Cbl–CIN85–endophilin complex mediates ligand-induced downregulation of EGF receptors

Philippe Soubeyran*†, Katarzyna Kowanetz*†, Iwona Szymkiewicz*†, Wallace Y. Langdon‡ & Ivan Dikic*

** Ludwig Institute for Cancer Research, Box 595, Husargatan 3, Uppsala, S-75124, Sweden*
‡ Department of Pathology, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia
† These authors contributed equally to this work

Cbl is a multi-adaptor protein involved in ligand-induced downregulation of receptor tyrosine kinases. It is thought that Cbl-mediated ubiquitination of active receptors is essential for receptor degradation and cessation of receptor-induced signal transduction[1–5]. Here we demonstrate that Cbl additionally regulates epidermal growth factor (EGF) receptor endocytosis. Cbl rapidly recruits CIN85 (Cbl-interacting protein of 85K; ref. 6) and endophilins (regulatory components of clathrin-coated vesicles[7–10]) to form a complex with activated EGF receptors, thus controlling receptor internalization. CIN85 was constitutively associated with endophilins, whereas CIN85 binding to the distal carboxy terminus of Cbl was increased on EGF stimulation. Inhibition of these interactions was sufficient to block EGF receptor internalization, delay receptor degradation and enhance EGF-induced gene transcription, without perturbing Cbl-directed receptor ubiquitination. Thus, the evolutionary divergent C terminus of Cbl uses a mechanism that is functionally separable from the ubiquitin ligase activity of Cbl to mediate ligand-dependent downregulation of receptor tyrosine kinases.

Recent genetic evidence has indicated that the C terminus of SLI-1, the Cbl orthologue in *Caenorhabditis elegans*, is necessary for negative regulation of LET-23-induced vulval development in nematode worms[11]. The C terminus of Cbl is divergent from SLI-1, and contains a larger proline-rich region and a distal C-terminal tail composed of an acidic box domain and a ubiquitin-associated (UBA) domain[1]. To identify proteins that bind specifically to the C terminus of Cbl, we screened for clones interacting with either the proline-rich sequences (residues 480–680) or the distal part (residues 680–906) of Cbl in the yeast two-hybrid system. Among six double positive clones, one clone coding for CIN85 bound to the distal tail of Cbl (Supplementary Information Fig. 1a). CIN85, also known as Ruk[12] and SETA[13], is an adapter protein containing three Src-homology 3 (SH3) domains, a proline-rich region and a coiled-coil domain[6]. The SH3 domains of CIN85 bound to the distal C terminus of Cbl but not to the proline-rich region of Cbl in pull-down assays (Supplementary Information Fig. 1b, c), and in co-precipitation studies in mammalian cells (Fig. 1a). We also observed increased complex formation between CIN85 and Cbl in EGF-stimulated HeLa and Chinese hamster ovary (CHO) cells (Fig. 1b), as well as in human embryonic kidney (HEK) 293T cells transfected with Cbl and CIN85 constructs (Supplementary Information Fig. 1d). CIN85 binding to Cbl was largely attenuated in a phosphorylation-defective Cbl mutant (Y700/731/774F) (data not shown and ref. 6). Similarly, Cbl association with CMS, a homologue of CIN85, is regulated by tyrosine phosphorylation of Cbl[14]. Therefore, EGF-induced phosphorylation of Cbl may cause a conformational change in its distal C terminus, thus exposing polyproline motifs that serve as binding sites for the SH3 domains of CIN85 or CMS. Notably, a ligand-inducible complex between EGF receptor and CIN85 was detected in cells expressing Cbl or oncogenic Cbl with impaired RING-finger domain (Cbl-70Z), but not Cbl deleted for CIN85 binding sites (Cbl-655) (Fig. 1c). These data indicate that increased Cbl phosphorylation and binding to EGF receptors, but not its ubiquitin ligase activity, contribute to the recruitment of CIN85 in EGF receptor complexes.

CIN85 was also shown to associate with several adaptor proteins[12,15,16], and could thereby facilitate the assembly of a larger Cbl-associated network on growth-factor stimulation. To identify additional CIN85-associated proteins, we screened for clones able to interact with the C terminus of CIN85 in the yeast two-hybrid system. Seven double positive clones were identified as endophilin A1, A2 or A3, each containing the intact SH3 domain (not shown), suggesting that the SH3 domain of endophilins binds to CIN85. These results were confirmed *in vitro* by the ability of the SH3 domain of endophilin A1 to bind to the isolated proline-rich

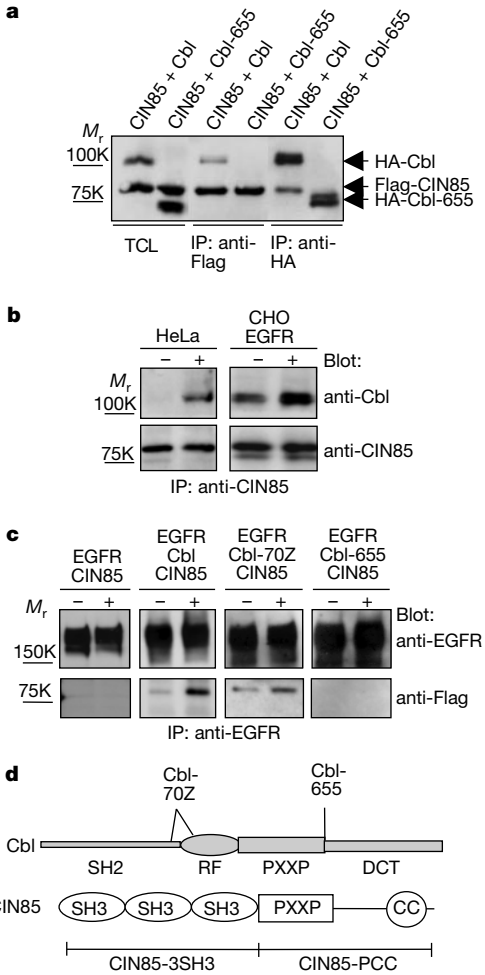

Figure 1 CIN85 is recruited to Cbl–EGF receptor complexes on EGF stimulation. **a**, Lysates (TCL) of HEK 293T cells expressing indicated proteins were immunoprecipitated (IP) with anti-HA or anti-Flag antibodies, and immunoblotted with a mixture of anti-HA/Flag antibodies. M_r, relative molecular mass. **b**, Lysates of unstimulated (–) or stimulated (EGF 50 ng ml^{-1} for 5 min at 37 °C) cells were subjected to immunoprecipitation with an antibody against CIN85 (CT), and blotted with anti-Cbl or anti-CIN85 (CT) antibodies. **c**, Lysates of HEK 293T cells expressing designated proteins were subjected to immunoprecipitation with anti-EGFR and immunoblotting with anti-EGFR or anti-Flag (M5) antibodies. Anti-Flag antibodies recognized Flag-tagged CIN85. **d**, Structure of Cbl and CIN85 and schematic representation of constructs used in these studies. SH2, Src homology 2 domain; RF, RING-finger ubiquitin ligase domain; PXXP, polyproline region; DCT, distal C terminus of Cbl; 3SH3, three SH3 domains; CC, coiled-coil. Cbl-655 is a deletion of the DCT of Cbl; Cbl-70Z is an internal deletion of 17 amino-acid residues in the beginning of the RING finger.

sequence of CIN85, and vice versa (Supplementary Information Fig. 2a, b). In addition, endogenous or overexpressed Cbl co-precipitated with endophilin A1 only in the presence of CIN85, indicating that CIN85 acts as a linker between Cbl and endophilins (Fig. 2a). CIN85 was constitutively bound to endophilin A1 (Fig. 2b), whereas its binding to Cbl was increased in EGF-stimulated cells (Figs 1b and 2b), thereby leading to EGF-induced co-precipitation between Cbl and endophilin A1 (Fig. 2b) and EGF receptors and endophilin A1 (Fig. 2c). Importantly, we were able to demonstrate a ligand-inducible association of endogenous CIN85 and endophilins with the Cbl–EGFR complex in HeLa and MDA cells (Fig. 2d). CIN85 and endophilins co-precipitated with—in addition to phosphorylated EGF receptors and Cbl—several unidentified tyrosine phosphorylated proteins from EGF-treated cells (Fig. 2d). Taken together our data indicate that CIN85 constitutively associates with endophilins, whereas its potency to bind to Cbl is increased on EGF stimulation. In this way, CIN85 recruits endophilins in the complex with activated EGF receptors.

Endophilins were previously implicated in the control of clathrin-mediated endocytosis[7–10]. They bind to the regulatory components of endocytic vesicles[17,18] and modulate plasma membrane invagination by intrinsic acyl-transferase activity[7] and by direct binding to lipid bilayers[19]. Both CIN85 and endophilins were found diffusely distributed in the cytoplasm of resting CHO cells, whereas they co-localized with EGF receptors in endocytic vesicles after EGF stimulation (Supplementary Information Fig. 3a, b). We next tested the importance of a Cbl–CIN85–endophilin complex for EGF receptor ubiquitination and endocytosis. Dominant interfering forms of CIN85 containing either isolated SH3 domains (CIN85-3SH3) that associate with Cbl (Supplementary Information Fig. 1c) or a proline-rich region with a coiled-coil domain (CIN85-PCC) that binds to endophilin A1 (Supplementary Information Fig. 2a, b), were analysed for their ability to modulate EGF receptor polyubiquitination and subsequent receptor degradation. Cbl-mediated polyubiquitination of the EGF receptor was intact in the presence of dominant interfering forms of CIN85 (Fig. 3a). In contrast, the expression of CIN85-3SH3 or CIN85-PCC, but not CIN85, delayed Cbl-mediated EGF receptor degradation (Fig. 3b). These data suggest that CIN85 is dispensable for polyubiquitination of EGF receptors, whereas it may be critical for the regulation of receptor endocytosis and lysosomal degradation. Furthermore, these results indicate that polyubiquitinated EGF receptors can be uncoupled from a degradative fate if parallel Cbl/CIN85-mediated events are perturbed.

We investigated further the impact of expression of dominant interfering mutants of CIN85 on EGF receptor endocytosis and downregulation. Expression of Cbl together with EGF receptors led to a significant increase in the rate of ligand internalization and the

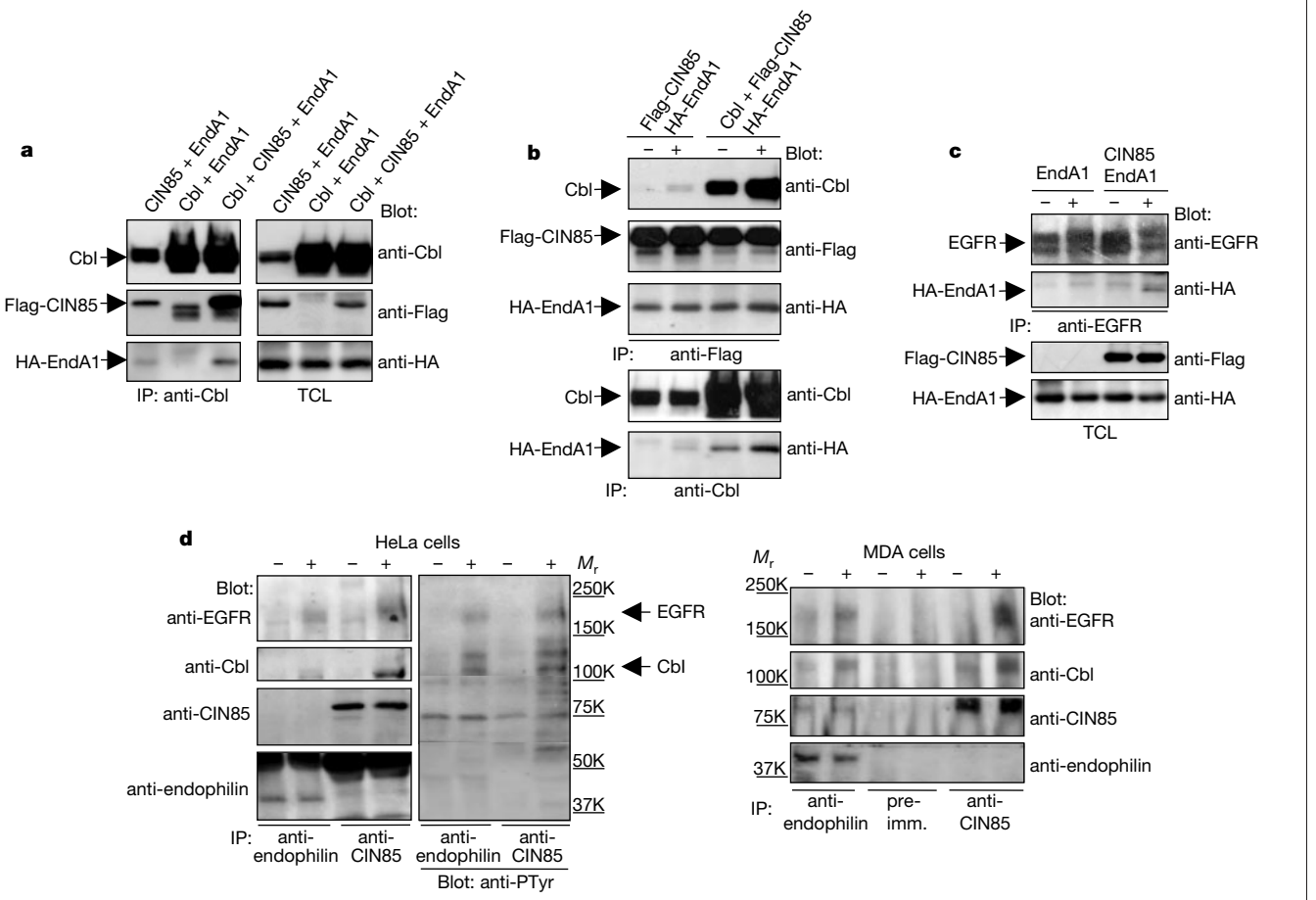

Figure 2 CIN85 links endophilins with the Cbl–EGFR complex. **a**, Lysates (TCL) of HEK 293T cells expressing designated proteins were subjected to immunoprecipitation (IP) and immunoblotting with indicated antibodies. EndA1, endophilin A1. **b**, **c**, HEK 293T cells expressing indicated proteins were left unstimulated (−) or stimulated with EGF (50 ng ml^{-1}) for 5 min at 37 °C (+). Lysates were immunoprecipitated with anti-Flag (M2), anti-Cbl (RF) and anti-EGFR antibodies, and blotted with corresponding antibodies. **d**, HeLa and MDA cells were starved (−) or stimulated with EGF (50 ng ml^{-1}) for 5 min at 37 °C (+), and lysates were subjected to immunoprecipitation with antibodies against endophilins (mixed S-20 and P26), an anti-CIN85 (CT) serum or preimmune serum, followed by immunoblotting with the indicated antibodies. Co-precipitation between CIN85 and endophilins was observed only after long exposures owing to low immunoblotting sensitivity of anti-endophilin and anti-CIN85 antibodies. Membranes were stripped and reblotted with anti-phosphotyrosine (PY99) antibodies.

clearance of EGF receptors from the cell surface when compared with cells transfected with EGF receptors alone (Fig. 3c, d). However, Cbl-accelerated receptor internalization and downregulation was efficiently blocked by expression of CIN85-3SH3 and CIN85-PCC (Fig. 3c, d), but not by expression of CIN85 (Fig. 3c), single SH3 domains of CIN85 or polyproline motifs that do not bind to endophilins (Supplementary Information Fig. 4). The principal function of CIN85 was further demonstrated by the inhibitory effect of CIN85-3SH3 or CIN85-PCC on the endogenous machinery involved in EGF receptor internalization and downregulation in CHO cells (Fig. 3c, d). These inhibitory effects of CIN85-3SH3 and CIN85-PCC were also visualized by immunofluorescence (Fig. 3e), quantified and expressed as a percentage of CHO cells internalizing EGF receptors (Supplementary Information Fig. 3c). It was recently demonstrated that Cbl binds and ubiquitinates EGF receptors at the plasma membrane, and remains associated with EGF receptors along the endocytic pathway where it may participate in sorting internalized receptors for lysosomal degradation[20,21]. Our results indicate that Cbl, by recruiting CIN85 and endophilins in complex with EGF receptors, also has an important function in the regulation of receptor internalization.

Finally, we tested whether dominant interfering forms of CIN85 can attenuate the inhibitory effect of Cbl on EGF receptor signalling pathways, by measuring EGF-induced transcription of the serum response element (SRE). Expression of Cbl decreased—in a dose-dependent manner—transcription of SRE triggered by EGF (Fig. 4a), whereas co-expression of CIN85-3SH3 or CIN85-PCC, but not CIN85, led to a reduction in the ability of Cbl to block EGF-induced SRE activity (Fig. 4a). Therefore, the full inhibitory effect of Cbl on EGF receptor signalling depends on its interactions with a functional CIN85 protein. The fact that CIN85-PCC, which does not bind to Cbl, was also sufficient to reduce the inhibitory effects of Cbl supports the idea that CIN85-PCC interacting proteins mediate, in part, the inhibitory effects of Cbl on EGF receptor signalling.

Identification of ligand-inducible interactions between the EGF receptors and a CIN85–endophilin complex has shed new light on the mechanisms involved in the regulation of receptor endocytosis, as well as receptor-mediated signalling[4,5]. Here we have defined a

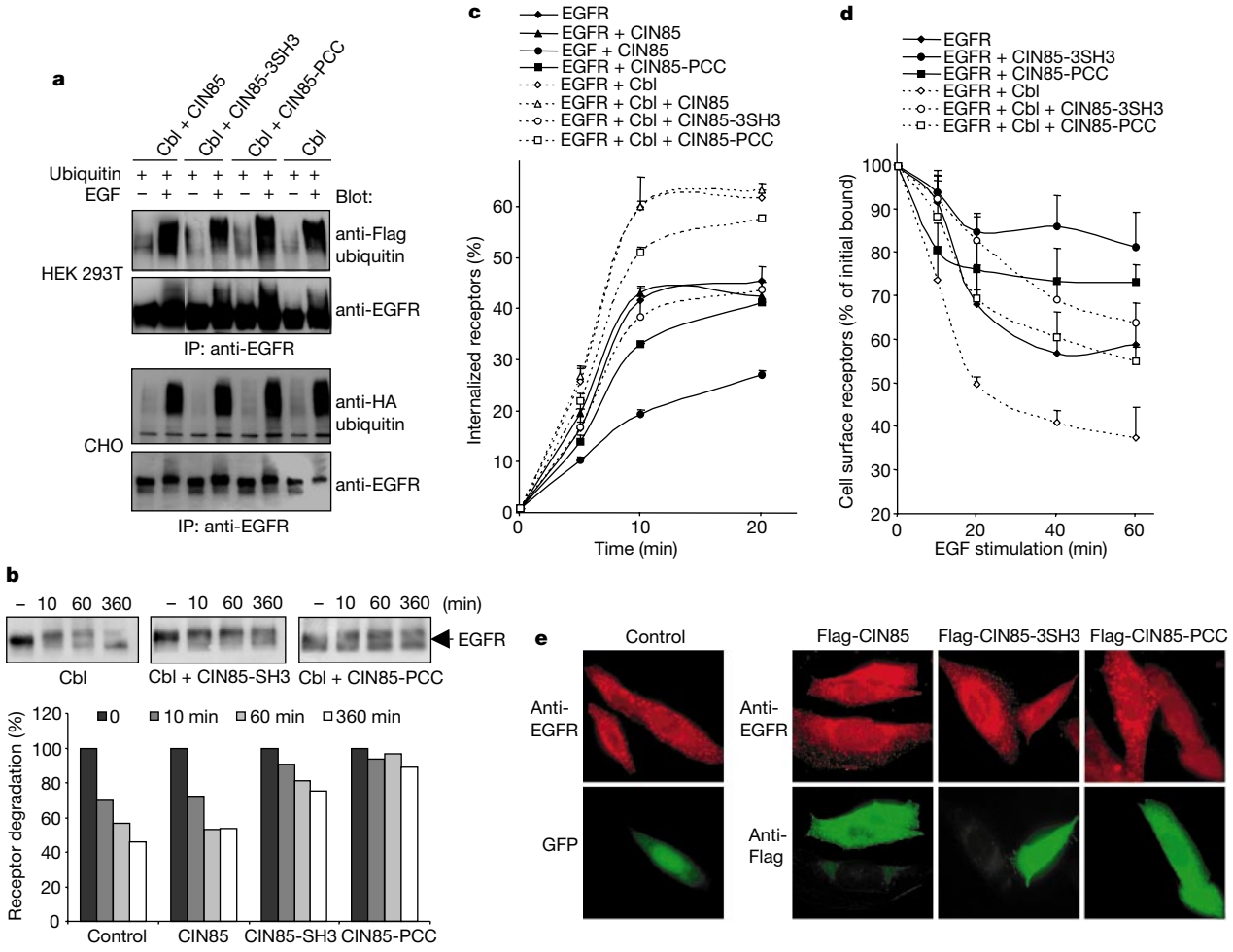

Figure 3 Effects of dominant interfering forms of CIN85 on Cbl-mediated ubiquitination, endocytosis and degradation of EGF receptors. **a**, HEK 293T or CHO cells were transfected with plasmids encoding EGFR and Flag- or HA-tagged ubiquitin in addition to Cbl alone or Cbl with CIN85, CIN85-3SH3 or CIN85-PCC constructs. Cells were treated with EGF (50 ng ml^{-1}) for 10 min at 37 °C (+), and immunoprecipitates of EGFR were blotted with anti-Flag, anti-HA or anti-EGFR antibodies. **b**, HEK 293T cells expressing EGFR and designated proteins were stimulated for the indicated time periods, and lysates were subjected to immunoblotting with anti-EGFR antibodies (upper panel). The levels of EGFR in cells were quantified and expressed as a percentage of remaining receptors for each time point. **c**, Ligand internalization in CHO cells transfected with EGFR alone or together with the indicated proteins was performed as described in Methods. The results are expressed as a percentage between internalized and total cell-associated radioactive EGF. **d**, The level of surface receptors following time-lapse EGF stimulation was measured by a downregulation assay. The results are expressed as a percentage of the ^{125}I-labelled EGF bound to cell surface receptor after stimulation by non-labelled EGF for the indicated times. **e**, CHO EGFR cells expressing the indicated proteins were subjected to immuno fluorescence analysis using anti-EGFR and anti-Flag (M2), followed by anti-rabbit TRITC (tetramethylhylrhodamine isomer R) and anti-mouse fluorescein isothiocyanate antibodies. A representative picture of the inhibition of receptor internalization and vesicle formation in cells expressing CIN85-3SH3 and CIN85-PCC is shown. GFP, green fluorescent protein.

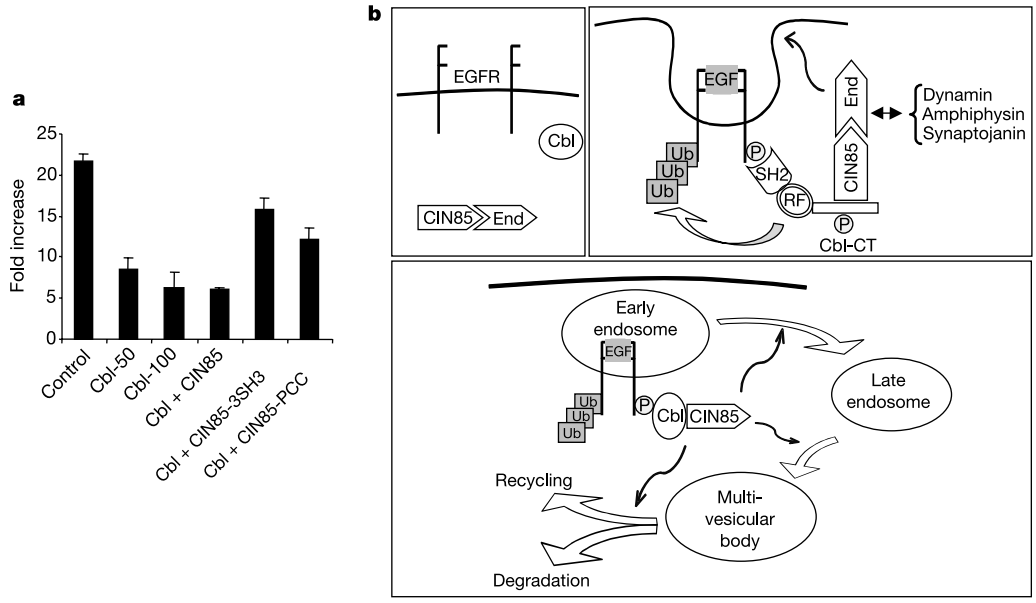

Figure 4 Role of CIN85 in the regulation of EGF-induced gene transcription. **a**, HEK 293T cells were transfected with reporter genes *SRE-Luc* (10 ng) and *lacZ* (20 ng), together with pRK5-EGFR (10 ng) alone (control) or with increasing amounts of Cbl (50 ng or 100 ng). In addition, vectors coding for CIN85 (100 ng), CIN85-3SH3 (100 ng) or CIN85-PCC (100 ng) were co-transfected with Cbl (100 ng). EGF-induced increase in luciferase activity was quantified and expressed as average ± s.d. **b**, Schematic model of EGF receptor downregulation. In resting cells (top left), CIN85 and endophilins (End) are constitutively associated and are not bound to Cbl or EGF receptors. Upon ligand-induced activation of EGF receptors (top right), Cbl binds to phosphorylated receptors and promotes receptor ubiquitination (Ub). Cbl is also tyrosine phosphorylated in this complex, leading to translocation of CIN85/endophilin in the vicinity of active EGF receptors, whereby endophilins together with dynamin, amphiphysin and synaptojanin regulate clathrin-mediated endocytosis. Cbl is associated with EGF receptors along the endocytic pathway, and is implicated in sorting internalized receptors for lysosomal degradation (bottom). CIN85 may control EGF receptor trafficking by its ability to bind to multiple adaptor proteins, and thus promote the assembly of Cbl-linked signalling networks involved in cross-talk between endosome and the actin cytoskeleton.

regulatory role for Cbl, which is functionally separable from its ubiquitin ligase activity, and involves the recruitment of CIN85–endophilin complexes to activated EGF receptors (Fig. 4b). Translocation of endophilins in the vicinity of active EGF receptors promotes membrane invagination and thus receptor internalization, by acting together with other regulatory components of endocytic vesicle formation, including the GTPase dynamin[7,9,17]. Accordingly, it has been demonstrated that overexpression of a dominant interfering form of endophilin prevents c-Met receptor endocytosis and enhances receptor-mediated biological responses[22]. The present study has also indicated a role for CIN85 in the control of post-membrane events such as targeting receptors for degradation and regulation of gene transcription (Figs 3b and 4a). CIN85 may control these processes by binding to multiple adaptor proteins (for example, Crk[15], p130Cas[15] and the p85 subunit of phosphatidylinositol-3 kinase[12]) involved in cross-talk between endosome and the actin cytoskeleton. The ability of CIN85 to assemble larger Cbl-associated signalling networks places it in a position to coordinate multiple steps in the endocytosis of tyrosine kinase receptors. Defining how this process is regulated in living cells will be the challenge for future investigations. □

Methods

Materials and plasmids

EGF was purchased from Intergen and ^{125}I-labelled EGF was from Amersham Biosciences. pSR-HA-Cbl, pSR-HA-Cbl-70Z, pSR-HA-Cbl-655 and pSR-HA-Cbl-480 or pRK5-Cbl were described previously[23,24]. Haemagglutinin (HA)-tagged endophilin A1 in pcDNA3, Flag-tagged CIN85 in pcDNA3, Flag-tagged ubiquitin in pcDNA3.1, and pYTH9 vector was provided by P. De Camili, S. Kajigaya, K. Tanaka and P. Aspenström, respectively. Description of CIN85 constructs is provided in Supplementary Information. CHO, MDA and HeLa cells were purchased from American Type Culture Collection; CHO cells stably expressing EGFR were provided by J. Borst and A. de Melker.

Yeast two-hybrid system screening

Yeast screening was performed using the Gal4-based Matchmaker two-hybrid system with the human fetal brain, thymus and T-cell libraries (Clontech). The yeast retransformation and the filter lift assay were done as described in the Matchmaker system manual.

GST binding assays and immunochemical analyses

Glutathione *S*-transferase (GST) binding assays, immunoprecipitation and immunoblotting were performed as described[25,26]. The following antibodies were used: anti-HA (12CA5; Roche), anti-Flag (M2 and M5; Sigma), mouse monoclonal anti-Cbl (C40320; Transduction Laboratories), rabbit polyclonal (RK2) anti-EGFR antibodies (a gift of J. Schlessinger), anti-endophilin A1 (S-20; Santa Cruz), pan-endophilin antibodies (Nuts) (a gift of P. De Camilli), anti-endophilin (P26) antibodies (a gift of N. Migone), rabbit polyclonal anti-Cbl antibodies raised against the GST fusion protein containing the RING finger of Cbl (RF), and rabbit polyclonal anti-CIN85 (CT) antibodies raised against the last 21 amino acids of human CIN85. Immunofluorescence studies were carried out as described[26], with specific details provided in Supplementary Information.

Ligand internalization and receptor downregulation assays

Ligand internalization and downregulation assays were performed as reported[21,27]. Briefly, cell monolayers of transfected CHO cells were incubated for 1 h at 4 °C with ^{125}I-labelled EGF, washed twice with binding buffers, and then incubated at 37 °C for the indicated time intervals. Cells were transferred on ice and washed with either cold binding buffer or mild acidic buffer to remove surface-bound radiolabelled EGF. We quantified the remaining radioactivity in cells after cell lysis. Each point was measured in quadruplicate and expressed as a percentage (average ± s.d.) of internalized versus total cell-associated radioactive EGF. To measure EGF receptor downregulation, CHO cells were transfected in triplicates in 24-well plates, and 48 h later cells were either left unstimulated or incubated with EGF (50 ng ml^{-1}) at 37 °C for the indicated time intervals, subjected to acid wash to remove bound EGF, and subsequently incubated with ^{125}I-labelled EGF for 1.5 h at 4 °C. The amount of surface-bound radioactive EGF was determined by a γ-counter and expressed as a percentage of surface-labelled receptors compared with unstimulated cells. Details of receptor ubiquitination and degradation assays are provided in Supplementary Information.

Reporter gene assays

HEK 293T cells were grown in collagen-coated 24-well dishes and transfected using LipofectAMINE reagent (GIBCO BRL). After 36 h, cells were stimulated with EGF, lysed in 1 × lysis buffer (Pharmigen), and mixed with luciferase assay reagent (Pharmigen). The luciferase activity was measured by a luminometer (Wallace) and normalized against β-galactosidase activity for each point. EGF-induced increase in luciferase activity was quantified for every pair in the triplicate and was expressed as the average induction ± s.d.

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

Received 8 November 2001; accepted 7 January 2002.

1. Thien, C. B. & Langdon, W. Y. Cbl: many adaptations to regulate protein tyrosine kinases. *Nature Rev. Mol. Cell Biol.* **2,** 294–307 (2001).
2. Joazeiro, C. A. *et al.* The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286,** 309–312 (1999).
3. Levkowitz, G. *et al.* Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* **4,** 1029–1040 (1999).
4. Waterman, H. & Yarden, Y. Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. *FEBS Lett.* **490,** 142–152 (2001).
5. Di Fiore, P. P. & De Camilli, P. Endocytosis and signaling. An inseparable partnership. *Cell* **106,** 1–4 (2001).
6. Take, H. *et al.* Cloning and characterization of a novel adaptor protein, CIN85, that interacts with c-Cbl. *Biochem. Biophys. Res. Commun.* **268,** 321–328 (2000).
7. Schmidt, A. *et al.* Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature* **401,** 133–141 (1999).
8. Ringstad, N. *et al.* Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. *Neuron* **24,** 143–154 (1999).
9. Simpson, F. *et al.* SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nature Cell Biol.* **1,** 119–124 (1999).
10. Gad, H. *et al.* Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. *Neuron* **27,** 301–312 (2000).
11. Yoon, C. H., Chang, C., Hopper, N. A., Lesa, G. M. & Sternberg, P. W. Requirements of multiple domains of SLI-1, a *Caenorhabditis elegans* homologue of c-Cbl, and an inhibitory tyrosine in LET-23 in regulating vulval differentiation. *Mol. Biol. Cell* **11,** 4019–4031 (2000).
12. Gout, I. *et al.* Negative regulation of PI 3-kinase by Ruk, a novel adaptor protein. *EMBO J.* **19,** 4015–4025 (2000).
13. Bogler, O. *et al.* SETA: a novel SH3 domain-containing adapter molecule associated with malignancy in astrocytes. *Neuro-oncology.* **2,** 6–15 (2000).
14. Kirsch, K. H. *et al.* The adapter type protein CMS/CD2AP binds to the proto-oncogenic protein c-Cbl through a tyrosine phosphorylation-regulated Src homology 3 domain interaction. *J. Biol. Chem.* **276,** 4957–4963 (2001).
15. Watanabe, S. *et al.* Characterization of the CIN85 adaptor protein and identification of components involved in CIN85 complexes. *Biochem. Biophys. Res. Commun.* **278,** 167–174 (2000).
16. Borinstein, S. C. *et al.* SETA is a multifunctional adapter protein with three SH3 domains that binds Grb2, Cbl, and the novel SB1 proteins. *Cell Signal* **12,** 769–779 (2000).
17. Ringstad, N., Nemoto, Y. & De Camilli, P. The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. *Proc. Natl Acad. Sci. USA* **94,** 8569–8574 (1997).
18. Micheva, K. D., Ramjaun, A. R., Kay, B. K. & McPherson, P. S. SH3 domain-dependent interactions of endophilin with amphiphysin. *FEBS Lett.* **414,** 308–312 (1997).
19. Farsad, K. *et al.* Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J. Cell Biol.* **155,** 193–200 (2001).
20. de Melker, A. A., van Der Horst, G., Calafat, J., Jansen, H. & Borst, J. c-Cbl ubiquitinates the EGF receptor at the plasma membrane and remains receptor associated throughout the endocytic route. *J. Cell Sci.* **114,** 2167–2178 (2001).
21. Levkowitz, G. *et al.* c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* **12,** 3663–3674 (1998).
22. Petrelli, A. *et al.* The endophilin–CIN85–Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature* **416,** 187–190 (2001).
23. Galisteo, M. L., Dikic, I., Batzer, A. G., Langdon, W. Y. & Schlessinger, J. Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. *J. Biol. Chem.* **270,** 20242–20245 (1995).
24. Scaife, R. M. & Langdon, W. Y. c-Cbl localizes to actin lamellae and regulates lamellipodia formation and cell morphology. *J. Cell Sci.* **113(2),** 215–226 (2000).
25. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. & Schlessinger, J. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383,** 547–550 (1996).
26. Ivankovic-Dikic, I., Gronroos, E., Blaukat, A., Barth, B. U. & Dikic, I. Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nature Cell Biol.* **2,** 574–581 (2000).
27. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L. & Beguinot, L. Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. *J. Biol. Chem.* **271,** 13377–13384 (1996).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank S. Giordano and Y. Yarden for communication of unpublished results, and L. Claesson-Welsh and C.-H. Heldin for critical reading of the manuscript. We also thank other members of the Dikic laboratory for valuable comments and help with various reagents. This work was supported by a Marie Curie Fellowship of the European Community programme to P.S., and the Boehringer Ingelheim Fonds and the Swedish Strategic Funds to I.D.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to I.D. (e-mail: Ivan.Dikic@licr.uu.se).

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

Annalisa Petrelli*†, Giorgio F. Gilestro†, Stefania Lanzardo*, Paolo M. Comoglio†, Nicola Migone*‡ & Silvia Giordano†‡

** CNR-CIOS and Department of Genetics, Biology and Biochemistry, University of Torino, 10126 Torino, Italy*
† Division of Molecular Oncology, Institute for Cancer Research and Treatment (IRCC), University of Torino Medical School, I-10060 Candiolo, Torino, Italy
‡ These authors contributed equally to this work

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

enzyme is not required for this proton pumping in the reductive phase. Proton pumping only occurs on injection of the second electron. This is notable because in the oxidative phase of the cycle a single electron seems to be sufficient, and may indicate that more redox energy per electron is available in the oxidative part of the cycle. The usage of the proton transfer pathways is partially different. The charge-compensating proton appears to be taken up by means of the K-pathway in the E to R transition (in contrast to the prediction of ref. 4), but through the D-pathway in the F to O transition (Fig. 2b and ref. 21). The D-pathway, however, is required for proton pumping in both cases. The mechanism of proton-pumping itself remains to be elucidated. □

Methods

The proteoliposomes of wild-type or D124N cytochrome *c* oxidase were prepared as described[9]. For the spectroscopic measurements, 500 µl proteoliposomes containing 6 µM or 20 µM enzyme were mixed with 500 µl of 50 mM HEPES/KOH buffer, pH 7.4, 100 mM β-D-glucose in an anaerobic cuvette, degassed and overlaid with argon. After recording a reference spectrum for the oxidized form of the enzyme (O), a 100-fold molar excess of hydrogen peroxide and 40 µg glucose oxidase were added to form state F. Next, we added 25 µg catalase, and the cuvette was flushed with carbon monoxide in the dark. We recorded optical absorbance spectra every 90 s. For comparison the same procedure was used with 10 µM solubilized D124N mutant enzyme in the presence of 0.05% dodecyl-β-D-maltoside as detergent.

The photopotential was measured as described[9]. Proteoliposomes were adsorbed to a planar lipid membrane (protein concentration in the cuvette approximately 100 nM), and the potential was measured across the proteoliposome/planar membrane system. The states E or F were prepared as described above for the spectroscopic measurements. Next, the cytochrome *c* oxidase was reduced upon laser-flash excitation of tris(2,2′-bipyridyl) ruthenium, a photoactivatable electron donor.

Received 30 July 2001; accepted 18 February 2002.

1. Wikström, M. K. Proton pump coupled to cytochrome *c* oxidase in mitochondria. *Nature* **266,** 271–273 (1977).
2. Wikström, M. Identification of the electron transfers in cytochrome oxidase that are coupled to proton-pumping. *Nature* **338,** 776–778 (1989).
3. Michel, H. The mechanism of proton pumping by cytochrome *c* oxidase. *Proc. Natl Acad. Sci. USA* **95,** 12819–12824 (1998).
4. Michel, H. Cytochrome *c* oxidase: catalytic cycle and mechanisms of proton pumping—a discussion. *Biochemistry* **38,** 15129–15140 (1999).
5. Verkhovsky, M. I., Jasaitis, A., Verkhovskaya, M. L., Morgan, J. E. & Wikström, M. Proton translocation by cytochrome *c* oxidase. *Nature* **400,** 480–483 (1999).
6. Ferguson-Miller, S. & Babcock, G. T. Heme/copper terminal oxidases. *Chem. Rev.* **96,** 2889–2907 (1996).
7. Michel, H., Behr, J., Harrenga, A. & Kannt, A. Cytochrome *c* oxidase: structure and spectroscopy. *Annu. Rev. Biophys. Biomol. Struct.* **27,** 329–356 (1998).
8. Nilsson, T. Photoinduced electron transfer from tris(2,2′-bipyridyl)ruthenium to cytochrome *c* oxidase. *Proc. Natl Acad. Sci. USA* **89,** 6497–6501 (1992).
9. Ruitenberg, M. *et al.* Single-electron reduction of the oxidized state is coupled to proton uptake via the K pathway in *Paracoccus denitrificans* cytochrome *c* oxidase. *Proc. Natl Acad. Sci. USA* **97,** 4632–4636 (2000).
10. Mitchell, R. & Rich, P. R. Proton uptake by cytochrome *c* oxidase on reduction and on ligand binding. *Biochim. Biophys. Acta* **1186,** 19–26 (1994).
11. Iwata, S., Ostermeier, C., Ludwig, B. & Michel, H. Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* **376,** 660–669 (1995).
12. Thomas, J. W., Puustinen, A., Alben, J. O., Gennis, R. B. & Wikström, M. Substitution of asparagines for asparate-135 in subunit I of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli* eliminates proton-pumping activity. *Biochemistry* **32,** 10923–10928 (1993).
13. Thomas, J. W., Lemieux, L. J., Alben, J. O. & Gennis, R. B. Site-directed mutagenesis of highly conserved residues in helix VIII of subunit I of the cytochrome *bo* ubiquinol oxidase from *Escherichia coli*: an amphipathic transmembrane helix that may be important in conveying protons to the binuclear center. *Biochemistry* **32,** 11173–11180 (1993).
14. Verkhovsky, M. I., Tuukkanen, A., Backgren, C., Puustinen, A. & Wikström, M. Charge translocation coupled to electron injection into oxidized cytocrome *c* oxidase from *Paracoccus denitrificans*. *Biochemistry* **40,** 7077–7083 (2001).
15. Witt, S. N. & Chan, S. I. Evidence for a ferryl Fea3 in oxygenated cytochrome *c* oxidase. *J. Biol. Chem.* **262,** 1446–1448 (1987).
16. Vygodina, T. V. & Konstantinov, A. A. H_2O_2-Induced conversion of cytochrome *c* oxidase peroxy complex to oxoferryl state. *Ann. NY Acad. Sci.* **550,** 124–138 (1988).
17. Moody, A. J. Ligation and electronation states of cytochrome *c* oxidase in relation to other oxidases and peroxidases. *Biochem. Soc. Trans.* **19,** 617–622 (1991).
18. Greenwood, C., Wilson, M. T. & Brunori, M. Studies on partially reduced mammalian cytochrome oxidase. Reactions with carbon monoxide and oxygen. *Biochem. J.* **137,** 205–215 (1974).
19. Brzezinski, P. & Malmström, B. G. The reduction of cytochrome *c* oxidase by carbon monoxide. *FEBS Lett.* **187,** 111–114 (1985).
20. Oliveberg, M. & Malmström, B. G. Internal electron transfer in cytochrome *c* oxidase: evidence for a rapid equilibrium between cytochrome *a* and the bimetallic site. *Biochemistry* **30,** 7053–7057 (1991).
21. Konstantinov, A. A., Siletsky, S., Mitchell, D., Kaulen, A. & Gennis, R. The roles of two proton input channels in cytochrome *c* oxidase from *Rhodobacter sphaeroides* probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer. *Proc. Natl Acad. Sci.USA* **94,** 9085–9090 (1997).
22. Jasaitis, A., Verkhovsky, M. I., Morgan, J. E., Verkhovskaya, M. L. & Wikström, M. Assignment and charge translocation stoichiometries of the major electrogenic phases in the reaction of cytochrome *c* oxidase with dioxygen. *Biochemistry* **38,** 2697–2706 (1999).

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the Max-Planck-Gesellschaft for financial support. We are grateful to B. Ludwig for providing the D124N mutant.

Correspondence and requests for materials should be addressed to H.M. (e-mail: michel@mpibp-frankfurt.mpg.de) or K.F. (e-mail: fendler@mpibp-frankfurt.mpg.de).

errata

A laboratory analogue of the event horizon using slow light in an atomic medium

Ulf Leonhardt

Nature **415**, 406–409 (2002).

In Table 1 of this Letter, the average particle number for slow light was incorrectly expressed as: $\frac{1}{(e^{\pi\mu}-e^{-\pi\mu})^2}$. It should have read: $\frac{1}{(e^{\pi\mu}+e^{-\pi\mu})^2}$. □

Cbl–CIN85–endophilin complex mediates ligand-induced downregulation of EGF receptors

Phillippe Soubeyran, Katarzyna Kowanetz, Iwona Szymkiewicz, Wallace Y. Langdon & Ivan Dikic

Nature **416**, 183–188 (2002).

In Fig. 3c of this Letter, the line (filled circles) labelled EGF+CIN85 should have been labelled EGFR+CIN85−3SH3, as in Fig. 3d. □

corrigendum

Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine

Tobias Krojer, Marta Garrido-Franco, Robert Huber, Michael Ehrmann & Tim Clausen

Nature **416**, 455–459 (2002).

In this Letter, the Protein Data Bank entry code for the DegP S210A crystal structure is incorrectly listed as 1KJ9. It should be 1KY9. We thank C. Zardecki for bringing this to our attention. □