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CYLD is a deubiquitinating enzyme that negatively regulates NF-κB activation by TNFR family members

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Familial cylindromatosis is an autosomal dominant predisposition to tumours of skin appendages called cylindromas. Familial cylindromatosis is caused by mutations in a gene encoding the CYLD protein of previously unknown function[1]. Here we show that CYLD is a deubiquitinating enzyme that negatively regulates activation of the transcription factor NF-κB by specific tumour-necrosis factor receptors (TNFRs). Loss of the deubiquitinating activity of CYLD correlates with tumorigenesis. CYLD inhibits activation of NF-κB by the TNFR family members CD40, XEDAR and EDAR in a manner that depends on the deubiquitinating activity of CYLD. Downregulation of CYLD by RNA-mediated interference augments both basal and CD40-mediated activation of NF-κB. The inhibition of NF-κB activation by CYLD is mediated, at least in part, by the deubiquitination and inactivation of TNFR-associated factor 2 (TRAF2) and, to a lesser extent, TRAF6. These results indicate that CYLD is a negative regulator of the cytokine-mediated activation of NF-κB that is required for appropriate cellular homeostasis of skin appendages.

Activation of NF-κB by cytokines depends on the activity of the IκB kinase (IKK) complex, which mediates phosphorylation of the NF-κB inhibitor IκB[2]. NEMO (also known as IKK-γ) is an essential component of the IKK complex. IKK-mediated phosphorylation of IκB causes its degradation by the ubiquitin–proteasome pathway and results in nuclear translocation and activation of NF-κB.

A yeast two-hybrid screen with NEMO as the bait identified complementary DNAs encoding either the alternatively spliced, 'full-length' CYLD of 953 amino acids or two truncated forms of the protein spanning amino acids 211–709 and 387–953. The interaction of CYLD with NEMO was specific, because full-length CYLD did not interact with two mutated forms of NEMO (C417R and D406V) that have an altered carboxy-terminal zinc-finger motif[3]. To determine whether this association occurs in mammalian cells, Flag-tagged CYLD and glutathione *S*-transferase (GST)-tagged NEMO were coexpressed in human embryonic kidney (HEK) 293T cells. Co-precipitation experiments indicated that CYLD and NEMO can indeed interact in mammalian cells after their coexpression (Fig. 1a, b). The amino-terminal region of CYLD mediates the interaction with NEMO, because deletion of the N-terminal 537 amino acids of CYLD abolished NEMO binding (Fig. 1c). We could not detect an interaction between endogenous CYLD and NEMO, either because of the possible transient nature of this interaction or because of the relatively low affinity of our antisera against CYLD.

The C-terminal 365 amino acids of CYLD contain motifs found in ubiquitin-specific proteases (UBPs), a subclass of deubiquitinating enzymes thought to be responsible for the removal of polyubiquitin chains from polypeptides[1,4]. Most pathogenic inactivating mutations of CYLD result in truncations or frameshift alterations of the C-terminal region of the molecule[1]. To determine whether CYLD can act as a UBP, Flag-tagged wild-type CYLD and three C-terminally truncated mutants of CYLD that are associated with familial cylindromatosis (encoding residues 1–932, 1–864 and 1–754 of CYLD; see Online Mendelian Inheritance in Man number 132700 at http://ncbi.nlm.gov/Omim/) were expressed in HEK 293T cells, immunoprecipitated and tested for their ability to cleave tetraubiquitin. Wild-type CYLD readily cleaved the tetrameric substrate to its monomer, dimer and trimer; however, the pathogenic mutations associated with familial cylindromatosis abolished the UBP activity of CYLD, indicating a correlation between tumorigenesis and loss of the deubiquitinating activity of CYLD (Fig. 1d, top). The deubiquitinating activity of CYLD did not require any other mammalian proteins, because it was detectable in bacteria (Supplementary Fig. 1). In addition, the deubiquitinating activity of CYLD was abolished by replacing the conserved catalytic residue Cys 601 with serine.

The interaction of CYLD with NEMO prompted us to investigate the potential involvement of CYLD in NF-κB activation. CD40, XEDAR and EDAR are members of the TNFR family implicated in the proper development of skin appendages from which cylindromas arise[3,5,6]. Expression of wild-type CYLD in HEK 293T cells inhibited the ability of coexpressed CD40 (Fig. 2a), XEDAR or EDAR (Supplementary Fig. 2) to activate the NF-κB pathway; by

contrast, the CYLD mutant associated with familial cylindromatosis that lacks 21 amino acids at its C terminus was markedly impaired in its ability to inhibit NF-κB activation by these receptors. CYLD(538–953), which is catalytically active but does not bind to NEMO, also failed to inhibit CD40-mediated activation of NF-κB, suggesting that the NF-κB inhibitory activity of CYLD is dependent on NEMO (Fig. 2b). In addition, wild-type CYLD inhibited activation of NF-κB induced by the Epstein-Barr virus oncoprotein LMP1, which mimics activated CD40 (ref. 7 and data not shown).

To test whether endogenous CYLD functions as an inhibitory factor of NF-κB, we used a short interfering RNA (siRNA) approach to reduce the expression of CYLD and determined the effects on the basal and CD40-ligand-induced activity of NF-κB. HEK 293T cells were transfected with an NF-κB-dependent luciferase reporter plasmid and either a human CYLD-specific siRNA (HCYLDsiRNA) or an unrelated siRNA (NSsiRNA). Transfection of HCYLDsiRNA resulted in a marked decrease both in endogenous CYLD messenger RNA (Supplementary Fig. 3a) and in the amount of overexpressed Flag-tagged CYLD protein (Supplementary Fig. 3b). Both the basal and the CD40-ligand-induced activity of NF-κB were significantly augmented on reduction of CYLD expression, reinforcing the notion that CYLD acts as a negative regulator of the NF-κB pathway (Fig. 2c).

To analyse the mechanism underlying the inhibition of TNFR-mediated NF-κB activation by CYLD, we studied the point in the signalling pathway at which CYLD interferes. TRAF2 and TRAF6 have been implicated in the activation of NF-κB by CD40, EDAR, XEDAR and LMP1 (refs 6, 8–13). TRAF2 and TRAF6 interact directly or indirectly with the cytoplasmic tail of the receptor. These proteins are activated by oligomerization, and this event can be induced either by their overexpression or by receptor activation. After receptor activation, the principal rate-limiting step of NF-κB induction is activation of the IKK complex, which can be also induced by overexpressing either catalytic subunit of IKK.

Wild-type CYLD inhibited TRAF2-mediated NF-κB activation markedly (by about 93%; Fig. 3a); however, the deubiquitinase-deficient mutants of CYLD (CYLD(1–932) and CYLD-C601S) were severely impaired in this activity (Fig. 3a and Supplementary Fig. 4). Wild-type CYLD had a weaker but still significant inhibitory effect (about 50% inhibition) on NF-κB activation by TRAF6, as compared with its effect on activation by TRAF2 (Fig. 3b). Notably, CYLD had no effect on IKKβ-mediated activation of NF-κB (Fig. 3c). These results indicate that the principal target of CYLD is located upstream of IKKβ. These findings are also consistent with

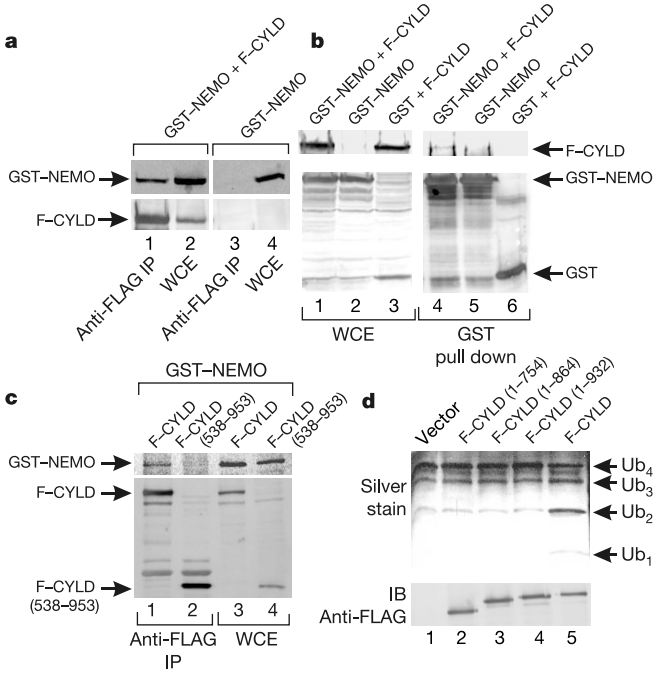

Figure 1 CYLD is a deubiquitinating enzyme that interacts with NEMO. **a**–**c**, HEK 293T cells were transfected with vectors expressing GST–NEMO or GST in the presence or absence of constructs expressing Flag-tagged (F–) CYLD or CYLD(538–953). Cell lysates were precleared and subjected to either immunoprecipitation with an antibody against Flag (anti-Flag IP) or a GST pull-down assay. Along with an aliquot of the whole-cell extract (WCE), the samples were subjected to immunoblotting with antisera against GST (**a** and **c**, top), the M5 monoclonal antibody against Flag (**a** and **c**, bottom) or antisera against GST and against CYLD (**b**). **d**, Flag-tagged full-length CYLD or truncations associated with familial cylindromatosis were immunoprecipitated with the M2 antibody against Flag from lysates of transfected HEK 293T cells and tested for their ability to cleave tetraubiquitin. The reaction mixture was analysed by tricine SDS–PAGE and silver staining (top). Arrows indicate the positions of tetrameric (Ub_4), trimeric (Ub_3), dimeric (Ub_2) and monomeric (Ub_1) ubiquitin. The amount of immunoprecipitated Flag-tagged proteins was determined by immunoblotting with the M5 antibody (bottom).

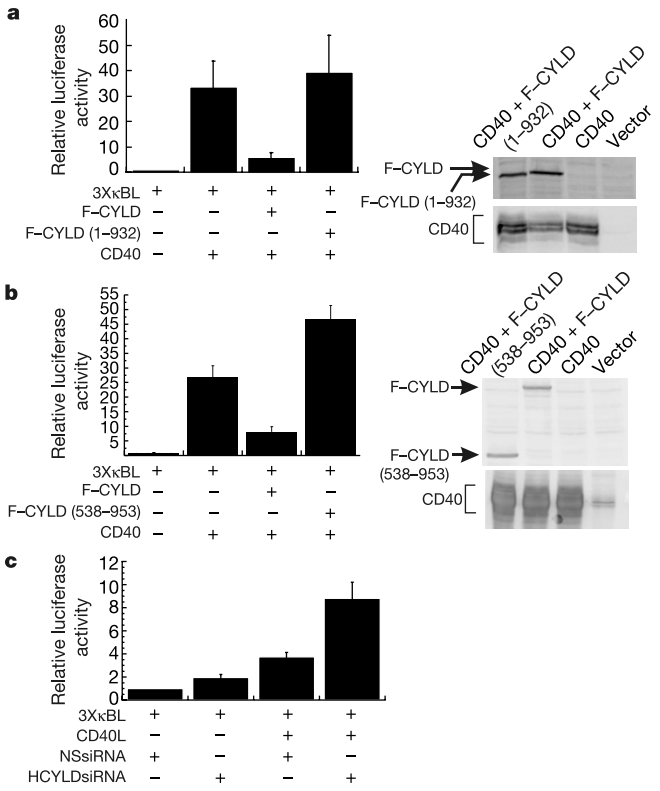

Figure 2 Effect of CYLD and truncated forms of CYLD on NF-κB activation by CD40. HEK 293T cells were transfected with the 3XκBL and the pGK–β-galactosidase reporter plasmids plus a CD40 expression vector in the presence or absence of vectors expressing Flag-tagged (F–) CYLD or CYLD(1–932) (**a**); a CD40 expression vector in the presence or absence of vectors expressing Flag-tagged CYLD or CYLD(538–953) (**b**); or the same amount of either a nonspecific siRNA (NSsiRNA) or a human CYLD-specific siRNA (HCYLDsiRNA) (**c**). Cell extracts were prepared either 24 h (**a**, **b**) or 48 h (**c**) after transfection, and their luciferase and β-galactosidase activities were determined. In **c**, the cells were left untreated or stimulated with CD40 ligand for 5 h before lysis. Results are the mean ± s.d. relative luciferase activity from three (**a**, **b**) or four (**c**) independent experiments. Representative immunoblots of whole-cell lysates from equal numbers of HEK 293T cells subjected to transfection in one representative experiment are shown on the right in **a** and **b**.

our observed cytoplasmic localization of CYLD tagged with either green fluorescent protein (GFP) or the Flag epitope in epithelial cells (data not shown).

The NF-κB-inducing activity of TRAF2 and TRAF6 depends on their RING-finger-dependent ubiquitin ligase activity and autoubiquitination[14,15]. The autoubiquitination of TRAF2 and TRAF6 depends on the activity of the ubiquitin-conjugating enzyme Ubc13. This enzyme mediates the formation of polyubiquitin chains linked through Lys 63, which do not mediate degradation of the polyubiquitinated protein[14–17]. A dominant-negative mutant of Ubc13 inhibited activation of NF-κB by CD40, XEDAR and EDAR, consistent with the idea that both Ubc13 and polyubiquitination through Lys 63 have a crucial role in the activation of NF-κB by these receptors (Supplementary Fig. 5a, b).

A possible mechanism for the inhibitory activity of CYLD on TNFR and TRAF-mediated NF-κB activation might be based on the CYLD-dependent deubiquitination of TRAF2 and/or TRAF6. We tested this idea by determining the extent of TRAF2 and TRAF6 autoubiquitination on expression of wild-type or UBP-deficient CYLD. Flag-tagged TRAF2 was coexpressed with haemaglutinin A (HA)-tagged ubiquitin in HEK 293T cells and immunoprecipitated under stringent conditions that would disrupt most protein–protein interactions. Polyubiquitination of TRAF2 was apparent by immunoblot analysis and was nearly abolished by the expression of a dominant-negative mutant of Ubc13, consistent with previous reports[14,16] of non-degradative Lys-63-linked polyubiquitination of TRAF2 under similar conditions (Fig. 4a and Supplementary Fig. 5).

Expression of wild-type CYLD reduced TRAF2 polyubiquitination to roughly background levels (Fig. 4a). By contrast, the CYLD(1–932) deubiquitinase-deficient mutant caused a small increase in TRAF2 polyubiquitination, consistent with a possible dominant-negative effect (Fig. 4a). Deubiquitination of TRAF2 by CYLD did not affect the steady-state quantities of protein (data not shown). In addition, the proteasome inhibitor ALLN did not affect the ubiquitination or steady-state amounts of TRAF2 under the conditions of our experiments (data not shown). These findings are consistent with the notion that CYLD targets the Lys-63-linked non-degradative polyubiquitination of TRAF2, which may be related to its ability to activate NF-κB[14]. The effect of CYLD on TRAF2 ubiquitination was specific. because expression of CYLD did not affect the overall extent of ubiquitination in the cell

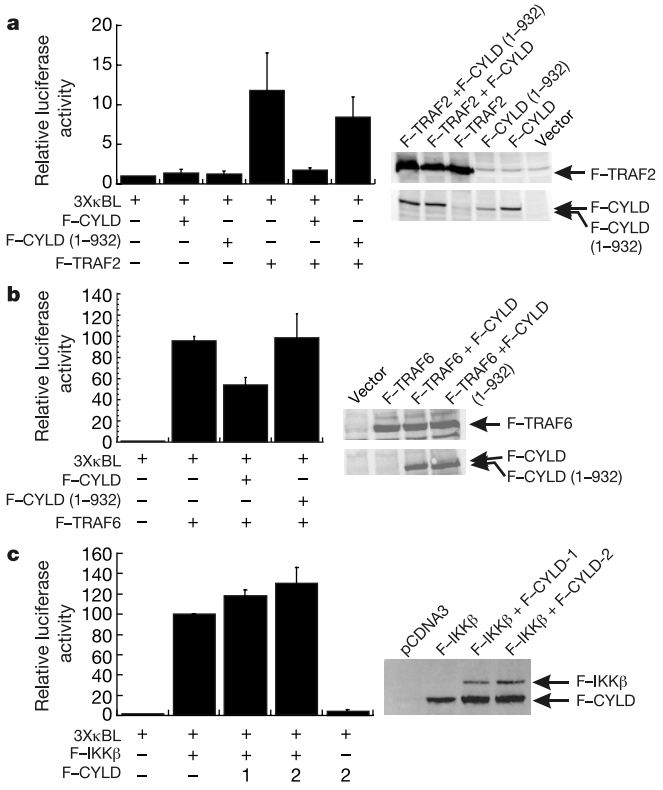

Figure 3 Effect of CYLD and CYLD(1–932) on NF-κB activation by TRAF2, TRAF6 and IKKβ. HEK 293T cells were transfected with the 3XκBL and the pGK–β-galactosidase reporter plasmids plus vectors expressing Flag-tagged (F–) TRAF2, CYLD or CYLD(1–932) (**a**); Flag-tagged TRAF6, CYLD or CYLD(1–932) (**b**); and Flag-tagged IKKβ and the indicated amounts (in μg) of the Flag-tagged CYLD expression vector (**c**). After 24 h, cell extracts were prepared and the luciferase and β-galactosidase activities were determined. Results are the mean ± s.d. relative luciferase activity from three independent experiments. Representative immunoblots of whole-cell lysates from an equal number of HEK 293T cells subjected to transfection are shown on the right.

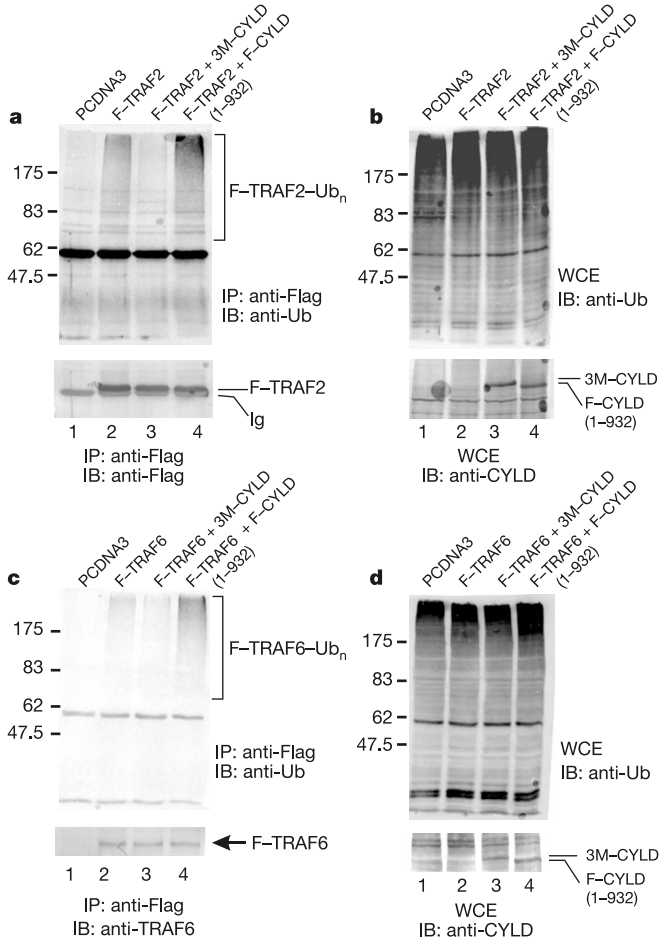

Figure 4 Effect of CYLD and CYLD(1–932) on TRAF2 and TRAF6 polyubiquitination. HEK 293T cells were transfected with expression vectors for HA-tagged ubiquitin, Flag-tagged (F–) TRAF2, TRAF6 and CYLD(1–932), and Myc-tagged CYLD (3M–CYLD). Proteins immunoprecipitated with the M2 antibody against Flag from lysates of transfected cells prepared 24 h after transfection were analysed by immunoblotting with antisera against ubiquitin (**a** and **c**, top) and then reprobed with the M2 antibody against Flag (**a**, bottom) or an antibody against TRAF6 (**c**, bottom). The positions of polyubiquitinated Flag-tagged TRAF2 (F–TRAF2-Ub$_n$) and TRAF6 (F–TRAF6-Ub$_n$), and M2 immunoglobulin (Ig) are shown. Whole-cell extracts (WCE) of the transfected cells were subjected to SDS–PAGE and immunoblotting with a monoclonal antibody against ubiquitin to determine the overall extent of ubiquitination (**b** and **d**, top). The same blots were reprobed with antisera against CYLD (**b** and **d**, bottom).

(Fig. 4b), nor did it reverse the E6-mediated degradation of p53 by ubiquitination (data not shown). Wild-type CYLD caused a weak but reproducible reduction in TRAF6 polyubiquitination (Fig. 4c), whereas CYLD(1–932) had a small positive effect (Fig. 4c). These experiments suggest that CYLD inhibits the NF-κB activation pathway, at least in part, by removing Lys-63-linked polyubiquitin from TRAF2 and TRAF6. In addition, CYLD might inhibit the NF-κB pathway by deubiquitinating IκB, although we do not have direct evidence to support this mechanism.

Our studies have identified a regulatory mechanism of the NF-κB pathway that relies on the deubiquitinating activity of CYLD. This protein mediates an inhibitory action on the NF-κB pathway by reversing the ubiquitination of TRAF2 and TRAF6, possibly through a transient interaction with NEMO. Binding of CYLD to NEMO probably enables CYLD to position itself on IKK holoenzyme that has been translocated onto the cytoplasmic tail of the activated TNFR, thereby allowing CYLD to deubiquitinate receptor-bound TRAF proteins[18–20]. CYLD might be activated by binding to the ubiquitin moiety of target proteins, as this has been reported for another deubiquitinase[21]. Our results indicate that increased or prolonged activation of NF-κB caused by a loss of CYLD is important in the generation and growth of cylindromas, possibly because of the inhibition of apoptosis[22,23]. Because of the localized and accessible nature of these tumours, inhibitors of the NF-κB pathway such as steroidal and nonsteroidal anti-inflammatory compounds are candidate therapeutic agents for cylindromas. □

Methods

Plasmid construction

Plasmids were obtained or constructed by standard methods and details are given in the Supplementary Information.

Yeast two-hybrid screen

A plasmid expressing a fusion of GAL4 DNA-binding domain to full-length human NEMO (pBridgeNEMO) was used as the bait plasmid. We carried out the yeast two-hybrid screen as described[24].

Cell lines, transfection assays and reporter assays

The HEK 293T cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (D10, Invitrogen) at 37 °C in 5% CO_2. HEK 293T cells were transfected with ExGEN500 (Fermentas) or Lipofectamine 2000 (Invitrogen) transfection reagents. We determined the activation of NF-κB by using an NF-κB-dependent luciferase reporter plasmid 3XκBL (ref. 25). Normalization for transfection efficiency in luciferase reporter assays was done by including a constant amount of a β-galactosidase expression plasmid (pGK–β-gal)[26] in all transfections and measuring β-galactosidase activity. Luciferase and β-galactosidase activities were assayed 1–2 d after transfection. Relative luciferase activities are expressed as fold activation relative to the activity of the NF-κB-dependent luciferase reporter (3XκBL) alone, and were calculated by dividing the values for luciferase activity by the corresponding values for β-galactosidase activity. For luciferase-reporter assays, cells were lysed in reporter lysis buffer (Promega) and luciferase activity was assayed with the luciferase assay system (Promega) according to the manufacturer's instructions. We measured β-galactosidase activity with the Galacton-Plus substrate system (Tropix) according to the manufacturer's instructions.

Deubiquitination assays

Wild-type or mutated Flag-tagged CYLD proteins were immunoprecipitated from HEK 293T cell lysates prepared with Nonidet P-40 (NP-40) lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM phenyl methylsulphonyl fluoride) from cells that were pretreated for 4 h with 20 μM ALLN. The immunoprecipitated proteins were incubated with 1.25 μg of tetraubiquitin (Affinity Research Products) in 15 μl of 50 mM Tris-HCl buffer (pH 7.2) containing 1 mM dithiothreitol (DTT) for 2 h at 37 °C. Reaction mixtures were analysed by SDS-PAGE, followed by silver staining or immunoblotting with a monoclonal antibody against Flag.

GST pull-down assays, immunoprecipitation and immunoblotting

Cells were lysed for 30 min on ice in 500 μl of NP-40 lysis buffer for the GST pull-down assay shown in Fig. 1c and the immunoprecipitations shown in Fig. 1b, d, or in 500 μl or 1,000 μl of RIPA buffer (NP-40 lysis buffer containing 1 mM DTT, 0.1% SDS and 1% deoxycholic acid) for the other immunoprecipitations. We carried out GST pull-down assays and immunoprecipitations as described[27]. Immunoblots were developed with the ECL-plus reagent (Amersham Biosciences) according to the manufacturers instructions. Antibodies were obtained from Santa Cruz Biotechnology, with the exception of the M5 and M2 antibodies against Flag, which were obtained from Sigma. We purchased sheep secondary antibodies against mouse and donkey secondary antibodies against rabbit conjugated to horseradish peroxidase from Amersham Pharmacia.

RNA-mediated interference

RNA-mediated interference for downregulating CYLD expression was done by the transfection of double-stranded RNA oligonucleotides, details of which are given in the Supplementary Information.

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