml EGF for 30 min. Insets 1–3 show higher-magnification images of the boxed areas in the overview pictures. Bars, 5 μm. (D) Quantitation of three independent ILV sorting assays as described in C. Localization of EGFR was assessed in >30 cells, and the data are represented as mean ± SE.

EGFR in early endosomes due to the missorting of the receptor to the endosomal limiting membrane (Figures 3 and 6). Of importance, depletion of CIN85 induced the same EGFR trafficking defects as did inhibition of CIN85 phosphorylation, supporting an important role of CIN85 in EGFR sorting as well as degradation (Supplemental Figure S3, B–I). Taken together, these data provide novel insights into the regulation of CIN85 action and indicate that this adaptor protein plays an active role in EGFR sorting, most likely in concert with Rab5 and cbl at the early endosome (Figures 6 and Supplemental Figure S2). Identifying Rab5 as an interaction partner of CIN85 also underlines the importance of CIN85 at the early endosome. Thus this interaction could ensure that the endocytosed EGFR, likely in complex with cbl and CIN85 as reported previously (Dikic, 2002), is recruited to the early endosome while being retained to maximize the ubiquitination process for subsequent lysosomal sorting (Supplemental Figure S4, A and B). This

idea is also strongly supported by a recent report by Ronning *et al.* (2011) in which they demonstrated that CIN85 constitutively interacts with Hrs, which led them to speculate that in this context, CIN85 might help in promoting assembly of ESCRT complexes to facilitate EGFR sorting and degradation—a concept also proposed by Zhang *et al.* (2009). Further studies will be needed to determine the exact mechanism by which CIN85 regulates EGFR sorting at early endosomes.

Because the CIN85 phosphorylation, and potentially its interaction with cbl, occurs 30 min after EGF, it is possible that a participation in the initial receptor ubiquitination step is minimal. It does remain unclear, however, why these processes/modifications would even be partially initiated at the plasma membrane since they are not essential for EGFR internalization. From the findings in this study and others, we view CIN85 as a critical component of a positive feedback loop at the early endosome that participates in the activation of a cbl/EGFR ubiquitination complex (Supplemental Figure S4B). In this model, src-phosphorylated CIN85 increases the Ub-ligase activity of cbl at the early endosome, potentially by regulating src-mediated cbl phosphorylation as already shown for cbl-c (Ryan *et al.*, 2010). This increased cbl activation would then promote a more complete EGFR ubiquitination that promotes EGFR sorting into MVBs and targeting to the lysosome for degradation. Loss of CIN85 phosphorylation, for example, by overexpressing the phospho-defective CIN85-4F mutant, would cause a reduction in src-mediated cbl phosphorylation/activation and subsequent EGFR ubiquitination, leading to receptor missorting.

The CIN85–cbl interaction regulates EGFR ubiquitination and sorting

As previously mentioned, several reports have implicated the CIN895-cbl complex in EGFR endocytosis and down-regulation, although it is unclear whether the CIN85–cbl interaction per se is essential for proper receptor ubiquitination and sorting. A previous report showed that the CIN85–cbl interaction is dispensable for EGFR ubiquitination (Soubeyran *et al.*, 2002), whereas others reported that depletion of CIN85 results in a decrease in EGFR ubiquitination (Ronning *et al.*, 2011) without providing mechanistic insights into how and where this effect occurs. In contrast, we observed a substantial decrease in EGFR ubiquitination when the CIN85–cbl complex was disrupted (Figure 6). We emphasize that our data do not exclude the possibility that EGFR ubiquitination could be initiated at the plasma membrane, as there is not a complete loss of this modification upon disruption of the CIN85–cbl complex. However, these findings agree with other studies showing that EGFR ubiquitination is not important for its internalization (Huang *et al.*, 2007). In general, the timing of EGFR ubiquitination is still not completely defined and might depend on the cell type and experimental conditions. For example, the discrepancy between the mentioned studies and the data presented here could be explained by the different time courses of the observed ubiquitination, as others measured the EGFR ubiquitination profile in HEK293T and CHO cells just 10 min after EGF stimulation compared with 30 min after EGF in HeLa cells in the present study. It is likely, then, that the different studies observed distinct phases/peaks of EGFR ubiquitination, as described by Pennock and Wang (2008). Most important, we present a mechanistic insight into how CIN85 could regulate EGFR ubiquitination, namely by regulating the EGFR–cbl interaction. Taken together, our findings suggest that the CIN85–cbl complex contributes significantly toward the second wave of EGFR ubiquitination that occurs downstream of the initial ubiquitination in the endocytic event.

In addition, the data in the present study strongly support the concept that the CIN85–cbl interaction is required for proper EGFR trafficking and degradation, as prevention of CIN85 phosphorylation and thus CIN85–cbl complex formation greatly affects both processes. This is consistent with our previous findings that disruption of the CIN85–cbl complex by overexpression of another CIN85 mutant defective in cbl binding (CIN85B W → Y) also causes retention of EGFR in early endosomes (Schroeder *et al.*, 2010). Thus, interfering with the CIN85–cbl complex either by mutating the SH3-binding site (CIN85B W → Y) or preventing phosphorylation (CIN85-4F; Figure 2) results in the same physiological outcome. Our findings also agree with the published role for cbl on the early endosome, where it controls early endosome fusion and exit of the EGFR from the early endosome (Ravid *et al.*, 2004; Visser Smit *et al.*, 2009). Several unanswered questions need to be addressed in the future, however. For example, how is the CIN85–cbl complex targeted to the early endosome? What are the implications of CIN85 phosphorylation for EGFR-dependent signaling? In particular, how does phospho-CIN85 activate cbl? Does phospho-CIN85 also affect cbl–EGFR binding? Answering these questions will lead to better understanding EGFR trafficking and might help to develop new tools to specifically block distinct steps in EGFR trafficking.

MATERIALS AND METHODS

Cell culture and transfection

HeLa and HuH7 (human hepatocellular carcinoma) cells were incubated in MEM, supplemented with 10% fetal bovine serum (FBS), 1.5 g/l sodium bicarbonate, 50 U/mg penicillin plus 50 µg/ml streptomycin, 1× nonessential amino acids, and 1 mM sodium pyrophosphate. SYF cells were cultured in DMEM supplemented with 10% FBS and 50 U/mg penicillin plus 50 µg/ml streptomycin. Cells were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Generation of the knockdown 3T9 fibroblasts

Control (firefly luciferase) and CIN85-specific shRNA duplexes (Srivatsan and Shaw, unpublished data) were cloned into the pFLRu lentivirus as previously described (Feng *et al.*, 2010). Viral supernatants were generated in 293T cells by transfection of the lentiviral plasmids with Lipofectamine 2000 (Invitrogen) and the packaging plasmids as described (Feng *et al.*, 2010). Pooled supernatants, harvested at 24 and 48 h posttransfection, were applied to 3T9 fibroblasts with 8 µg/ml polybrene and spun at room temperature for 2 h at 2000 rpm. Supernatants were replaced with fresh medium immediately after centrifugation. Transduction efficiency was assessed 48 h later by flow cytometry, and yellow fluorescent protein–positive cells were sorted using a FACSAria II high-speed cell sorter (BD, Franklin Lakes, NJ).

Cells were cultured in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 1× nonessential amino acids, and 50 U/mg penicillin plus 50 µg/ml streptomycin. Cells were transfected for immunofluorescence analysis using Lipofectamine 2000 according to the manufacturer's instructions.

Protein purification and glutathione *S*-transferase pull down

Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 cells and purified using glutathione-coated beads (Amersham-Pharmacia Biotech, GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. Purified GST-src kinase was purchased from Cell Signaling Technology (Beverly, MA).

Pharmacological inhibition of src

Cells were pretreated with 5–10 μM SU6656 or DMSO as control for 2 h before immunoprecipitation of CIN85 for further analysis.

Immunoprecipitation

HeLa 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 either in hypotonic lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM EDTA, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, protease inhibitors) or RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors), sonicated, and centrifuged for 10 min at 14,000 rpm at 4°C. Between 500 and 800 μg of lysate was added to 5 μg of antibody and incubated for 2 h at 4°C. Antibody-bound complexes were precipitated by adding protein A– or G–coated beads (Sigma-Aldrich [St. Louis, MO] and Santa Cruz Biotechnology [Santa Cruz, CA], respectively) for 1 h at 4°C, washed five times, and subjected to Western blot analysis.

EGFR ubiquitination

HeLa cells were cotransfected with HA-ubiquitin (HA-Ub) and CIN85wt or mutants as indicated, serum starved overnight, and then stimulated with 50 ng/ml EGF for the indicated time points. Cells were lysed in a NP-40/*N*-ethylmaleimide (NEM) buffer (30 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 4 mM EDTA, pH 8, 10 mM NaF, 1 mM Na_3VO_4, 1 mM NEM, protease inhibitors), sonicated, and centrifuged for 10 min at 14,000 rpm at 4°C. From 0.5 to 1 mg of lysate was added to 5 μg of EGFR antibody and immune precipitated as described earlier.

Surface biotinylation after EGF stimulation

EGFR surface biotinylation assays were performed as described previously (Schroeder *et al*., 2010).

Immunofluorescence, image acquisition, and manipulation

Indirect immunofluorescence, image acquisition, and manipulations were performed as described previously (Schroeder *et al*., 2010).

RhEGF uptake, quantification, and statistical analysis

RhEGF trafficking assays and their quantitation were performed as described in Schroeder *et al*. (2010). Briefly, cells were serum starved overnight in low serum medium (0.2% FBS), pretreated with 50 μg/ml cycloheximide (CHX) for 1 h at 37°C, and then incubated with 100 ng/ml RhEGF plus CHX at 4°C for 45 min, pulsed for 15 min at 37°C to allow endocytosis, and then washed and chased for the indicated time points with low serum medium plus CHX. Cells were fixed and subjected to immunofluorescence analysis.

MVB sorting and quantitation

HeLa cells were cotransfected with GFP-tagged, constitutively active Rab5 (GFP-Rab5Q79L) and either FLAG-CIN85wt or FLAG-CIN85-4F, serum starved overnight, and then stimulated with 50 ng/ml EGF for 30 min. Endogenous EGFR was stained with an EGFR antibody, and the expressed proteins were detected using a FLAG antibody. For quantitation, each Rab5-positive structure that had a clear distinction between the lumen and the limiting membrane was taken into account. Under each experimental condition, the localization of EGFR in either the lumen or the limiting membrane of the Rab5 structure was assessed. For each condition ≥30 cells were counted, and the statistical analysis was performed as described before (Schroeder *et al*., 2010).

Antibodies and reagents

The CIN85 antibody used for immunoprecipitation was generated against amino acids 291–400 of CIN85 and kindly provided by Daniel Billadeau (Mayo Clinic, Rochester, MN). All other antibodies were purchased from the following companies: anti-CIN85 from Upstate (Millipore, Billerica, MA), anti-GFP from Roche (Indianapolis, IN), anti-actin and anti-GST from Sigma-Aldrich, anti-EGFR (polyclonal), anti-FLAG, and anti-HA from Cell Signaling Technology, anti-cbl (monoclonal) from BD Transduction Laboratories (Lexington, KY), anti-lamp1 (rat) and anti-EGFR (monoclonal) from Santa Cruz Biotechnology, anti-pTyr, clone 4G10, from Millipore, and anti-Hrs from Bethyl Laboratories (Montgomery, TX). GFP-Rab7 was kindly provided by Richard Pagano (Mayo Clinic); GFP-Rab5wt and GFP-Rab5Q79L were provided by Bruce Horazdovsky (Mayo Clinic). Rhodamine-EGF was purchased from Invitrogen, and human EGF and cycloheximide were purchased from Sigma-Aldrich.

Plasmids

GST-CIN85ABC and FLAG-CIN85wt were described previously (Schroeder *et al*., 2010). The CIN85-4F mutant (CIN85 Y10/109/271/278F) and GFP-tagged active Rab5 (GFP-Rab5Q79L) were generated by site-directed mutagenesis using *Pfu* turbo (Calbiochem, La Jolla, CA) according to the manufacturer's instructions. mCherry-CIN85 was cloned into the *Hin*dIII/*Xho*I sites of mCherry-C3 and verified by PCR and sequence analysis.

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