Expression of transfected proteins was monitored for every experiment by immunoblotting. We repeated the experiments three times.

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The endophilin–CIN85–Cbl complex mediates ligand-dependent downregulation of c-Met

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Ligand-dependent downregulation of tyrosine kinase receptors is a critical step for modulating their activity. Upon ligand binding, hepatocyte growth factor (HGF) receptor (Met) is polyubiquitinated[1] and degraded[2]; however, the mechanisms underlying HGF receptor endocytosis are not yet known. Here we demonstrate that a complex involving endophilins, CIN85 and Cbl controls this process. Endophilins[3] are regulatory components of clathrin-coated vesicle formation. Through their acyltransferase activity they are thought to modify the membrane phospholipids and induce negative curvature and invagination of the plasma membrane during the early steps of endocytosis[4]. Furthermore, by means of their Src-homology 3 domains, endophilins are able to bind CIN85, a recently identified protein that interacts with the Cbl proto-oncogene[5]. Cbl, in turn, binds and ubiquitinates activated HGF receptor, and by recruiting the endophilin–CIN85 complex, it regulates receptor internalization. Inhibition of complex formation is sufficient to block HGF receptor internalization and to enhance HGF-induced signal transduction and biological responses. These data provide further evidence of a relationship between receptor-mediated signalling and endocytosis, and disclose a novel functional role for Cbl in HGF receptor signalling.

Cell stimulation with growth factors usually leads to increased receptor tyrosine kinase activity, intracellular substrate recruitment, receptor internalization by accelerated endocytosis through clathrin-coated pits, and finally, degradation[6–8]. Several data sets suggest that ligand-dependent internalization may be a principal process regulating the duration and propagation of the signal initiated by tyrosine kinase receptors, thereby preventing overstimulation that could potentially lead to cellular transformation.

To clarify the molecular mechanisms connecting receptor-mediated signalling to endocytosis by means of clathrin-coated pits, we searched for proteins interacting with endophilins, by using the yeast two-hybrid system. A bait construct, encoding the full-length human endophilin A3, fused in-frame with the Gal4 DNA-binding domain, was used to screen a human brain complementary DNA library. Approximately 500,000 transformants were analysed, leading to isolation of 27 positive colonies. Sequence analysis of the prey plasmids revealed that five of them encoded for dynamins (well known interactors of endophilins)[9], whereas three clones of different length represented overlapping cDNAs for CIN85 (Fig. 1a). CIN85 is a member of a newly discovered subfamily of adaptor molecules including CMS[10] and CD2AP[11], and was recently shown to bind the Cbl proto-oncogene product[5]. CIN85 contains three amino-terminal Src-homology 3 (SH3) domains, followed by a proline-rich domain containing two sequences (PKKPPPP and PKKPRPP) that are optimal consensus sites for endophilin SH3 binding[12] and a carboxy-terminal coiled-coil domain, essential for dimerization[13]. As shown in Fig. 1b, pull-down experiments confirmed that a recombinant glutathione *S*-transferase (GST) fusion protein encoding full-length endophilin A3 was able to interact with

CIN85, and that the SH3 domains of the known endophilins are necessary and sufficient for this binding. Co-immunoprecipitation experiments demonstrated that endophilin A3 and CIN85 also associate in mammalian cells (Supplementary Information Fig. 1). As endophilin A3 binds CIN85, which in turn interacts with Cbl[5], we investigated whether these three proteins can form a ternary complex in cells. As shown in Fig. 1c, in transfected cells endophilin A3, CIN85 and Cbl associate in a complex; moreover, a dominant interfering form of CIN85, which is unable to interact with Cbl (CIN85-PCC, containing only the proline-rich and the coiled-coil domains), prevents complex formation.

Cbl binds to several activated tyrosine kinase receptors, acting either as a transducer[14] or as a ubiquitin ligase, and thus has a role in receptor downregulation[15]. In particular, the role of Cbl has been studied in signal transduction activated by HGF receptor[16], the heterodimeric tyrosine kinase with a relative molecular mass of 190,000 (190K) that is encoded by the Met proto-oncogene[17]. After HGF binding, signalling is elicited through receptor interactions with the major substrate Gab1 (ref. 18) and several SH2-containing proteins[19], one of which is the Cbl adaptor protein, known to bind to the Met tail[16] and to have a positive role in mitogen-activated protein (MAP) kinase activation[20]. After ligand stimulation, Met is rapidly polyubiquitinated[1] and degraded[2]; however, the molecular mechanisms involved in this process are unknown. We therefore investigated whether Cbl has a role in HGF receptor ubiquitination.

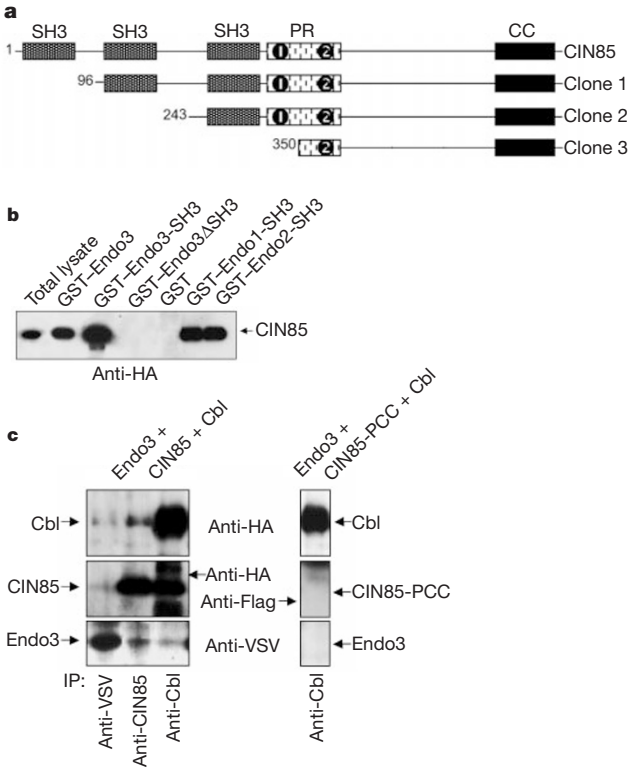

Figure 1 Endophilins interact with CIN85 and Cbl. **a**, Schematic representation of full-length CIN85 and the three clones obtained from the yeast two-hybrid screening. PR, proline-rich; CC, coiled-coil. Numbers inside the proline-rich domain indicate putative consensus sequences for endophilin SH3 binding. **b**, Pull-down experiments performed on lysates of cells transfected with HA-CIN85. CIN85 interacts with full-length endophilin A3 (GST–Endo3), the SH3 domains of endophilin A1, A2 and A3, but not with endophilin A3 lacking the SH3 domain (GST–Endo3ΔSH3). **c**, Endophilin A3 associates in a complex with CIN85 and Cbl. Lysates of HEK 293T cells co-transfected with VSV-endophilin A3, CIN85 (HA-wild type (WT) or Flag-CIN85-PCC) and HA-Cbl were immunoprecipitated with the indicated antibodies. Expression of a CIN85 mutant unable to bind Cbl (CIN85-PCC) impairs complex formation.

As shown in Fig. 2a, Cbl binds to the receptor (right panel) and induces its ubiquitination (left panel). On the contrary, a Cbl mutant devoid of ubiquitin ligase activity (Cbl-70Z) interacts with Met, but is unable to induce its ubiquitination (left panel). Notably, this mutant does not act in a dominant interfering manner with respect to preventing Met downregulation (Supplementary Information Fig. 2B). The interaction between Cbl and Met requires the presence of Y1356 in the receptor docking site[16]. We show here that a Cbl mutant lacking the SH2 domain is still able to associate with Met, although to a lesser extent, and to ubiquitinate the receptor (Supplementary Information Fig. 2A). It is possible that the interaction between this Cbl mutant and Met might occur indirectly by means of a bridge made by the SH3 domain of the adaptor protein Grb2 and the Cbl proline-rich region. This alternative modality of interaction has recently been observed also for Cbl and the EGF receptor (Y. Yarden, personal communication).

We then investigated whether the association of endophilin, CIN85 and Cbl was modulated by HGF. Indeed, under physiological conditions, Cbl binds to ligand-activated HGF receptor, becomes tyrosine phosphorylated, and recruits the CIN85–endophilin complex (Fig. 2b). Furthermore, endophilins are constitutively associated to HGFR in a cell line (GTL16) where Met is basally activated[17] (Supplementary Information Fig. 3).

Endophilins are known to be regulatory elements of the machinery controlling endocytosis through clathrin-coated pits[3,21–23]. To verify whether the Cbl–CIN85–endophilin complex recruited to Met has a role in receptor internalization, we impaired complex formation by using dominant interfering forms of each component of the complex. As shown by immunofluorescence analysis (Fig. 3a, b), the HGF–HGFR complex is not internalized in cells expressing mutants of Cbl (Cbl-480, a mutant that is unable to interact with CIN85; I. Dikic, personal communication), CIN85 (CIN85-PCC) and endophilin (Endo-SH3, a mutant still able to bind CIN85 but devoid of enzymatic activity). Indeed, in these cells, ligand-induced

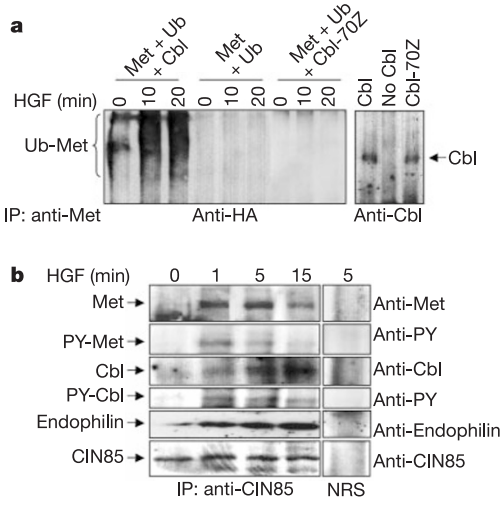

Figure 2 Cbl ubiquitinates Met and recruits the CIN85-endophilin complex. **a**, Activated Met interacts with Cbl and undergoes ubiquitination. HEK 293T cells were co-transfected with Met and HA-tagged ubiquitin (Ub) either in the absence or in the presence of wild-type Cbl (Cbl) or of a Cbl mutant devoid of ubiquitin ligase activity (Cbl-70Z). Cells were stimulated with HGF and immunoprecipitated with anti-Met antibodies. Activated Met associates with Cbl (right panel) and becomes polyubiquitinated (left panel). Cbl-70Z interacts with Met but it is not able to induce Met ubiquitination. **b**, Ligand-activated Met recruits the Cbl–CIN85–endophilin complex. HeLa cells were stimulated with HGF. Cell lysates were immunoprecipitated with either anti-CIN85 antibodies or pre-immune serum (NRS). Upon HGF-binding, Met becomes tyrosine phosphorylated, and binds to and phosphorylates Cbl, inducing the association with CIN85 and endophilin. PY-Met, tyrosine phosphorylated Met; PY-Cbl, tyrosine phosphorylated Cbl.

HGF receptor downregulation is strongly impaired—the amount of receptor is increased even in basal conditions and remains almost unaltered on HGF stimulation (Fig. 3c). Moreover, HGFR tyrosine phosphorylation is prolonged. Of note, in the presence of dominant interfering forms of either CIN85 or endophilin, the interaction between Cbl and Met is not impaired, Cbl becomes tyrosine phosphorylated (Supplementary Information Fig. 4), and Cbl-induced HGFR ubiquitination is unaffected (Supplementary Information Fig. 2A and data not shown).

To assess the signalling capacity of HGFR not undergoing downregulation, we evaluated its ability to recruit signal transducers in cells expressing the interfering form of endophilin. We found that when the receptor is not internalized, it remains tyrosine phosphorylated for a longer time (Fig. 3c), increasing binding and phosphorylation of its major substrate Gab1 (Fig. 4a). This increase of substrate recruitment and phosphorylation leads to enhancement of HGF-induced motility (Fig. 4b). We also observed a basal increase of motogenic properties, probably due to the fact that blocking of ligand-induced endocytosis may affect several receptors[24], and thus enhance cell responsivity to serum chemotactic molecules.

To explain our observations we propose the following model. Upon ligand activation, HGF receptor becomes tyrosine phosphorylated, binds and phosphorylates Cbl, which in turn targets the receptor to clathrin-coated pits by recruiting the CIN85–endophilin complex. This complex drives plasma membrane invagination and vesicle formation, thus resulting in negative modulation of signal transduction and biological responses. Our results also show that Cbl has a dual role in receptor signalling: it acts as a positive transducer, by triggering signals from the activated receptor, and as a negative modulator, by ubiquitinating the receptor and activating the endocytic machinery through recruitment of CIN85 and endophilin. Moreover, this role of Cbl in induction of endocytosis can be functionally separated from its E3 ligase activity. As a similar role for the Cbl–CIN85–endophilin complex has been demonstrated for downregulation of EGF receptors[24], the mechanism we describe seems to represent a general way to downregulate activated tyrosine kinase receptors. □

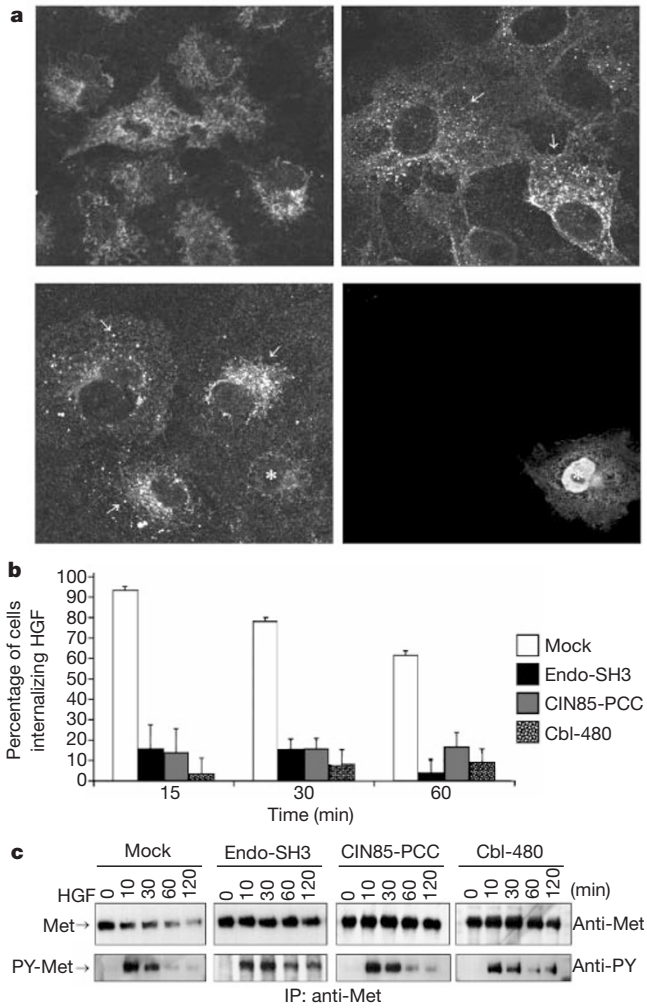

Figure 3 Expression of dominant interfering mutants of Cbl, CIN85 and endophilin impairs HGF receptor internalization and downregulation. **a**, Confocal microscopy images of COS7 cells transfected with Mock (top panels) or endophilin A3 SH3 (bottom panels), treated (top right and bottom panels) or not (top left panel) with HGF (30 min). The top and bottom left panels show staining with anti-HGF antibodies; the bottom right panel (same field as in bottom left, asterisk) is stained to determine endophilin SH3 expression. Ligand internalization (fluorescent dots, some of which are indicated by arrows) is abrogated in cells expressing endophilin SH3 domain. Magnification is at ×100. **b**, Quantitative analysis of immunofluorescence experiments. COS7 cells were transfected with Cbl, CIN85 or endophilin mutants. Immunofluorescence was performed as in **a**. The percentage of cells containing fluorescent dots was evaluated. Experimental points were carried out in triplicate. **c**, HEK 293T cells expressing the same mutants were stimulated with HGF. The same amount of total proteins was immunoprecipitated with anti-Met antibodies. In these cells, HGFR downregulation is impaired, and receptor tyrosine phosphorylation is prolonged.

Methods

Plasmid construction and yeast two-hybrid screening

For details of plasmid construction and yeast two-hybrid screening see Supplementary Information.

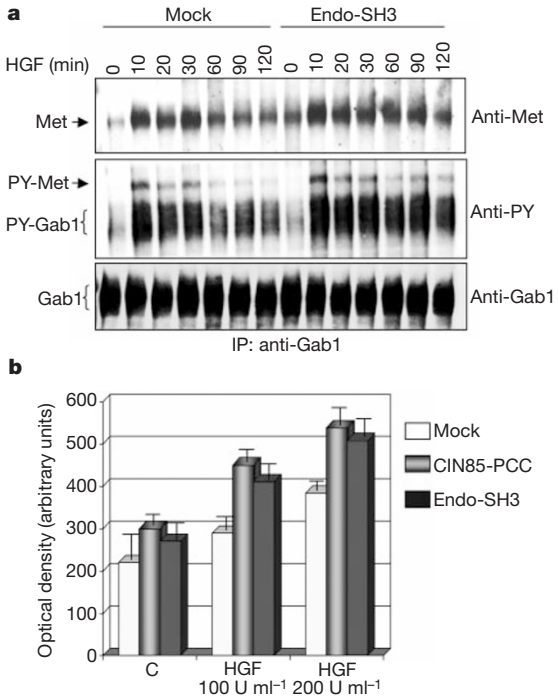

Figure 4 Impairment of Met downregulation enhances signal transduction and biological responses. **a**, A dominant interfering form of endophilin A3 enhances HGF-induced signal transduction. 293T cells transfected with Mock or Endo-SH3 were stimulated with HGF. Equal amounts of proteins were immunoprecipitated with anti-Gab1 antibodies. Endo-SH3-expressing cells show an increased association of Gab1 with Met and prolonged tyrosine phosphorylation. **b**, Dominant interfering forms of CIN85 or endophilin A3 increase cell motility. HEK 293T cells were transfected with Mock, CIN85 or endophilin mutants, and chemotactic ability of HGF was evaluated in a modified Boyden chamber assay. For experimental details see Methods.

Transfection, immunoprecipitation and western blotting

Human embryonic kidney (HEK) 293T and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were co-transfected with 3 μg of each plasmid and cultured for 48 h. For *in vivo* stimulation experiments, cells were grown for 48 h in spent medium and then stimulated with HGF (400 U ml^{-1}) at 37 °C, for the indicated times. Ubiquitination experiments were performed as described[15].

For immunoprecipitations, cells were lysed with EB buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) in the presence of protease inhibitors and 1 mM Na-orthovanadate. After immunoprecipitation with the appropriate antibodies, high stringency washes were performed (EB plus 1 M LiCl).

Western blots were performed according to standard methods. In western blot and immunoprecipitations the following antibodies were used: PY20 monoclonal antibody (Transduction Laboratories), anti-Met antibodies (described in ref. 25), anti vesicular stomatitis virus (VSV)-G (Sigma), anti-Cbl and anti-haemagglutinin (HA) (Santa Cruz Biotechnology), anti-Gab1 (Upstate Biotechnology), and anti-Flag (Sigma). Monoclonal anti-endophilin A3 and polyclonal anti-endophilins antibodies (able to recognize endophilins A1, A2 and A3) were a gift of E. Lantelme and C. Giachino. Anti-CIN85 (CT) antibody and pre-immune serum were provided by I. Dikic. Final detection was done with ECL system (Amersham).

GST pull-down assay

The host strain *Escherichia coli* XL-1 was transformed with plasmids encoding GST fusion proteins, and protein expression was induced with isopropyl-β-D-thiogalactopyranoside. Bacterial cells were lysed in phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors. Fusion proteins were captured on glutathione–Sepharose beads and incubated with crude lysates of transfected HEK 293T cells at 4 °C for 2 h. The beads were washed extensively in lysis buffer, and then bound proteins were eluted by boiling in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer, separated by SDS–PAGE, and processed for western blotting.

Motility assay

For motility assay, 10^5 cells were seeded on the upper side of a Transwell chamber on a porous polycarbonate membrane (8.0-μm pore size); the lower chamber of the Transwell was filled with DMEM containing 5% FBS, either in the absence or in the presence of HGF (100 or 200 U ml^{-1}). After 24 h of incubation, cells attached to the upper side of the filter were mechanically removed. Cells that migrated to the lower side were fixed and stained with crystal violet. The stained cells were solubilized in 10% acetic acid, absorbance at 560 nm was measured in a micro-plate reader.

Immunofluorescence

COS7 cells were transfected using Lipofectamine (Life Technologies) and then seeded on polylisine-coated coverslips. Cells were treated with HGF (400 U ml^{-1}) for different times, then fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and finally co-stained using biotin-conjugated anti-HGF (R&D), anti-VSV to detect endophilin A3 SH3 domain, anti-Flag to detect CIN85-PCC, and anti-HA to detect Cbl-480. Images were taken using a Bio-Rad Confocal Microscopy System.

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Involvement of receptor-interacting protein 2 in innate and adaptive immune responses

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Host defences to microorganisms rely on a coordinated interplay between the innate and adaptive responses of immunity[1]. Infection with intracellular bacteria triggers an immediate innate response requiring macrophages, neutrophils and natural killer cells, whereas subsequent activation of an adaptive response through development of T-helper subtype 1 cells (T_H1) proceeds during persistent infection[1]. To understand the physiological