The deubiquitinating enzyme CYLD controls apical docking of basal bodies in ciliated epithelial cells

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CYLD is a tumour suppressor gene mutated in familial cylindromatosis, a genetic disorder leading to the development of skin appendage tumours. It encodes a deubiquitinating enzyme that removes Lys63- or linear-linked ubiquitin chains. CYLD was shown to regulate cell proliferation, cell survival and inflammatory responses, through various signalling pathways. Here we show that CYLD localizes at centrosomes and basal bodies via interaction with the centrosomal protein CAP350 and demonstrate that CYLD must be both at the centrosome and catalytically active to promote ciliogenesis independently of NF-κB. In transgenic mice engineered to mimic the smallest truncation found in cylindromatosis patients, CYLD interaction with CAP350 is lost disrupting CYLD centrosome localization, which results in cilia formation defects due to impairment of basal body migration and docking. These results point to an undiscovered regulation of ciliogenesis by Lys63 ubiquitination and provide new perspectives regarding CYLD function that should be considered in the context of cylindromatosis.
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entrioles are conserved microtubule-based organelles displaying structural asymmetry, with the mother centriole bearing two sets of appendages at its distal end. In resting cells, the mother centriole can turn into a so-called basal body and dock to the plasma membrane through the distal appendages, to complete primary cilia formation. In addition to these primary cilia, multiple motile cilia are present in some mammalian epithelia (for example, brain ventricles, respiratory tract and fallopian tubes). In contrast to cycling cells, these multiciliated cells have the ability to assemble hundreds of centrioles that anchor to the plasma membrane and grow cilia.

In all cases, ciliogenesis is a multistep process that includes basal body assembly, maturation and migration, transition zone formation, docking to the cell surface and further axonemal extension, which requires intraflagellar transport.

Primary cilia are considered as cell antennas that receive extracellular signals and transmit them to the cell bodies, thus coordinating cell growth, polarity and differentiation. Among their role in sensing the environment, cilia are required to sense the environment, cilia are required to coordinate cell growth, polarity and differentiation. Among

the numerous centrosomal proteins, CAP350 serves as a platform to anchor several proteins to the centrosome. FOP (FGFR1 oncogene partner) localization at the centrosome depends on its association with CAP350 and is required to recruit EB1 at the centrosome. The complex CAP350/FOP was shown to be required for microtubule anchoring to the centrosome. In addition, as a microtubule binding protein, CAP350 stabilizes the microtubule network as well as the microtubules of the centriolar barrel, allowing the maintenance of a continuous pericentrosomal golgi ribbon. Interestingly, removal of EB1 from the centrosome by expression of a carboxy-terminal fragment of CAP350 as well as depletion of FOP inhibits primary cilia formation.

CYLD was originally identified as a gene mutated in familial cylindromatosis, a genetic condition that predisposes patients to the development of skin appendages tumors, referred to as cylindromas. Patients with cylindromatosis carry heterozygous germ-line mutations in the CYLD gene. During their life, some cells undergo a loss of heterozygosity with the disappearance of the wild-type (WT) CYLD allele. The remaining mutated allele leads to the expression of a truncated protein, thus resulting in the development of cylindromas. Therefore, this mode of tumour formation defines CYLD as a tumour suppressor. The CYLD gene encodes a deubiquitinating (DUB) enzyme, which removes lysine 63-linked polyubiquitin chains (K63-linked ubiquitin) as well as linear chains from target proteins. Interestingly, most of the mutations of CYLD found in human cylindromas are predicted to cause C-terminal truncations and catalytic inactivation of the DUB domain.

The CYLD protein was shown to regulate cell survival and inflammatory responses mainly through inhibiting nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinase signalling. In addition, CYLD was also shown to negatively regulate the Wnt/β-catenin signalling pathway by removing K63-linked ubiquitin of Dishevelled (Dvl), and more recently CYLD was found to interact with the centrosomal protein CEP192 (ref. 16), which plays a critical role in centrosome biogenesis.

The tumour suppressing function of CYLD has been studied, in vivo, using Cyld knockout (KO) mice. Surprisingly, these mice do not spontaneously develop tumours but their skin is more sensitive to tumorigenic chemicals compared with WT mice. Intriguingly, transgenic mice, which carry a complete deletion of the DUB domain (CyldΔD) die perinatally from respiratory dysfunction and exhibit immature lungs characterized by hyperplastic mesenchyme. The striking difference between those two phenotypes led us to use a knockin mouse model engineered to recapitulate more closely the smallest truncation found in patients by expressing a truncated CYLD protein lacking the last 24 C-terminal amino acids of its catalytic domain (CyldΔ24).

To further characterize how CYLD may contribute to ciliogenesis, we highlight the interaction between CYLD and the centrosomal CAP350 protein, which leads to CYLD localization to the centrosome. In addition, we demonstrate that CyldΔ24 mice show impaired apical migration and docking of basal bodies in multiciliated cells with a concomitant loss of CYLD centrosomal localization and CAP350 interaction. To understand the mechanism by which CYLD impairs basal body migration/docking, we expressed several constructs of the protein and show that CYLD overexpression in the cytosol prevents primary cilia formation, while its specific expression at the centrosome leads to primary cilia formation. Conversely, expression of the catalytically inactive CYLD at the centrosome abolishes ciliogenesis. This demonstrates that both centrosome localization and CYLD DUB activity are required for cilia formation. Finally, we demonstrate by inhibiting the NF-κB pathway that catalytically inactive CYLD abolishes ciliogenesis in an NF-κB-independent manner. Altogether, these results point to an unravelled role of Lysine 63 ubiquitination in ciliogenesis.

Results

CYLD interacts with CAP350 at the centrosome/basal bodies. We identified CYLD as a potential interacting partner of CAP350, by performing immunoprecipitation directed against CAP350 followed by mass spectrometry analysis. Eleven peptides were found in the 110-kDa band that matched sequences throughout the tumour suppressor protein CYLD (Fig. 1a and Supplementary Fig. 1). We confirmed the presence of CYLD in the CAP350 immunoprecipitate using a monoclonal antibody directed against the catalytic domain of CYLD (Fig. 1b), thus establishing CAP350/CYLD interaction. To further map the interacting domains of CAP350 and CYLD, we transfected Myc-tagged DNA constructs encoding various portions of CAP350 with full-length Flag-tagged CYLD into HEK 293T cells and performed immunoprecipitation with an anti-Flag antibody. The CYLD interacting domain was mapped to the C-terminal domain of CAP350 (Fig. 1c). As full-length CAP350 is insoluble, we overexpressed the CAP350 C-terminal domain with either a CYLD ΔN (AA394-956) or CYLD ΔC (AA1-585) construct. We found that the CAP350 C-terminal domain interacts with a CYLD ΔN (AA394-956) protein but not with the CYLD ΔC (AA1-585) protein, suggesting that it interacts with the DUB domain of CYLD (Fig. 1d). In addition, cells co-expressing both proteins show a co-localization of CAP350-GFP and CYLD-Flag to the centrosome and to the microtubules (Supplementary Fig. 2a). Such localization has already been observed for CAP350 overexpression alone, as previously described, while CYLD-Flag expressed alone was present in the cytosolic fraction (Supplementary Fig. 2b). These results suggest that CAP350 directs CYLD localization to perform specific functions in cells.

CYLD localizes to the centrosome and basal bodies. This CAP350/CYLD interaction prompted us to investigate the localization of endogenous CYLD. To that end, we generated and affinity purified an antibody directed against CYLD (CYLD 089). Staining with this antibody showed that CYLD is enriched at the centrosome throughout the cell cycle (Fig. 2a,b). In addition, a faint labelling of the nucleus was observed in interphase cells. Such a nuclear staining has previously been observed for several

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Figure 1 | CYLD interaction with CAP350. (a) Schematic representation of the CYLD protein with the three CAP-Gly domains and the catalytic DUB domain (593–956). The locations of the 11 peptides found by mass spectrometry are underlined. In the lower panel, representation of the truncated protein expressed in CyldΔ932 mice, which lacks the last 24AA of the DUB domain. (b) IP experiments were performed on RPE1 cells using pre-immune (PI, as control), polyclonal anti-CAP350 (CAP), or monoclonal anti-CYLD (CYLD) IgGs. The immunoprecipitated proteins and non-associated components are referred to as ‘Immunoprecipitates’ and ‘unbound fraction’, respectively. IP products were analysed by WB analysis using antibodies directed against CAP350 and CYLD (polyclonal) as indicated. CAP, CAP350. (c) Full-length CYLD-Flag and three CAP350 fragments as schematized above were expressed in HEK293. CYLD-Flag immunoprecipitates the C-terminal domain of CAP350. (d) CAP350 C-terminal domain and either CYLD ΔC (1–585) and CYLD ΔN (394–956) were expressed in HEK293. CYLD ΔN immunoprecipitates the C-terminal domain of CAP350. Un.Fr, unbound fractions; IP, Immunoprecipitates.
ciliary or centrosomal proteins\textsuperscript{20}. During metaphase, CYLD was detected along some spindle microtubules and at the spindle poles (Fig. 2b), reminiscent of CAP350 staining\textsuperscript{8}. To further ascertain this centrosomal localization, we used a biochemical approach. Western blot (WB) analysis with two antibodies against CYLD identified a major band at 110 kDa, which was specifically enriched in the centrosomal fraction of KE37 cells but also weakly detected in Triton X-100-soluble and -insoluble fractions (Fig. 2c). Consistent with these observations, purified centrosomes were strongly labelled by CYLD antibodies (Fig. 2d). We conclude that CYLD is a centrosomal protein.

To confirm the centrosomal localization of CYLD, we investigated its localization in multiciliated cells that contain numerous basal bodies. Serum starvation of cultured ependymal cells leads to the development of numerous motile cilia growing from these basal bodies\textsuperscript{21}. Therefore, we cultured ependymal cells

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**Figure 2 | CYLD localizes to the centrosome and basal bodies.** (a) Double labelling of RPE1 cells with polyclonal affinity-purified CYLD antibody (CYLD 089) and the monoclonal γ-tubulin antibody GTU88, which recognizes the centrosome. (b) CYLD staining in RPE1 during metaphase (upper panel) and telophase (lower panel) with antibodies directed against CYLD and γ-tubulin. During metaphase, CYLD remains localized to the centrosome but is also present along the spindle as previously described for CAP350 (ref. 8). During telophase, CYLD antibodies label the centrosome and some dots around it. Staining of the midbody and the central spindle was never observed. (c) WB analysis of CYLD in Triton X-100-soluble (S) and insoluble (I) protein fractions and a highly enriched centrosomal fraction (CTR) from KE37 cells. (d) Immunostaining of isolated centrosomes with polyclonal affinity-purified CYLD antibody (CYLD089) and CTR453. (e) Deconvoluted images of cultured ependymal cells stained with antibodies against CAP350 and TAP 952. (f) Deconvoluted images of cultured ependymal cells stained with antibodies against CYLD and TAP 952. Note that CYLD stained not only basal bodies but also cilia tips. Scale bars, 10 μm.
from mouse brains and stained them with antibodies against CAP350 and CYLD. Antibodies directed against glycylated tubulin, such as TAP952 (ref. 22) were used to decorate the cilia. Numerous dots located at the cilia base were labelled by antibodies directed against CAP350 or CYLD (Fig. 2e,f). A co-staining with antibodies directed against γ-tubulin suggests that both proteins localize at the basal bodies of multiciliated cells (Supplementary Fig. 3). Surprisingly, CYLD antibodies, but not CAP350 antibody, stained the tips of the cilia and weakly the cilia themselves (Fig. 2f). Altogether, these results suggest that CYLD localizes at the centrosome and basal bodies but also at the ciliary tips.

**Truncated CYLD inhibits primary cilia formation.** The presence of CYLD at the basal bodies and at the ciliary tips prompted us to investigate the role of this protein in ciliogenesis. Therefore, we turned to CyldΔ932 mice24, which express the smallest truncation of CYLD causing the human pathology (a deletion of the last 24 amino acids) leading to a catalytically inactive protein24 (Fig. 1a). As previously described in mice with complete deletion of CYLD catalytic domain (CyldΔ9/58)19, CyldΔ932 mutants die perinatally from respiratory distress with pulmonary defects (Supplementary Fig. 4) reminiscent of the phenotype observed in several mouse models exhibiting defects in signalling as Wnt5a25, Vangl2 (ref. 26) and GMAP210 (ref. 27). As these mutants show defects in cilia or in PCP signalling, we decided to investigate primary cilia formation in primary mouse embryonic fibroblasts (MEFs) derived from CyldΔ932 or CyldΔ932 littermate embryos. We first verified that proliferation of cells of both genotypes was comparable as proliferation rate is likely to modify cell’s behaviour in respect to ciliation. Results of fluorescence-activated cell sorting (FACS) analysis or Ki67 staining (a marker of proliferative cells) show that both WT and mutant MEFs proliferate similarly (Supplementary Fig. 5a–c). As CYLD is a tumour suppressor, and primary cilia usually form in the G0/G1 phase of the cell cycle28, we also wanted to determine whether both cell types were able to undergo cell cycle arrest on serum starvation. Again, after FACS analysis and Ki67 staining we found no difference in the ratio of non-cycling cells between the two conditions (Supplementary Fig. 5a–c). Although both cell types proliferate similarly and are able to stop cycling on serum starvation, primary cilium staining showed that only 32% of CyldΔ932 cells present a primary cilium compared with 68% of WT MEFs (Fig. 3a,b), suggesting that truncated CYLD mildly affects primary cilia formation.

**Truncated CYLD inhibits multiple motile cilia formation.** We next examined motile cilia formation in trachea of WT and CyldΔ932 E18.5 embryos from several litters by scanning electron microscopy. As ciliogenesis in the trachea occurs proximo-distally29, we imaged and counted the ciliated cells in every field from the larynx to the beginning of the main bronchi to avoid bias due to spatial differences. For each litter, the number of multiciliated cells was at least 50% lower in the mutants than in WT embryos. In addition, the motile cilia of mutant multiciliated cells were generally shorter and scarce (Fig. 4a). As a control of the specificity of this result, we took advantage of the previously published Cyld knock-out mouse model18,30,31. As CyldΔ932 mice live without any major health issues, we were not expecting any defects in cilia formation. Indeed, there was no difference in the morphology and number of multiciliated cells between the WT and CyldΔ932 embryos (Fig. 4a, graph). These results show that CYLDΔ932, a truncated catalytically inactive protein, behaves as a dominant negative form of CYLD to inhibit formation of motile cilia in multiciliated cells, whereas complete absence of CYLD has no effect on cilia formation.

As the decreased number of multiciliated tracheal cells might be due to a developmental delay in the CyldΔ932 embryo, we used the ependymal culture system31 and compared motile cilia formation in parallel cell cultures obtained from WT and CyldΔ932 littersmates. There was no difference in the doubling time of cells with these different genotypes, but there was a dramatic difference in the number of multiciliated cells between WT and CyldΔ932 cells, independently of cell-density variations (Fig. 4b). Altogether, our results in trachea and ependymal cells culture
show that truncation of the catalytic domain of CYLD affects formation of motile cilia of multiciliated cells.

**Cyld** 

To determine the reason of the decrease in cilia formation in **Cyld** embryos, we studied basal body formation in ependymal cell cultures from each genotype at several differentiation time points by electron microscopy analysis of WT and **Cyld** embryos. We found that a significant number of basal bodies failed to position apically and dock at the plasma membrane in **Cyld** tracheal ciliated cells (Fig. 5b). They appeared to be positioned in different orientation (Fig. 5b, arrows). In addition, the microvilli, which are interspaced the axonema, appeared to be reduced in length as well as in number, compared with the WT samples from the same litter, reminiscent of actin organization or Rho GTPase activity defect.

**Centriole maturation and satellites are not affected.** The failure of basal bodies to anchor could be explained by either a defect in centriole maturation or in centriolar satellite organization. Indeed, centriole maturation is characterized by the acquisition of subdistal and distal appendages, which form the ciliary basal feet and transition fibres, respectively. Both of them are required for cilia formation. Furthermore, centriolar satellites

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**Figure 4 | CYLD**

(a) Left: cilia analysis by scanning electron microscopy of tracheas isolated from WT and **Cyld** embryos at E18.5. Representative fields of trachea from WT embryos (upper) or **Cyld** mutant (lower) are shown, with a magnified image on the right. Right: quantification of the percentage of ciliated cells in the trachea from WT or mutant embryos. Analysis was performed on five litters from **Cyld** embryos and one litter from **Cyld** KO mice. The number of WT and mutant embryos is indicated at the top of each bar. The mean was determined when possible and the s.d. is indicated. Scale bars, 10 μm for the low magnification and 1 μm for the high magnification. (b) Left: staining of a large field of cultured ependymal cells isolated from WT (left) or **Cyld** (right) from P0 animals 15 days after differentiation with GT335 for cilia and DAPI for nuclei. Note the decreased number of multiciliated cells in the mutant. Scale bars, 10 μm. Right: quantification of the percentage of multiciliated cells in WT or **Cyld** cell cultures. Multiciliated cells in each culture were counted and reported relative to the number of nuclei. In mutant cell cultures, the number of multiciliated cells was reduced by 50%. (n for WT = ~2,500 cells of 5 embryos; n for **Cyld** = ~2,500 cells of 5 embryos). P-value <0.0001 after χ²-test. Error bars show s.d.
are believed to drive cargo to the centrosome and basal bodies\textsuperscript{35}, and are also required for ciliogenesis\textsuperscript{36}. To ascertain the presence of centriolar satellites and appendages, we stained ependymal cells with antibodies directed against PCM1 and OFD1, two proteins of centriolar satellites\textsuperscript{37,38}, as well as with antibodies against Cep164 and CCDC123 (Cep123), two proteins located within the distal appendages\textsuperscript{37,38}, and ODF2, a protein located both at distal and subdistal appendages\textsuperscript{39}. All antibodies decorated centriolar satellites or basal bodies irrespective of the genotype (Fig. 6), suggesting that basal body maturation and centriolar satellite organization are not significantly modified in \textit{Cyld}\textsuperscript{Δ932} mice.

\textbf{\textit{Cyld}\textsuperscript{Δ932} does not interact with \textit{CAP350}.} To gain molecular insight into the defects associated with \textit{Cyld}\textsuperscript{Δ932} expression, we tested whether \textit{CYLD}\textsuperscript{Δ932} protein interacted with \textit{CAP350} by immunoprecipitation of \textit{CAP350} in WT and \textit{Cyld}\textsuperscript{Δ932} MEFs. In parallel experiments, \textit{CAP350} antibody immunoprecipitated \textit{CYLD} (revealed by a polyclonal antibody directed against the amino-terminal domain) in WT but not \textit{Cyld}\textsuperscript{Δ932} MEFs (Fig. 7), suggesting that the truncated version of \textit{CYLD} is unable to interact with \textit{CAP350}. It follows that \textit{CYLD}\textsuperscript{Δ932} would not be localized at the centrosome and, indeed, we did not detect any \textit{CYLD} at the centrosome or basal bodies in \textit{Cyld}\textsuperscript{Δ932} mutant ependymal cells (Fig. 7b,c), while \textit{CAP350} remained at the centrosome. It is worth noting that \textit{CYLD} was still localized at the ciliary tip in mutant ependymal cells. These observations indicate that the lack of interaction between \textit{CYLD}\textsuperscript{Δ932} and \textit{CAP350} results in abnormal \textit{CYLD} localization.

\textbf{CYLD localization and activity are required for ciliogenesis.} As the impaired ciliogenesis observed in \textit{Cyld}\textsuperscript{Δ932} mice might be driven by the absence of \textit{CYLD} at the centrosome, or by its compromised catalytic activity or both, we decided to investigate the effect of \textit{CYLD} activity and localization on primary cilia formation. This was performed by overexpressing \textit{CYLD}\textsuperscript{WT} as well as its catalytically inactive version \textit{CYLD}\textsuperscript{H871/N} (ref. 24) in RPE1 cells. Overexpressed \textit{CYLD}\textsuperscript{WT} or \textit{CYLD}\textsuperscript{H871/N} protein is mainly cytosolic with no obvious enrichment at the centrosome (Fig. 8a). We observed a dramatic decrease in primary cilia formation in cells expressing GFP-\textit{CYLD}\textsuperscript{WT}, \textit{CYLD}\textsuperscript{WT}-Flag or \textit{CYLD}\textsuperscript{H871/N}-Flag (Fig. 8a) with 23%, 17% and 24% of ciliated cells, respectively, while non-transfected cells or green fluorescent protein (GFP)-transfected cells showed \~90%.

As this result could be due to aberrant \textit{CYLD} localization, we decided to force \textit{CYLD} expression at the centrosome using the centrosomal targeting domain, PACT\textsuperscript{40}. The targeting of this GFP-PACT-\textit{CYLD}\textsuperscript{WT} to the centrioles affected ciliogenesis only
slightly, resulting in ~80% of ciliated cells (Fig. 8b). By contrast, the targeting to the centrioles of CYLD with a point mutation, which impaired the catalytic activity of CYLD (GFP-PACT-CYLD<sup>WT</sup>), showed only 57% of ciliated cells. A similar decrease was also observed (Fig. 8b, graph) when using the truncated and catalytically inactive version of CYLD (GFP-PACT-CYLD<sup>Δ932</sup>), Cep164 (36%), but no CYLD localize at the centrosome. These results demonstrate that both the localization of CYLD at the centrosome/basal body and its deubiquitinase activity are essential for its function in ciliogenesis.

Ciliogenesis defects are independent of NF-κB activation. As CYLD is a negative regulator of the NF-κB signalling pathway, we were wondering whether the defective ciliogenesis observed on the trachea and ependymal cells of mutant mice or after targeting at the centrosome catalytically inactive CYLD, might be due to a deregulation of the NF-κB pathway. Therefore, we decided to co-transfect RPE1 cells with either GFP-PACT-CYLD<sup>WT</sup> or GFP-PACT-CYLD<sup>Δ932</sup> with or without a non-degradable form of IκB acting a Super-repressor of NF-κB (IκBα Super-Repressor (IκBα-SR))<sup>61</sup> to block NF-κB activation. As shown on Fig. 8d, the addition of the tumour necrosis factor (TNF) induced activation of NF-κB either in the presence of serum or after 48 h serum depletion, while the presence of IκBα-SR completely blocked this activation. In parallel, cells were fixed and analysed for the
presence of a primary cilium. Expression of IκBα-SR did not impact the ciliation process either in GFP-PACT-CYLDWT-expressing cells as shown in Fig. 8e, suggesting that the defects in the ciliogenesis process due to the presence of GFP-PACT-CYLDΔ932 at the centrosome is independent of NF-κB signalling.

Discussion
In this study we demonstrate that part of the CYLD pool is located at the centrosome/basal bodies, through its interaction with CAP350. This interaction is attested by several observations. First of all, we identified CYLD by mass spectrometry as a partner of CAP350 and confirmed their interaction in endogenous immunoprecipitation experiments; Furthermore, we demonstrate by co-overexpression that the C-terminal portion of CYLD, including the DUB domain, interacts with the C-terminal domain of CAP350. Second, CAP350 overexpression directs the centrosomal localization of overexpressed CYLD; finally, CYLD no longer localizes at the centrosome when its interaction with CAP350 is abolished. It is worth noting that CYLD has already been found in interaction with another centrosomal protein CEP192 in co-overexpressed conditions, reinforcing the centrosomal localisation of CYLD16. Nevertheless, the relationship
between CAP350/CEP192 and CYLD will require further investigations.

This specific centrosomal localization combined with the staining observed at the tip of motile cilia suggests that CYLD plays a role in cilium assembly/disassembly. Interestingly, localization of CYLD at the cilia tips is reminiscent of the enrichment observed for several other proteins such as EB1/EB3 (ref. 42), some IFT proteins and, more recently, cep104/FAP256 (ref. 44). This localization of CYLD is probably independent of its DUB activity, as the staining at the cilia tip is still observed in Cyld<sup>−/−</sup> ependymal cells. As a matter of fact, an interaction between CYLD and EB1 has been recently reported<sup>43</sup>. In any case, this dual CYLD localization at the centrosome and at the cilia tip suggests that CYLD has distinct functions depending on the partners it interacts with.

Our results show that CYLD participates in ciliogenesis by allowing basal bodies to migrate and dock to the plasma membrane. However, not all motile cilia are affected, as CYLD<sup>−/−</sup> mutants do not display any left–right patterning defects, suggesting that the function of the node is unaffected by CYLD truncation. In addition, we demonstrate that CYLD has to be both catalytically active and located at the centrosome to promote ciliogenesis. Therefore, we propose a model (Fig. 9) in which ciliogenesis would be only possible if a centrosomal protein, or CAP350 itself, is deubiquitinated. In the WT situation, CYLD would be located at the centrosome/basal body via CAP350 and be able to hydrolyse K63- or linear-linked ubiquitin chains from its substrate, enabling ciliogenesis to proceed. In the mutant situation, CYLD would not be present at the centrosome, resulting in impaired ciliogenesis. On CYLD overexpression, extra CYLD in the cytoplasm would delocalize its targets and prevent ciliogenesis. However, when CYLD is overexpressed and targeted to the basal body by the PACT domain, it can deubiquitinate its target and, consequently, ciliogenesis occurs. Conversely, cells overexpressing the catalytically inactive CYLD<sup>H87I/N</sup> or CYLD<sup>A932</sup> fused with the PACT domain do not retain the ability to develop cilia. All these results argue for the involvement of K63-ubiquitination/deubiquitination in ciliogenesis. Importantly, we demonstrate that this role is NF-κB independent. Recently, several reports have suggested an inhibition of ciliogenesis caused by activation of the NF-κB pathway. First, Lattea et al.<sup>46</sup> have shown that the activation of this pathway by the expression of constitutively active IKK2 inhibits cilia formation. However, it is not clear whether the cilia formation defect is a consequence of a direct cell-autonomous NF-κB activation rather than activation of inflammatory processes. Moreover, it could also be a direct effect of active IKK2 in ependymal cells, independently of NF-κB, as IKK2 has NF-κB-independent functions<sup>47</sup>. As a matter of fact, IKK2 has been found inhibiting ciliogenesis in an RNA interference screen<sup>48</sup>. Second, the pro-inflammatory cytokine TNF triggered a dose-dependent loss of primary cilia in mesenchymal stromal cells<sup>49</sup>, but no molecular explanation was provided. However, our study brings several arguments supporting the fact that this DUB acts on ciliogenesis independently of NF-κB activation as trachea from Cyld KO mice do not show any defects in motile cilia formation, whereas they are defective in NF-κB signalling<sup>41,48</sup>, and inhibition of the NF-κB pathway by the expression of a non-degradable form of IkBα acting as a Super-repressor<sup>41</sup> do not modify the ciliogenesis defect caused by expression of the CYLD<sup>A932</sup> protein at the centrosome.

Nevertheless, it is intriguing that Cyld<sup>KO</sup> mice are perfectly viable without any problem in motile cilia formation, whereas the mice expressing a truncated version of CYLD die at birth with cilia defects. These results suggest some redundancy in the control of ciliogenesis by DUBs. In the Cyld<sup>KO</sup> mice another DUB may compensate for the function of CYLD, whereas in the Cyld<sup>A932</sup> mice the mutant CYLD protein probably acts as a dominant negative by delocalizing its substrate, thus preventing the action of the substitute DUB. USP21 is a good candidate for carrying this function, as it has recently been shown to localize at the centrosome and affect primary cilium formation<sup>50</sup>. Moreover, it shares a similar target with CYLD in the regulation NF-κB signalling pathway<sup>51</sup>.

The involvement of the deubiquitinas CYLD in basal body migration/docking has to be integrated in a more general role of ubiquitination processes in ciliogenesis or centrosomal function. At first, ubiquitinated proteins have been reported to be present in cilia and flagella<sup>25</sup>, and the K63-specific E2 ubiquitin-conjugating enzyme Ubc13 is present in the ciliome of various species<sup>52–55</sup>. Next, protein ubiquitination was involved in flagellar disassembly<sup>52</sup>. Finally, the E3 ubiquitin ligase MIB1 (ref. 56) was identified in a centrosome proteome<sup>57</sup> and the deubiquitinase USP21 is shown to be required for primary cilium formation<sup>50</sup>. In addition, another deubiquitinase Usp33 has recently been shown to localize at the centrosome by its binding with CP110 and its activity antagonizes Scribble<sup>58</sup> (ubiquitination of CP110 (ref. 58).

How may CYLD regulate ciliogenesis? Our data show that CYLD is required to allow basal body migration and docking at the plasma membrane. We also show that centriole maturation...
proteins as well as some centriolar satellite proteins are still present in our mutants, suggesting that the anchoring defect is not a result of basal body structural defects. However, we cannot rule out that ubiquitination/deubiquitination processes might affect centriole appendages and centriolar satellite functions.

Interestingly, a target of CYLD, Dvl, has been shown in Xenopus multiciliated cells to be essential for the positioning of the apical basal bodies and for the activation of the Rho GTPase. In addition, the RhoGEF LARG, co-immunoprecipitates with CYLD. Therefore, we propose that CYLD would allow basal body migration by deubiquitinating, a substrate working with Dvl, and LARG to activate RhoA GTPase and subsequently remodel actin. The recent report that phosphorylated CYLD redistributes to the dorsal ruffles supports this hypothesis of a link between CYLD and the actin network.

In conclusion, our work identifies CYLD as a new regulator of ciliogenesis. Two recent studies have recently reported that impaired ciliogenesis impacts skin homeostasis and affects the pilosebaceous unit from which cylindroma are believed to originate. This convinced us that future studies designed to precisely characterize the molecular pathways regulated by CYLD during ciliogenesis might provide novel insights into how CYLD deregulation results in cylindroma in the cylindromatosis pathology.

**Methods**

**Mice.** All animals were housed in specific pathogen-free conditions at Institut Curie according to French and European Union laws. All animal procedures were conducted in accordance with European, national and institutional guidelines and protocols, and were approved by local government authorities (Authorization for animal testing N°91-518, Direction départementale des services vétérinaires de l’Essonne).

**Cell culture.** Human retinal pigment epithelial (RPE1) cells were obtained from ATCC and grown in DMEM/F12 medium supplemented with 10% FCS. Human lymphoblastic KE37 cells obtained from M. Bornens’s laboratory were grown in RPMI medium supplemented with 10% FCS. MEFs were obtained after trypsinization of dissected embryos at embryonic day 13.5 (E13.5) and grown in DMEM medium supplemented with 10% FCS and non-essential amino acids. Ependymal cell cultures were maintained as described previously. Human embryonic kidney cells (HEK293T) from ThermoScientific were transiently transfected with plasmid DNA using the calcium phosphate precipitation method. RPE1 cells were transiently transfected with plasmid DNA using X-tremeGENE HP (Roche) and fixed 24–48 h after transfection.

**Cellular fractionation.** Centrosomes were isolated from KE37 cells as described previously. Briefly, cells were pretreated for 1 h with Nocodazole, and Cytochalasin D (1 μg/mL). Cells were washed in PBS and resuspended in 8% sucrose in ten times diluted PBS before cell lysis in lysis buffer (1 mM HEPES, 0.5 mM MgCl₂, 0.5%NP40, 1 mM phenylmethanesulphonyl fluoride and antiproteases). After centrifugation at 2,500 g for 10 min at 4°C, supernatants were mixed with equal volume of 20 mM HEPES and treated with DNase. Concentrated centrosomes were overlaid on a discontinuous sucrose gradient (70%, 50% and 40%) and the supernatant is filtered through a SW28 tube and centrifuged for 1 h 15 min at 26,000 r.p.m. Fractions were collected and analysed for centrosomes.
One percent Triton X-100 soluble or insoluble cell fractions were isolated from KE37 or RPE1 cells as described by Tasin et al.68

Plasmid construction. Full-length CAP350 and CYLD cDNAs were subcloned into a GFP vector (GFP-C3 from Clontech) for expression of GFP-tagged proteins. Different fragments of CAP350 cDNAmyc-tagged corresponding to AA 1-893, into a GFP vector (GFP-C3 from Clontech) for expression of GFP-tagged proteins. The protein spots were excised from the gel after coloration with colloidal Coomassie blue (G250; Bio-Rad), reduced using dithiothreitol and alkylated using iodoacetamide. The proteins were subjected to digestion with trypsin (Sigma). The extracted peptides were analysed by matrix-assisted laser desorption-ionization–time of flight mass spectrometry (Voyager DEFT PRO, Applied Biosystems). Peptide masses obtained by this analysis were used to search the National Center for Biotechnology Information database to identify the full-length proteins.

Scanning electron microscopy. Tracheas were dissected from embryos at embryonic day 18.5 (E18.5), fixed overnight in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.2 and post-fixed with 2% osmium tetroxide (Electron Microscopy Sciences). Samples were dehydrated in a gradient ethanol series and dried with hexamethyldisilazane (HMDS) or the CO2–critical point dryer Leica CPD-030. Specimens were coated with 10 nm gold/palladium in a Gatan Ion Beam Coater 681 and observed in a field emission scanning electron microscope JSM-6700F (JEOL, Japan). Image processing and analyses were performed with ImageJ software.

Transmission electron microscopy. Tracheas were dissected from embryos at E18.5, fixed overnight in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.2 and post-fixed with 2% osmium tetroxide. Samples were dehydrated in a graded ethanol series and embedded in Epox (Electron Microscopy Science). Longitudinal 200-nm-thick sections were prepared with an ultramicrotome (Leica) and mounted on copper grids (200 mesh). The samples were observed in a 200-kV field emission gun electron microscope with an in-column energy filter (omega filter) JEM 2200 FS (JEOL, Japan).

Cilia counting and statistics. As ciliated cells in the trachea differentiate proximo-distally, we counted the cells in a series of jointed pictures taken from the larynx to the main bronchi to avoid any bias based on differentiation time. For a better visualization, results were normalized. This was achieved by taking the number of ciliated cells of WT embryos as 100%. In MEF cell cultures, primary cilia formation is not always homogeneous. Therefore, we took the pictures for counting in a randomized manner, observing the centrosome. The same field was then examined for the presence of cilia.

Most of the experiments needing statistical validation involved paired observations on two variables for which we wanted to assess the independence of two populations. In those cases, the events being considered were mutually exclusive and the total probability was one. All observations were independent. Therefore, we used Fisher’s exact test or, when the sample sizes were large enough to avoid a type II error following Cochran’s rule, we performed Pearson’s z2-test without Yates’ correction. All calculations were made with GraphPad Prism 5. Statistical significance in the fluorescence intensity experiment (Fig. 8) was assessed via a Mann–Whitney test. The P-value is based on the exact value given by the software. All error bars show standard error.

In one experiment, it was not possible to run any statistical test because of the nature of the data. To count ciliated cells in the tracheas of embryos, we had to compare individuals in the same litter to avoid bias arising from possible developmental differences between litters. Consequently, there were too few embryos in each litter to perform statistical testing.

FACS analysis. Cell cycle profiles of cycling as well as serum-starved WT or Cyld−/− MEFs were determined by analysing total DNA content using propidium iodide staining (1 h incubation of ethanol-fixed cells in PBS containing 25 μg ml−1 propidium iodide, 100 μg ml−1 RNase A, and 0.1% Triton X-100). Cells were subjected to flow cytometry by using a FACS Calibur analyser (BD Biosciences) and the results were analysed with FlowJo.

NF-κB inhibition by IkBα-SR. RPE1 cells were transfected by GFP-PACT-CYL7, GFP-PACT-CYLΔN or GFP-PACT-CYLΔDD plasmids in the presence of an IgκB-Luc reporter plasmid with or without a plasmid expressing a non-degradable form of IkBα (IkBα-SR)41. Cells were serum starved 24 h after transfection to induce cilia formation and fixed 48 h after serum depletion for cilia analysis. To measure the effect of IκBα-SR, cells were stimulated with 10 ng ml−1 of TNF-α just before removing the serum (24 h after transfection) or 3 h before completing the experiment. Cell extracts were prepared and IgκB-Luciferase-derived activity measured using a LB 9507 Berthold Luminoimeter.

References


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**Author contributions**

A.-M.T. conceived and directed the project. T.E. and A.-M.T. designed and performed the experiments. A.-M.T., T.E. and G.C. discussed the project and wrote the manuscript. Y.Z. and A.J. performed the experiments on the CYLD KO mice; M.E., M.B. and M.P. generated and provided the CYLD<sup>Δ932</sup> mice.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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