Cylindromatosis Tumor Suppresser Protein (CYLD) Deubiquitinase is Necessary for Proper Ubiquitination and Degradation of the Epidermal Growth Factor Receptor

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Cylindromatosis tumor suppressor protein (CYLD) is a deubiquitinase, best known as an essential negative regulator of the NFκB pathway. Previous studies have suggested an involvement of CYLD in epidermal growth factor (EGF)-dependent signal transduction as well, as it was found enriched within the tyrosine-phosphorylated complexes in cells stimulated with the growth factor. EGF receptor (EGFR) signaling participates in central cellular processes and its tight regulation, partly through ubiquitinination cascades, is decisive for a balanced cellular homeostasis. Here, using a combination of mass spectrometry-based quantitative proteomic approaches with biochemical and immunofluorescence strategies, we demonstrate the involvement of CYLD in the regulation of the ubiquitination events triggered by EGF. Our data show that CYLD regulates the magnitude of ubiquitination of several major effectors of the EGFR pathway by assisting the recruitment of the ubiquitin ligase Cbl-b to the activated EGFR complex. Notably, CYLD facilitates the interaction of EGFR with Cbl-b through its Tyr15 phosphorylation in response to EGF, which leads to fine-tuning of the receptor’s ubiquitination and subsequent degradation. This represents a previously uncharacterized strategy exerted by this deubiquitinase and tumor suppressor for the negative regulation of a tumorigenic signaling pathway. Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.066423, 1433–1446, 2017.

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Epidermal Growth Factor Receptor (EGFR) belongs to the family of receptor tyrosine kinases (RTKs) and plays a crucial role in the maintenance of a correct cellular homeostasis, controlling central processes such as cell proliferation, migration, differentiation or survival (reviewed in (1)). EGFR consists of an extracellular domain for the recognition of the ligands, a single pass transmembrane region and an intracellular tyrosine kinase-containing domain. The binding of a ligand, such as Epidermal Growth Factor (EGF), promotes the dimerization of the receptor and the subsequent activation of its kinase activity, which further leads to the autophosphorylation of tyrosine residues on the intracellular region of the EGFR (1, 2). These modified residues act as docking sites for recruiting SH2 or PTB domain-containing signaling proteins (3, 4), hence the stimulation triggers the association of large intracellular complexes that support rapid spread and amplification of the signal, eventually resulting in a specific cellular output (5).

To facilitate an adequate response in intensity and duration, the downstream events following the activation of the receptor necessitate tight negative regulation that counteracts the positive signals. In this regard, the attachment of ubiquitin moieties to the EGFR plays a key role in directing its internalization and further endocytic trafficking that eventually leads to its lysosomal degradation or recycling (6, 7). Hence, the regulated turnover of EGFR is pivotal for a correct cellular output. E3 ubiquitin ligases of the casitas B-lineage lymphoma (Cbl) family play a crucial part in this chain of events. Cbl proteins are recruited to activated receptors, either binding directly to their phosphorylated tyrosine residues or through the assistance of other adaptor proteins (8), thereby

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ubiquitinating the EGFR, which can in turn be recognized and sorted by the endocytic machinery (9, 10). In this context, further level of signaling modulation can be reached by ubiquitin hydrolases (deubiquitinases, DUBs), which may oppose the activity of Cbl ligases by removing ubiquitin moieties from the receptor (11, 12). These latter enzymes can thereby have a decisive role for the final cellular response.

Cylindromatosis protein (CYLD) is a tumor suppressor that displays a specific ubiquitin hydrolase activity for K63-linked polyubiquitin chains (13). Negative regulatory actions of CYLD have been reported for several signaling paradigms, the most explored to date being its role in the NF-kB pathway (14–16). CYLD Contributes to Ubiquitination and Degradation of EGFR displays a specific ubiquitin hydrolase activity for K63-linked polyubiquitin chains (13). Negative regulatory actions of CYLD in the control of essential cellular processes as apoptosis (14–16), inflammation (17), proliferation (18) and tumorigenesis (19). The downregulation of this DUB has been linked with oncogenesis in different cellular contexts including melanoma (20), myeloma (21), uterine cervix carcinoma (22), hepatocellular and colon carcinoma (23).

To our knowledge, the investigation of CYLD and its spectrum of actions have mostly been carried out through targeted approaches that explored, one at a time, the different regulatory strategies of this DUB on specific, discrete targets. However, advances in quantitative mass spectrometry (MS)-based proteomics allow studies of signaling cascades in a system-wide, unbiased manner for uncovering novel molecular characters with a key part in the transduction mechanisms. Currently, MS-based quantitative proteomics represents a powerful tool for the comprehensive characterization of signaling networks (24, 25), including their dynamics changes (26–28), protein interactions (29, 30) and numerous post-translational modifications (31). Indeed, the first evidences pointing to an involvement of CYLD in the molecular events downstream the EGFR were obtained by MS-based proteomics (32).

Following addition of EGF, the presence of CYLD among the tyrosine-phosphorylated complexes showed a very rapid and strong increase, which could indicate a role for this deubiquitinase in the RTK signaling and make CYLD-related ubiquitination events in EGFR pathway worth investigating.

Here, we conducted a global analysis of the cellular ubiquitinome by employing a recently described approach termed StUbEx (Stable Tagged Ubiquitin Exchange) followed by MS-based quantitative proteomics (33). This, in combination with protein and peptide pull-down assays, uncovered an unexpected role for CYLD as a key factor for proper ubiquitination of the EGFR and downstream signaling components. Namely, ubiquitination state of the activated receptor is compromised in the absence of CYLD, which results in a decreased receptor degradation. We demonstrate herein that CYLD interacts with the ubiquitin ligase Cbl-b, contributing to the recruitment of the later to the EGFR in response to EGF stimulation. Our findings provide a yet uncharacterized and intriguing strategy of CYLD for the negative modulation of signaling pathways, hence reinforcing and expanding the mechanisms underlying its role as a tumor suppressor.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human cervix epithelial adenocarcinoma HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, and 1% L-Glutamine. Ovarian and laryngeal carcinoma cells (Skov3 and Hep2, respectively) were cultured using the same medium. For labeling experiments, cells were grown in light, medium or heavy DMEM media, containing either L-arginine (Arg0) and L-lysine (Lys0), L-arginine-15N2 14C0 (Arg6) and L-lysine-15N2 (Lys4) or L-arginine-15N2 13C0 14N0 (Arg10) and L-lysine-15N2 13C0 15N0 (Lys8), respectively, as previously described (27). For EGF stimulation, cells were grown to a confluency of 70%, starved without serum for 16 h and stimulated with 150 ng/ml of EGF for the indicated times. When using inhibitors, those were added to the starving medium 30 min prior the stimulation and present while treating with the growth factor. EGFR inhibitors (Iressa and CI1033) and Akt inhibitor MK2206 were purchased from Selleckchem (Houston, TX) whereas MEK inhibitor U0126 was from Promega (Madison, WI). Where indicated, 10 μg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO) was used to block the de novo protein synthesis.

**RNAi-based Silencing and Rescue Expression of Wild Type and Y15F Mutant of CYLD**—RNAi constructs for RNAi silencing of CYLD and generation of stable cell lines were performed using a lentiviral system as described before (33). We used the following targeting sequences for RNAi: 5’-GCAATATGACGAGTTAGTA-3’ for shControl and 5’-GGTGGAGACCTCTGGTAA-3’ for shCYLD. Depletion of CYLD was confirmed by real-time PCR and immunoblotting (see Supplemental Data for details). A sequence-verified CDNA clone of CYLD was acquired from I.M.A.G.E. consortium (IRAU969D0565D). RNAi-resistant CDNA of CYLD was created by mutating 8 nucleotides (indicated in capitals below) in the sequence targeted by the shRNA, but maintaining the amino acid code. The following primer was used for this purpose: 5’-ttaaagagtcaagagctcagtGGTGTGAAAATGTTTAAAG-3’. Using methods of conventional cloning, the RNAi-resistant cDNA was then sub-cloned into the shCYLD vector with the Flag-epitope sequence at the N terminus and under control of EF1a promoter (construct named shCYLD-Flag-WT-CYLD). A mutant expressing CYLD with tyrosine exchanged with phenylalanine at position 15 was created by conventional methods of site-directed DNA mutagenesis (construct named shCYLD-Flag-Y15F-CYLD). HeLa cells with CYLD KD (shCYLD) were used to create clones expressing shCYLD-Flag-WT-CYLD and shCYLD-Flag-Y15F-CYLD constructs with the lentiviral delivery method as described above.

**Immunoblotting, Immunoprecipitation, and Immunofluorescence**—Antibodies used for immunoblotting were from Santa Cruz Biotechnology (Dallas, TX) (CYLD, Cbl-b), BD Transduction Laboratories (San Jose, CA) (c-Cbl), Millipore (Billerica, MA) (EGFR), Enzo Life Sciences (Farmingdale, NY) (Ubiquitin), Sigma (α-Tubulin), or Cell Signaling Technology (Danvers, MA) (phospho-Akt Ser473, Akt, phosphor-Erk1/2 Thr202/Tyr204, Erk).

For immunoprecipitation experiments, proteins were extracted in ice-cold modified RIPA buffer (50 mm Tris/HCl pH 8.0, 150 mm NaCl, 1% (v/v) Nonidet P-40, 0.25% Sodium Deoxycholate) supplemented with proteases and phosphatases inhibitors (both from Roche). The antibodies used for immunoprecipitation of phosphorylated-tyrosine containing complexes were from Millipore (clone 4G10) and from Cell Signaling Technology (P-Tyr-100). Antibodies sc-120 and 06-847, from Santa Cruz Biotechnology and Millipore respectively, were used for the immunoprecipitation of the EGFR. CYLD and Cbl-b proteins were immunoprecipitated using the antibodies sc-137139, sc-1704.
and sc-8006, from Santa Cruz Biotechnology. For control immunoprecipitations, an antibody against the GFP protein (sc-9996, Santa Cruz Biotechnology) was used. For immunoprecipitation of the Flag-tagged WT and Y15F CYLD, lysates were prepared in RIPA buffer, further incubated for 30 min with 1% SDS to disrupt protein-protein interactions and then diluted to 0.05% SDS with RIPA buffer to perform the immunoprecipitation using Anti-Flag M2 Affinity Agarose Gel (Sigma-Aldrich).

For immunofluorescence, HeLa cells were transfected with GFP-Rab7 plasmid and lipoemfectase as described previously (34). Antibody sc-120 from Santa Cruz was used for detection of EGFR (see supplemental Data for details).

StUbEx and Peptide Pull-down Analyses—For the enrichment of ubiquitinated proteins, shControl and shCYLD HeLa cells containing the StUbEx construct (33) were grown in the presence of doxycycline 60 h prior the experiment. Cells were subjected to serum starvation and stimulated with 150 ng/ml of EGF as indicated. To study the four conditions of interest, two parallel triple SILAC experiments were performed per replica (we carried out two biological replicas, thus corresponding to a total of four triple SILAC experiments; the experimental design of one replica is exemplified in Fig. 2A). Cells were lysed in a 50 mM phosphate buffer pH 8.0 containing 6 mM Guanidine-HCl and 500 mM NaCl. The subsequent enrichment of ubiquitinated proteins was performed as described previously (33).

For immunoprecipitation and peptide pull-down of CYLD, cells were lysed in ice-cold modified RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.25% Sodium Deoxycholate) containing proteases and phosphatases inhibitors (both from Roche). Lysates were cleared and protein extracts from the differently labeled conditions were quantified. CYLD antibody (Santa Cruz Biotechnology) coupled to protein A Sepharose beads was used for the immunoprecipitation. The peptide pull-down experiments were performed as described (35), using the following sequences: SGSQEKVTSP-pY-WEERIF and the unmodified counterpart SGSQEKVTSPYWEERIF. An empty-beads pull-down was carried out in the light condition to discriminate unspecific background proteins (see supplemental Data for details).

NanoLC Tandem Mass Spectrometry (LC-MS/MS) and Data Analysis—Peptides were separated by reversed-phase in an EASY-nLC 1000 (Thermo Scientific) coupled to a mass spectrometer equipped with a nanoelectrospray ion source. The samples were analyzed in either an LTQ Orbitrap Velos or a Q Exactive (Thermo Scientific). For the chromatographic separation, solvent A was 0.5% acetic acid, solvent B was 80% acetonitrile in 0.5% acetic acid. The peptides were eluted from an analytical in-house packed column of ReproSil Pur C18AQ, 3 μm resin (Dr Maisch GmbH), at a flow rate of 250 nL/min. The mass spectrometers were operated in positive ionization mode, in a top 12 data-dependent manner, at a resolution of 70,000 (at m/z 400) and an AGC target of 1e6 for the MS survey, scanning from 300 to 1750 m/z. For the MS/MS analysis, resolution was set to 150–200ms (37, 38). Fragmentation was performed in HCD mode with normalized collision energy of 28. PRM data analysis was carried out on Skyline™ 3.6.0 software (39). Spectral libraries were built using MaxQuant ms/ms search files. Resolving power and mass analyzer were set respectively to 60,000 and Orbitrap. Area under the curve (AUC) values relative to the 4–6 most intense product ions for each peptide were exported and plotted in a matrix which was used for further statistical analysis (supplemental Table S8). Total MS2 fragments AUC values for EGFR recycling (3 biological replicas) and Cbl-b Co-IP (2 biological replicas) analysis were normalized using the housekeeping protein Filamin-A or the Co-IP antigen Cbl-b, respectively. All the Skyline data, MaxQuant searches, and .raw files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository.

Experimental Design and Statistical Rationale—Four triple SILAC experiments corresponding to two full biological replicas were performed for the described StUbEx experiments. Two biological replicates were performed for peptide pull-down experiments per experiment, swapping medium and heavy SILAC conditions to avoid any technical bias. A control condition, in which the light SILAC protein extract was incubated with empty beads, was included in both pull-down experiments, helping to discriminate background and contaminant signals. In the case of CYLD immunoprecipitation, two biological replicates we performed as well.

The visualization of overlapping protein identifications was performed using the BioVenn web application (http://www.cmbi.ru.nl/ cdd/biovenn/), whereas the significance of the changes in SILAC protein ratios were based on significance B calculations inbuilt in the MaxQuant/Perseus software and visualized with GOProX (40). The analysis of functional groups and GO term categories were carried out in MetaCore (Thomson Reuters), which relies on Fisher statistical test with an FDR adjusted p values.

RESULTS

CYLD is Enriched in Early Tyr-phosphorylated Complexes Upon EGF Stimulation—EGFR pathway drives central cellular processes and, hence, its fine-tuned regulation is crucial for the maintenance of a correct cellular homeostasis. Therefore, the characterization of the molecular events downstream this RTK constitutes a powerful strategy for understanding both healthy and pathological mechanisms of the cell. In previous
proteomics studies aiming at gaining insights into the molecular players involved in the EGF-induced response, we revealed a very strong increment of the deubiquitinase CYLD within the tyrosine-phosphorylated complexes formed upon EGF stimulation (32). To corroborate this finding, we performed a protein immunoprecipitation using antibodies against phosphorylated tyrosine residues followed by immunoblotting, thus confirming the involvement of CYLD within the complexes engaged in response to EGF. The presence of this DUB was very rapidly increased, being evident just 1 min after the addition of the ligand and declining at 15 min post-stimulation (Fig. 1A). Interestingly, this dynamic enrichment profile parallels that of the EGFR itself, which is nevertheless stronger. Furthermore, the signal corresponding to the enriched CYLD after the stimulation appeared to represent only a small proportion of the total amount of this protein expressed in the cell (input lysate, Fig. 1A, first lane on the right), indicating that merely a slight fraction of this deubiquitinase is engaged in the process.

To assess whether the enrichment of CYLD is dependent on EGFR activation, we performed the previous experiments in the presence of Canertinib (CI1033) or Gefinitib (Iressa), inhibitors of the EGFR kinase activity. CYLD enrichment was abrogated when the activity of the receptor was abolished (Fig. 1B) but not when inhibiting the two main signaling branches downstream of the EGFR: the MAPK and the PI3K-Akt pathways (supplemental Fig. S1A and S1B, respectively). These results, together with the very rapid enrichment of CYLD after the stimulation, indicated that the deubiquitinase could play a role in the very early signaling processes after EGFR activation, independently of the subsequent feedback events.

**CYLD Deficiency Impairs the Ubiquitination of Early Protein Effectors of the EGF Pathway**—Taking into account the ubiquitin hydrolase activity of CYLD and that many of the proteins in the EGFR signaling network undergo ubiquitination, including K63 polyubiquitination (41–43), we reasoned that CYLD could be responsible for deubiquitinating EGFR signaling pro-
TEINS following ligand stimulation. To examine that, we silenced the expression of CYLD, achieving at least 80% decrease at the mRNA and protein levels (Fig. 1C) and efficiently depleted CYLD from the EGF-dependent tyrosine-phosphorylated complexes (as shown in supplemental Fig. S1C). We next explored the impact of the silencing on the ubiquitination status of the proteins involved in the EGF pathway. Surprisingly, we found that the downregulation of the deubiquitinase did not lead to an increase, but rather diminished the overall ubiquitination signal of the engaged proteins after EGF addition (Fig. 1D). To gain deeper insight into this intriguing observation, we resolved to explore the EGF-induced ubiquitination events upon CYLD downregulation in a system-wide, unbiased manner.

We used quantitative MS-based proteomics coupled to StUbEx, a strategy that has proven successful for large-scale investigation of ubiquitination events (33). Briefly, in the StUbEx system the endogenous ubiquitin is replaced by a dual tagged version containing a tandem 6xHis sequence and a Flag tag, whereas the total pool of ubiquitin in the cell remains at physiological levels. Ubiquitinated proteins can then be enriched through a two steps procedure, making use of the 6xHis and Flag tags contained within their ubiquitin moieties (33).

We combined StUbEx with shRNA silencing of CYLD and stable isotope labeling by amino acids in cell culture (SILAC) (44) to provide a quantitative proteomic view of the changes in protein ubiquitination upon the downregulation of the DUB (Fig. 2A). We performed 4 triple SILAC experiments corresponding to two biological replicates (each replica consisted of two triple SILAC experiments; Fig. 2A) and consistently quantified 1812 protein groups across them (FDR<0.01; Fig. 2B and supplemental Table S1). Only these proteins quantified in all four SILAC experiments were used for the subsequent analysis. Considering that the ubiquitinated proteome is of relatively low abundance in the cell (45), the obtained extent of quantified proteins was rather satisfactory. In addition, the quantitation reproducibility of the measurements was very high, with Pearson correlation values between the conditions common to all 4 SILAC experiments (medium over light) ranging between 0.92 and 0.96 (supplemental Fig. S2A and supplemental Table S1). Gene ontology (GO) analysis of the identified proteins demonstrates that there is no bias of the technique for any molecular network or pathway (supplemental Fig. S2B and supplemental Table S2), as their associated biological processes were mostly metabolic and organizational, representing the general functions of the cell. Moreover, the enriched proteins originated from different sub-cellular locations, covering from cytosol to nuclear and membrane organelles (supplemental Fig. S2C and supplemental Table S2), hence displaying no bias for a particular subcellular fraction.

To identify potential regulatory effects of CYLD in the molecular characters of the EGF pathway, we focused our analysis on proteins whose ubiquitination status significantly changed in response to the growth factor in the control cells and compared their corresponding enrichment to the CYLD-silenced condition. Applying very stringent criteria (significance B, p<0.001 for each replica), we detected a set of 11 protein groups with consistently increased EGF-dependent ubiquitination in control cells (Fig. 2C, supplemental Fig. S3A, S3B and supplemental Table S3), in agreement with previous studies on the EGF-dependent ubiquitinome (33, 43). Accordingly, the GO-term annotations for these 11 proteins in respect to function and localization were related to the EGFR/ErbB signaling pathways and the corresponding membrane structures and complexes (supplemental Fig. S3C, S3D and supplemental Table S4). Interestingly, all 11 proteins displayed much lower levels of enrichment in response to EGF from the CYLD-silenced cells (shCYLD) compared with the cells with normal CYLD expression (shControl) (Fig. 2C). To visualize more clearly the effect of CYLD downregulation on the ubiquitination status of these molecular effectors, we normalized the SILAC ratios from the different conditions to the shControl-unstimulated cells (Fig. 2D and supplemental Fig. S3E). The impact of CYLD deficiency on the EGF-induced ubiquitination changes appeared to be the most significant (significance B, p<0.001 in each replica) for EGFR, Cbl-b, UBASH3B and SHC1 (supplemental Table S5).

In summary, the unbiased investigation of the ubiquitination signaling through StUbEx and quantitative proteomics allowed us to decipher the effect of CYLD silencing in the ubiquitination events following EGF stimulation. Notably, the results suggested an indirect role of CYLD in the RTK pathway, as the downregulation of this DUB triggered an unexpected decrease in the EGF-dependent ubiquitination state of several major molecular characters of the pathway including the EGFR, Cbl-b, UBASH3B, and SHC1.

**CYLD is Phosphorylated on Tyr15 upon EGF Stimulation**—A great part of the signaling cascades subsequent to the RTKs activation rely on protein-protein interactions mediated through the phosphorylation of tyrosine residues and proteins containing PTB or SH2 domains (46, 47). Our previous results demonstrated the involvement of CYLD within the phospho-tyrosine protein complexes following EGF stimulation (Fig. 1A). However, this DUB does not contain any PTB or SH2 domains and, hence, we hypothesized that its EGF-dependent enrichment was caused by its own tyrosine phosphorylation. To examine that, we used a SILAC-based approach coupled to immunoprecipitation of CYLD from wild type (WT) HeLa cells that were either left unstimulated or treated with EGF for 6 min (Fig. 3A). To discriminate potential background signals, shCYLD cells were utilized for the light (Lys0/Arg0) condition in our experimental design. The SILAC ratios corresponding to the CYLD protein were more than 20-fold higher in the WT over CYLD-silenced cells, correlating well with our earlier estimation of ~80% silencing efficiency, whereas the enrichment of CYLD in the WT cells 6'EGF/unstimulated remained unchanged as expected (Fig. 3B and supplemental Table S6). Several serine-phosphorylated peptides derived from CYLD

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**A** Experimental workflow. SILAC-labeled shControl and shCYLD HeLa cells expressing 6xHis-Flag-tagged ubiquitin instead of endogenous ubiquitin were stimulated with EGF for 6 min, where indicated. Ubiquitinated proteins were enriched by sequential purification using nickel-affinity chromatography and anti-Flag antibodies, digested in-solution and analyzed by LC-MS/MS. Two biological replicas were performed for each of the indicated SILAC experiments (each biological replica corresponding to two paralleled triple SILAC experiments). B, Overlap of the identified and quantified proteins between the two biological replicas (left) and correlation of SILAC values between the channels common to all experiments (medium over light). See also supplemental Fig. S2. C, The group of 11 proteins with most significant ubiquitination changes in response to growth factor stimulation and the comparison of their corresponding EGF/Unstimulated ratios in control and CYLD-silenced cells. D, Ubiquitination status of the 11 proteins from panel C in the shControl and shCYLD cells, relative to their basal levels in the unstimulated shControl cells. See also supplemental Fig. S3.

**Fig. 2.** SILAC-based quantitative proteomics analysis of ubiquitinated proteins in control and CYLD-silenced cells using the StUbEx system. A, Experimental workflow. SILAC-labeled shControl and shCYLD HeLa cells expressing 6xHis-Flag-tagged ubiquitin instead of endogenous ubiquitin were stimulated with EGF for 6 min, where indicated. Ubiquitinated proteins were enriched by sequential purification using nickel-affinity chromatography and anti-Flag antibodies, digested in-solution and analyzed by LC-MS/MS. Two biological replicas were performed for each of the indicated SILAC experiments (each biological replica corresponding to two paralleled triple SILAC experiments). B, Overlap of the identified and quantified proteins between the two biological replicas (left) and correlation of SILAC values between the channels common to all experiments (medium over light). See also supplemental Fig. S2. C, The group of 11 proteins with most significant ubiquitination changes in response to growth factor stimulation and the comparison of their corresponding EGF/Unstimulated ratios in control and CYLD-silenced cells. D, Ubiquitination status of the 11 proteins from panel C in the shControl and shCYLD cells, relative to their basal levels in the unstimulated shControl cells. See also supplemental Fig. S3.
were detected in this experiment, however their phosphorylation status was unaffected by the EGF stimulation (supplemental Table S6). On the other hand, one of the identified modified peptides contained a tyrosine phosphorylated site, corresponding to the residue 15 (Fig. 3C). Manual inspection of the mass spectrometry signal showed a large increase of its abundance at the EGF-stimulated condition (Fig. 3D). Although the intensities of the pY15-containing peptide in the light and medium channels (corresponding to shCYLD cells and WT unstimulated cells, respectively) were not sufficiently above the signal-to-noise level to allow calculation of SILAC ratios, the profile of the corresponding signals evidenced a strong increment of at least 10-fold of the phosphorylated form in the EGF stimulated WT cells. To estimate the fraction of CYLD that responded to the growth factor treatment, we calculated the stoichiometry of pY15 according to the SILAC ratios obtained from the corresponding unmodified peptide and the protein, as described elsewhere (48). Assuming a pY15 6'EGF/unstimulated ratio of at least 10, our results suggested that, although at the basal state there is practically no presence of this modified site, the EGF stimulation promoted Tyr15 phosphorylation on ~4% or less of the total pool of CYLD in the cells (Fig. 3E). This data, together with our previous results (Fig. 1A), indicated again that only a small fraction of the total cellular pool of this DUB is involved in the EGF signaling pathway. Accordingly, CYLD appears to be ubiquitously present in the cell and has been described in several subcellular locations (49, 50), implying that it can therefore play distinct roles dependent on its molecular environment.

Cbl-b is Recruited to the pY15 of CYLD—Taking into account that phosphorylated tyrosine residues often act as docking sites for the interaction with SH2- or PTB domain-containing proteins (47), we next investigated a potential ability of CYLD pY15 to recruit molecular effectors belonging to the EGF pathway. We implemented a quantitative proteomics approach based on an affinity pull-down strategy using as bait the TYR15-containing peptide in phosphorylated or unmodified form (Fig. 4A). Two replicates were performed, swapping the medium and heavy SILAC conditions in order to avoid any technical bias of the method, and we quantified a...
total of 1451 protein groups in both replicas (Fig. 4B and supplemental Table S7). CHST3, UBASH3B and Cbl-b displayed the most selective binding to the phosphorylated Tyr15-containing peptide with more than 5-fold enrichment over the non-phosphorylated version of the peptide (Significance B, p < 0.001; Fig. 4C, 4D and supplemental Table S7). Notably, UBASH3B and Cbl-b are well known molecular characters involved in the ubiquitination-dependent EGFR signaling and both were already identified as proteins with a decreased EGF-dependent ubiquitination upon CYLD deficiency (Fig. 2). The presence of Cbl-b was of interest, as it contains a phospho-tyrosine binding domain and also represents a E3 ligase responsible for the ubiquitination of several effectors of the EGF-dependent signaling cascades, including the EGFR (51). Considering that the silencing of CYLD showed an unexpected impairment of the ubiquitination downstream the growth factor stimulation and that this DUB appears to recruit Cbl-b, we hypothesized that CYLD could be involved in the regulation of the EGF ubiquitination through its binding with Cbl-b. Notably, Cbl ligases have already been described not only to interact directly with the receptor (52, 53) but also to be recruited by accessory adapter proteins to the activated complexes following EGF stimulation (54). Here, our data provided cues suggesting such a role for the CYLD deubiquitinase as well.

**Downregulation of CYLD Impairs EGFR Ubiquitination Through a Deficient Recruitment of Cbl-b to the Receptor**—To corroborate the involvement of CYLD in the events described above, we first immunoprecipitated EGFR from control and CYLD silenced cells, followed by immunoblotting. In agreement with our quantitative proteomics analysis (Fig. 2), we observed a decrease of growth factor-dependent ubiquitination of the receptor in CYLD deficient HeLa cells (Fig. 5A).
further seen on Fig. 5A, the interaction of EGFR and Cbl-b was clearly impaired in CYLD-silenced cells, consolidating the notion that CYLD is in part responsible for the recruitment of this E3 ligase to the receptor. Accordingly, this also resulted in decreased levels of Cbl-b in the tyrosine-phosphorylated complexes upon EGF stimulation (Fig. 5B). In contrast, the engagement of the closely related E3 ligase c-Cbl, which is also strongly involved in EGFR signaling (53), was unaffected by the silencing of CYLD (Fig. 5B). Therefore, the impact of the DUB on the EGF-dependent ubiquitination events appears to be driven specifically through Cbl-b and not c-Cbl.

To further explore a potential interaction of CYLD, Cbl-b and EGFR we used series of co-immunoprecipitation experiments, which indeed demonstrate the existence of an endogenous complex among these proteins. The interactions of CYLD with Cbl-b and CYLD with EGFR appeared to occur already at basal levels in unstimulated cells and was further strengthened by the addition of EGF (Fig. 5C, 5D), whereas EGFR and Cbl-b did not interact in the absence of ligand (Fig. 5A). Furthermore, reduced amounts of EGFR were seen in the Cbl-b immunoprecipitated complexes from the CYLD-silenced cells (Fig. 5C), in correlation with the results from the EGFR co-immunoprecipitation experiments shown in Fig. 5A. Consistent with the proposed role of CYLD in assisting the interaction between the receptor and Cbl-b, we also observed enrichment of CYLD in the EGFR-immunoprecipitated complexes upon ligand stimulation (Fig. 5B). To further confirm these results and exclude potential cell type-specific effects, we performed equivalent experiments in two additional cell lines. We corroborated the interaction of CYLD with Cbl-b and its contribution to the ubiquitination of EGFR in ovarian and laryngeal carcinoma cells (Skov3 and Hep2, respectively), hence demonstrating the generic nature of this unexpected role of CYLD (Fig. 5E).

CYLD Deficiency Results in Deregulated Trafficking and Degradation of EGFR—The ubiquitination-dependent signaling events are crucial for proper endocytosis and intracellular trafficking of EGFR (42) and its subsequent lysosomal
degradation or recycling. Ubiquitin moieties are recognized by adaptor proteins containing ubiquitin-binding domains (UBDs) that sort the modified receptors through the endocytic pathway (55). Consequently, an altered ubiquitination of the EGFR can promote a de-regulated intracellular trafficking of activated receptors. The activities of the Cbl proteins constitute an essential pillar for the negative regulation of RTKs pathways and defective binding of these E3 ligases with the EGFR promotes a compromised ubiquitination and degradation of the receptor (56). Considering the effects of CYLD silencing on the EGFR ubiquitination, we reasoned that this DUB could accordingly impact the trafficking of the receptor following ligand stimulation.

To explore this option, we transfected HeLa cells with GFP-Rab7, a marker for late endosomes, prior to their stimulation with EGF at different time points. As seen in Fig. 6A, the EGFR was internalized and displayed partial co-localization with Rab7 both in the control and in the CYLD-silenced cells. Nevertheless, the trafficking pattern of the receptor throughout the cells differed noticeably. Although the control cells exhibited EGFR distribution in fewer discrete foci, the shCYLD cells presented a more scattered image of the receptor that spread over the cells (Fig. 6A; 30 and 60 min after EGF addition). We also noticed much stronger EGFR signal in CYLD-silenced cells at 2 h poststimulation, which could indicate a decreased degradation of the receptor (Fig. 6B).

We next monitored EGFR cellular levels by immunoblotting at different times after ligand stimulation, in combination with cycloheximide treatment to prevent contribution from the de novo protein synthesis. We observed that the amounts of the receptor after 60 and 120 min of EGF stimulation were indeed higher in the CYLD deficient cells (Fig. 6C). In addition, this effect was not because of an overall increased expression of the EGFR in the shCYLD cells. As seen by quantitative PCR

*Fig. 6. Impaired EGFR trafficking and degradation in CYLD-silenced cells. A and B, Immunofluorescence images of EGFR (red) in shControl and shCYLD HeLa cells expressing GFP-tagged Rab7 (green) and stimulated with EGF for indicated times. DAPI staining was used to visualize cellular nuclei (blue). Three independent images from the 2 h time point are shown in panel B, where the GFP-Rab7 (green) channel is not shown for better comparison of the EGFR (red) signals. C, Immunoblotting of EGFR on total lysates from shControl and shCYLD cells pre-treated with cycloheximide and stimulated with EGF for indicated times. Three different exposures of the image are shown. D, The mRNA levels of EGFR in shControl and shCYLD cells assessed by qPCR.*
analyses, the EGFR mRNA levels were lowered when the DUB is silenced (Fig. 6D). Altogether, these data indicate a specific stabilization of EGFR through a post-translational mechanism in CYLD-silenced cells, thus in agreement with our proposed role of CYLD in the regulation of receptor ubiquitination, important for its subsequent trafficking and degradation.

**CYLD PhosphoTyr15-deficient Mutant Mimics the Effects of CYLD Silencing**—To corroborate that the observed EGF-triggered effects upon CYLD silencing are indeed driven through the Tyr15 phosphorylation of the DUB, the following experiments were performed. Using RNAi-resistant constructs, we reconstituted in the CYLD-silenced HeLa cells either a wild type CYLD or a Y15F-CYLD mutant, in which tyrosine 15 was exchanged with phenylalanine, hence incapable of being phosphorylated at this position (Fig. 7A). The immunoprecipitation of the reconstituted CYLD proteins under stringent conditions demonstrated that the Y15F substitution results in a dramatic decrease of the tyrosine-phosphorylation of the DUB upon EGF stimulation (Fig. 7B), indicating Tyr15 as the major site of CYLD phosphorylation in response to EGF. Like the effects seen in the CYLD-silenced cells, we observed decreased ligand dependent ubiquitination of the EGFR in the cells reconstituted with either CYLD WT or CYLD Y15F mutant (Fig. 7C). To simultaneously measure to what extent the interaction of CYLD with Cbl-b is affected by the Y15F mutation and its consequence for the Cbl-b association with EGFR, we implemented MS procedure based on Parallel Reaction Monitoring (PRM) analysis coupled to immunoprecipitation of Cbl-b (supplemental...
Table S8). Compared with the CYLD WT cells, the association of the DUB with Cbl-b was strongly impaired in the CYLD-silenced cells as well as in the Y15F-CYLD expressing cells (Fig. 7D). Likewise, the recruitment of EGFR to Cbl-b was also diminished to more than 50% when the DUB was either downregulated or Y15F mutated (Fig. 7D). We next examined the levels of EGFR in the cells following 1 and 2 h treatment with the ligand and observed that the receptor is slightly stabilized in the Y15F-CYLD cells, like the CYLD-silenced cells (Fig. 7E). To obtain quantitative estimate of this observation we utilized PRM measurements, which also indicated a significant decrease in the degradation of EGFR when CYLD is either silenced in the cells or incapable of being phosphorylated on Tyr15 (Fig. 7F).

DISCUSSION

This study aimed to understand the engagement of the tumor suppressor CYLD deubiquitinase within the EGF-activated complexes and its impact on the ubiquitination events in the pathway. Here, we demonstrate that CYLD is necessary for proper EGF-dependent ubiquitination of several well-known effectors of the EGFRC pathway, including the receptor itself, UBASH3B and Cbl-b. We identified an EGF-dependent phosphorylation of Tyr15 on CYLD, a modification that created a docking site for the E3 ligase Cbl-b, thereby allowing the assembly of additional Cbl-b to the activated EGFR. Accordingly, the silencing of CYLD or the substitution of its Tyr15 with a phosphorylation-incapable residue promoted a decrease in the recruitment of the ligase to the receptor, triggering an impaired ubiquitination of EGFR, its posterior trafficking and degradation.

Although the function of CYLD in NFκB signaling is well explored (15, 16), its role in the RTKs signaling, particularly downstream of the EGFR, is less understood. Earlier work from our group initially pointed to a potential involvement of CYLD in the EGFR pathway (32). More recently, CYLD was revealed as a key character for integrating EGF and integrin-dependent signals for the formation of dorsal ruffles in fibroblasts (57). Interestingly, the crosstalk of these molecular networks also resulted in the tyrosine phosphorylation of CYLD, albeit through distinct molecular mechanisms compared with our current study. EGF stimulation and fibronectin-dependent activation of β1 integrin signaling were both required for the observed tyrosine phosphorylation of CYLD and although the exact sites of modification were not elucidated, it appeared not to involve Tyr15 (57).

In contrast, our study identified a specific role of CYLD, through its Tyr15 phosphorylation, in the molecular cascade downstream of EGFR. Of note, this site has previously been identified in a large-scale proteomic screen; nonetheless, the investigation of CYLD was out of the scope of the study and thus not explored (58). Here, we demonstrated that Cbl-b is recruited to the phosphorylated Tyr15 of CYLD and this interaction is necessary for the proper engagement of the ubiquitin ligase to the receptor upon EGF stimulation. Accordingly, we observed compromised recruitment of Cbl-b to the activated EGFR in CYLD-silenced or CYLD Y15F-expressing cells, which resulted in deficient ubiquitination and subsequent degradation of the receptor. It should be noted that previous research has shown that RNAi-based silencing of Cbl-b results in similar EGFR stabilization (59), and that the interaction of Cbl-b with EGFR as an important factor for the degradation of the activated receptor complexes (60).

Interestingly, the contribution of CYLD to the ubiquitination signaling downstream of this RTK appears to be driven specifically through the Cbl-b and not c-Cbl, as the later was not recruited to the CYLD pTyr15 and its engagement to the activated complexes was unaffected by the silencing of the DUB. These two closely-related proteins, despite exerting redundant functions, seem to be subjected to different regulatory mechanisms (51, 61, 62) and CYLD could indeed contribute to their differential modulation. In the context of EGF-dependent pathway, both Cbl-b and c-Cbl have been reported to directly interact with the activated EGFR through its phosphorylated tyrosine residues (51, 52). However, an additional association of the E3 ligases to the receptor could be facilitated by the Grb2 adaptor protein as well (54). Certainly, Grb2 deficiency promotes a reduced recruitment of c-Cbl to the EGFR with a subsequent impairment of its ubiquitination and trafficking (63). Grb2-mediated association of Cbl-b to EGFR is not explored, to our knowledge, being the interaction between Cbl-b and Grb2 only described in the context of TCR stimulation (64). It is possible that the recruitment of additional Cbl-b to activated EGFR is controlled by CYLD instead, similarly as Grb2 does for the related ligase c-Cbl. Future investigations on the importance of CYLD’s deubiquitinating activity in the process, the role of the basal association of CYLD with Cbl-b in the absence of EGF stimulation or the underlying mechanisms of differential recruitment of the two closely related ubiquitin ligases could provide further insights into the complex interplay that control the fine-tuned regulation of EGFR trafficking and degradation.

EGFR pathway represents a central molecular network in cellular physiology, controlling key processes such as proliferation and migration. To orchestrate adequate cellular responses, the EGF-dependent signaling cascades are subjected to a tight regulation that is frequently found altered in various human cancers. Understanding the molecular events controlling EGFR signaling constitutes the key for its management in case of alteration. Being best described as a tumor suppressor in the NFκB pathway, the role of CYLD in the EGFR axis remained however elusive. In the NFκB pathway, CYLD directly removes K63-polyubiquitin chains from key molecular effectors, such as TRAF2, TRAF6, and NEMO (15, 16). These K63-polyubiquitin chains facilitate protein-protein interactions that, hence, are inhibited by the action of CYLD, resulting in decreased signal transduction and diminished translocation of NFκB to the nucleus. We uncover herein an
additional molecular strategy utilized by CYLD for the negative regulation of another tumorigenic signaling network, namely the one controlled by EGFR. Here, CYLD appears to control the fine-tuned regulation of EGFR trafficking and degradation by assisting in the association of the E3 ubiquitin ligase Cbl-b with EGFR, thereby modulating the amplitude of ubiquitination on the receptor and several other major players in the EGFR network.

The unbiased proteomic surveys used in our work prove to be efficient for addressing complex regulatory mechanisms in a systematic manner, opening the line to a new understanding of the captivating and sometimes, like the case with CYLD, counterintuitive nature of the fine-tuned modulation of cellular signaling.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifiers PXD003423 and PXD006390.

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