CYLD negatively regulates cell-cycle progression by inactivating HDAC6 and increasing the levels of acetylated tubulin

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Introduction

Familial cylindromatosis is characterized by formation of multiple benign tumours originating from skin (Bignell et al., 2002). The tumour syndrome is caused by the loss of both CYLD alleles. The CYLD gene encodes a deubiquitinating enzyme, which removes lysine 48- or lysine 63-linked polyubiquitin chains from target proteins (Massoumi and Paus, 2007). Depending on the cellular context CYLD has been shown to negatively regulate NF-κB and/or JNK-signal-ling pathways resulting in suppression of cell proliferation and survival (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Reiley et al., 2004). The mechanism by which CYLD exerts its tumour-suppressor function in vivo has been analyzed in CYLD-null mice, which are highly susceptible to chemically induced skin tumours. The increased tumour incidence was attributed to the loss of an inhibitory interaction between CYLD and the proto-oncogene Bcl-3. The association of CYLD with Bcl-3, which results from activation and subsequent perinuclear translocation of the protein, leads to the removal of a lysine 63-linked ubiquitin chain from Bcl-3, which in turn inhibits the nuclear translocation and activity of Bcl-3 (Massoumi et al., 2006). In the absence of CYLD, Bcl-3 is able to translocate into the nucleus where it forms a complex with the NF-κB p50 and p52 isoforms. This results in activation of the cyclin-D1 promoter and increased proliferation and tumour growth (Massoumi et al., 2006). It is not clear, however, how CYLD translocates to the perinuclear region to capture Bcl-3 and whether this is the only mechanism by which CYLD regulates tumour cell proliferation.

In addition to the C-terminal ubiquitin C-terminal hydro-lase (UCH) domain, which executes the removal of ubiquitin chains, CYLD contains three cytoskeleton-associated protein-glycine-conserved (CAP-Gly) domains in the N-terminal portion of the protein. The exact function of CAP-Gly domains is not known, but their presence in various microtubule (MT)-binding proteins suggests that they enable binding to MTs (Riehemann and Sorg, 1993; Pierre et al., 1994; Bateman et al., 2002). The third CAP-Gly domain (CAP-Gly3) of CYLD has recently been shown to associate directly with the proline-rich sequences of NEMO/IKKγ, a pseudokinase, which together with IKKα and IKKβ triggers TNF-receptor-mediated NF-κB activation (Bateman et al., 2002). Interestingly, this CAP-Gly domain (Saito et al., 2004) differs structurally from the first two CAP-Gly domains (CAP-Gly1 and CAP-Gly2), which have been recently shown to associate directly with tubulin and promote tubulin polymerization (Gao et al., 2008). Whether the CAP-Gly domains in CYLD have any additional function besides tubulin binding is not known.

The stability of MTs is accompanied by post-translational modifications of α-tubulin such as acetylation, which usually occurs after MT assembly (Westermann and Weber, 2003). The level of α-tubulin acetylation in cells is accurately adjusted by the activities of tubulin acetyltransferases and deacetylases, which catalyze the acetylation and deacetylation of α-tubulin, respectively. Elp3 was identified very recently as α-tubulin acetyltransferase in neuronal cells (Crepe et al., 2003). The CAP-Gly domains of CYLD negatively regulate cell-cycle progression by inactivating HDAC6 and increasing the levels of acetylated tubulin.
Results

Interaction of CYLD with tubulin and MT

We have earlier shown that UV or TPA treatment of primary mouse keratinocytes triggers perinuclear accumulation of CYLD, which correlates with its ability to interact with its downstream targets (Massoumi et al., 2006). The present work aimed at understanding whether an MT-dependent mechanism was involved in the translocation of CYLD. To explore this we used primary mouse keratinocytes, primary human melanocytes, and different human malignant melanoma cells. Analysis of subcellular localization of CYLD in primary mouse keratinocytes revealed that TPA treatment caused a robust increase in perinuclear, acetylated α-tubulin (Figure 1A), and a colocalization of CYLD with acetylated MTs (Figure 1A). However, we could observe only partial colocalization of CYLD with tyrosinated α-tubulin in the cytoplasm (Supplementary Figure S1A). Interestingly, all melanoma cell lines we have tested so far lack detectable CYLD expression (Massoumi et al., 2009), while HDAC6 (histone deacetylase-6) and SIRT2 (the Silent Information Regulator Type-2) have been shown to deacetylate α-tubulin (Hubbert et al., 2002; North et al., 2003).

HDAC6 contains two intact HDAC catalytic domains, which execute deacetylation of α-tubulin (Zhang et al., 2006; Zou et al., 2006; Boyault et al., 2007a), and a ubiquitin-binding zinc-finger domain, which enables binding to ubiquitinated proteins (Seigneurin-Berny et al., 2001; Hook et al., 2002; Boyault et al., 2006). Even though HDAC6 contains both intrinsic nuclear import and export signals, it is mainly found in the cytoplasm (Verdel et al., 2000) where it localizes around the nucleus or at the leading edge of migrating cells (Hubbert et al., 2002). HDAC6 has also been shown to have a scaffold role for lymphocyte migration independent of its activity (Cabrero et al., 2006). Furthermore, the activity of HDAC6 promotes cell motility by regulation of membrane ruffle formation, macropinocytosis, and actin remodelling (Gao et al., 2007). Besides regulating cell motility, HDAC6 is also involved in the response of cells to various types of stress stimuli (Boyault et al., 2007b; Kawaguchi et al., 2003; Kwon et al., 2007), immune synapse organization, and the antigen-specific reorientation of the microtubule organizing center (MTOC) (Serrador et al., 2004).

In this paper we addressed the role of CYLD and specifically its N-terminal CAP-Gly domains in the regulation of cell proliferation both in primary keratinocytes and malignant melanoma cells. We found that CYLD associates with MTs and colocalizes primarily with acetylated tubulin. Activation of CYLD increases the levels of acetylated α-tubulin by interaction of the N-terminal domain of CYLD with the catalytic site of HDAC6. This interaction inhibits HDAC6-mediated tubulin deacetylation, allowing CYLD to translocate to the perinuclear region. Perinuclear localized CYLD induces a delay in the G1/S transition phase of the cell cycle through a Bcl-3-mediated pathway. In addition, CYLD is found in the midbody where it also associates with HDAC6 and regulates the rate of cytokinesis.

CYLD induces acetylation of α-tubulin and stabilization of MTs

As CYLD associates with MTs and colocalizes specifically with acetylated MTs, we hypothesized that CYLD might directly regulate α-tubulin acetylation to control its own localization. To investigate this we analysed the levels of acetylated tubulin in primary mouse keratinocytes. Both Cyld+/+ and Cyld−/− keratinocytes contained low levels of acetylated tubulin (Figure 2A). While TPA treatment did not change the total levels of α-tubulin, it increased the levels of acetylated α-tubulin in Cyld+/+ keratinocytes but not in Cyld−/− keratinocytes. (Figure 2A). This increase
was accompanied by enhanced association of CYLD with acetylated α-tubulin (Figure 2B). Importantly, re-expression of CYLD in Cyld−/− keratinocytes restored the ability of these cells to respond to TPA by increasing the levels of acetylated α-tubulin (Figure 2C). Reconstitution of melanoma cells with EGFP–CYLD induced an almost sixfold increase in the levels of acetylated α-tubulin (Figure 2D). Furthermore, transient transfection of melanoma cells with increasing concentrations of the EGFP–CYLD cDNA showed direct correlation between levels of CYLD expression and acetylated α-tubulin (Figure 2E and F). These results suggest that CYLD increases the level of acetylated α-tubulin in both TPA-stimulated keratinocytes as well as in untreated melanoma cells.

To determine whether the increase in α-tubulin acetylation depends on the deubiquitinase activity of CYLD, we transduced melanoma cells with catalytically inactive CYLD (CYLD C/S). CYLD C/S increased the acetylation of α-tubulin to the same extent as with full-length, wild-type CYLD (Figure 2G), indicating that the UCH domain of CYLD is not required for α-tubulin acetylation.

To investigate whether the association of CYLD with MTs influences their dynamic properties, we treated melanoma cells with nocodazole and monitored MT re-growth after nocodazole washout. Polymerised MT fibres in CYLD-expressing melanoma cells were evident already 10 min after the washout (Figure 2H) and acetylated α-tubulin after 30 min (Figure 2I). In EGFP-expressing melanoma cells, however, MT fibres were detected only 30 min after nocodazole washout (Figure 2H), and we failed to observe acetylation of α-tubulin even 2 h after nocodazole washout (Figure 2I).
This suggests that CYLD regulates the acetylation of tubulin and also influences the polymerization rate of MTs.

Next, we analysed the effect of CYLD on MT depolymerisation by treating EGFP–CYLD- or EGFP-expressing melanoma cells with increasing concentrations of nocodazole and found that in the presence of CYLD, MTs began to partially depolymerise at 5-μM concentrations (with some MT fibres still detectable) (Supplementary Figure S3A). Total
depolymerisation was achieved with 50 μM nocodazole. In sharp contrast, already 1 μM nocodazole induced partial depolymerisation of MT fibres in EGFP-expressing cells and 5 μM nocodazole was sufficient to induce complete depolymerisation (Supplementary Figure S3A). Furthermore, treatment with 1 μM nocodazole did not change the level of acetylated tubulin in EGFP–CYLD-expressing melanoma cells (Supplementary Figure S3B), while treatment with 50 μM of nocodazole resulted in almost complete deacetylation of α-tubulin (Supplementary Figure S3B). These results indicate that CYLD stabilizes the MT network and protects the MT network from nocodazole-induced depolymerisation.

**CYLD binding to HDAC6 increases α-tubulin acetylation**

To analyse whether CYLD regulates tubulin acetylation by inhibiting HDAC6, we treated keratinocytes and melanoma cells with different HDAC inhibitors: tubacin, a small-molecule inhibitor of HDAC6; trichostatin-A (TSA), a chemical inhibitor of class-I and II HDACs, including HDAC6; and sodium butyrate, a potent inhibitor of class-I and II HDACs except HDAC6. Sodium butyrate treatment did not affect the levels of acetylated tubulin either in keratinocytes or in melanoma cells (Figure 3A and Supplementary Figure S3C). In addition, TPA stimulation of Cyld+/+ keratinocytes led to an increase in acetylated α-tubulin levels both in the presence and absence of sodium butyrate (Figure 3A). In contrast, treatment of Cyld−/− keratinocytes with either TPA or TSA alone increased acetylated α-tubulin levels to a similar extent, while simultaneous treatment with both the compounds failed to induce a synergistic effect (Figure 3B). Moreover, TSA treatment of Cyld−/− keratinocytes increased the level of acetylated α-tubulin, while TPA treatment had no effect (Figure 3B). A similar effect was observed in keratinocytes treated with tubacin, which is a selective inhibitor for HDAC6-depleted cells and an additional signal induced by TPA.

**Association of CYLD and HDAC6 in the presence of TPA**

To analyse whether CYLD directly influences HDAC6 activity, we first performed co-immunoprecipitation and immunofluorescence experiments using primary keratinocytes. We observed both co-precipitation (Figure 4B) and colocalization (Figure 4C) of the proteins after TPA treatment, indicating that they form a protein complex in response to CYLD activation. The interaction between CYLD and HDAC6 was retained in the presence of nocodazole (Figure 4B), indicating that the interaction between CYLD and HDAC6 does not require intact MTs. Similarly, HDAC6 co-precipitated with EGFP–CYLD in melanoma cells, and this interaction was also retained in the presence of nocodazole (Supplementary Figure S4C). Furthermore, FLAG pull-down assays with a series of in vitro translated, FLAG-tagged CYLD-deletion mutants (Supplementary Figure S2B) showed that the N-terminal region of CYLD (CYLD1–212) harboured the ability to interact with purified HDAC6 protein (Figure 4D).

To define which domain(s) in HDAC6 is required for the interaction with CYLD, a series of HA-tagged HDAC6-deletion mutants (Zhang et al., 2003) were transiently transfected together with full-length, FLAG-tagged CYLD into HeLa cells. FLAG pull-down assays of CYLD demonstrated that the deletion mutants containing the HDAC6 domain DD1 or DD2 interacted with CYLD (Figure 4E). To confirm this interaction we expressed recombinant GST–HDAC6, His–CYLD, and GST alone as control (Supplementary Figure S5A). HDAC6 co-precipitated with His–CYLD-bound Ni-NTA agarose, indicating association of these two proteins (Figure 4F). Furthermore, GST pull-down assays using the N-terminal region of CYLD (GST–CYLD1–212) and the purified form of His-tagged, full-length HDAC6 confirmed an interaction between CYLD and HDAC6 (Figure 4G).
**CYLD inhibits HDAC6 activity by binding to its catalytic domains**

As it was shown earlier that DD1 and DD2 are critical for the catalytic activity of HDAC6 (Zhang et al., 2006), we hypothesized that binding of CYLD to these domains leads to HDAC6 inactivation. To test this hypothesis, we performed in vitro tubulin deacetylation assays. We incubated endogenous HDAC6 and/or EGFP–CYLD immunoprecipitates from...
melanoma cells in the absence or presence of TSA together with MAP-enriched polymerised MTs purified from bovine brain. Whereas HDAC6 alone induced deacetylation of α-tubulin, CYLD or TSA treatment could maintain acetylated α-tubulin even in the presence of HDAC6 (Figure 5A). To confirm the specificity of this effect, we transiently transfected COS cells
The N-terminal domain of CYLD is responsible for inhibition of HDAC6 activity. (A) MAP-enriched bovine tubulin (cytoskeleton) was polymerised into microtubules in the absence of taxol or glycerol by incubation for 30 min at 35°C. The polymerised microtubules were then incubated with endogenous HDAC6 and/or EGFP–CYLD immunoprecipitates from melanoma cells in the absence or presence of TSA (0.5 μM) at 37°C for 2 h. Samples were placed on ice for 15 min and the supernatant was collected by centrifugation and analysed by immunoblotting. (B) COS cells were transiently transfected with different concentrations of FLAG–CYLD (0.5, 1.0 and 3.0 μg) or HA–HDAC6 (1.0 μg) for 24 h. Polymerised microtubules were incubated with CYLD and/or HDAC6 eluted from the corresponding immunoprecipitates at 37°C for 2 h. Samples were placed on ice for 15 min and the supernatant was collected by centrifugation and analysed by immunoblotting. (C) COS cells were transiently transfected with FLAG-tagged, wild-type or truncation mutants of CYLD or HA-tagged HDAC6 constructs (1.0 μg of each for 24 h). Polymerised microtubules were then incubated with CYLD and/or HDAC6 eluted from the corresponding immunoprecipitates at 37°C for 2 h. Samples were placed on ice for 15 min and the supernatant was collected by centrifugation and analysed by immunoblotting. (D) Immunoprecipitation of endogenous HDAC6 from Cyld+/− and Cyld−− keratinocytes in the absence or presence of TPA (100 nM for 30 min) and immunoblotting against tubulin and HDAC6 in the absence or presence of TPA (100 nM for 30 min). The lysate (lower panel) shows equal amount of protein used for immunoprecipitation. (E) Immunoblot analysis of HDAC6, acetylated α-tubulin, and total tubulin in EGFP- or EGFP–CYLD-expressing melanoma cells before or after 24 h of transient transfection with HDAC6 siRNAs (0.2 μM). (F) Immunoblot analysis of HDAC6, acetylated α-tubulin, and total tubulin in untreated or TPA (100 nM for 30 min)-treated Cyld−/− keratinocytes before or after transient transfection with HDAC6 siRNAs.

with increasing concentrations of FLAG–CYLD or HA–HDAC6 cDNA. The polymerised MTs were then incubated with CYLD and/or HDAC6 immunoprecipitates after peptide elution. We found a direct correlation between the levels of CYLD and acetylated α-tubulin in vitro (Figure 5B). To identify the domain of CYLD responsible for inhibition of HDAC6, we transiently transfected COS cells with different fragments of CYLD (Supplementary Figure S2B) or HDAC6. The polymerised MTs were then incubated with immunoprecipitates of these fragments together with HDAC6. As expected, only the CYLD1–212 fragment, which mediates the association of CYLD with HDAC6 (Figure 4D), was able to inhibit HDAC6 activity and subsequent α-tubulin deacetylation (Figure 5C).

Next we tested whether TPA-mediated CYLD activation inhibits HDAC6 activity by regulating its interaction with tubulin. To this end, we immunoprecipitated HDAC6 from Cyld+/− and Cyld−− keratinocytes and found that TPA treatment, which was found to induce the interaction of CYLD and HDAC6, did not affect the association of HDAC6 with α-tubulin (Figure 5D). Similarly, EGFP–CYLD did not affect the association of HDAC6 with α-tubulin in the melanoma cells (Supplementary Figure S5B).

If loss of CYLD was directly responsible for elevated HDAC6 activity and increased deacetylation of α-tubulin, siRNA-mediated depletion of HDAC6 in EGFP-expressing melanoma cells or Cyld−− keratinocytes should restore α-tubulin acetylation. In line with this hypothesis, the levels of acetylated α-tubulin increased in melanoma cells upon HDAC6 depletion (Figure 5E). Furthermore, HDAC6-depleted Cyld−− keratinocytes showed significant increase in α-tubulin acetylation irrespective of whether they were untreated or treated with TPA (Figure 5F).

Delay in the G1/S-phase of the cell cycle and cytokinesis induced by CYLD

To determine whether CYLD, through its effects on MTs, can regulate the duration of the cell cycle, we used serum starvation in combination with contact inhibition to arrest keratinocytes in G1-phase and nocodazole treatment to synchronize melanoma cells in G2/M-phase, and then analysed cell-cycle progression using flow cytometry (Supplementary Figures S6 and 7). The release of synchronized, TPA-treated keratinocytes caused a delay in G1-to-S phase transition in Cyld+/− but not in Cyld−− keratinocytes (Figure 6A and Supplementary Figure S6). Similarly, release of synchronized, nocodazole-treated melanoma cells revealed a significant delay in G1-to-S phase progression and an accumulation in S-phase of EGFP–CYLD-expressing...
Figure 6 CYLD induces a delay in the G1/S phase of the cell cycle. (A) Cell-cycle distribution of Cyld+/+ and Cyld−/− keratinocytes after synchronization by serum starvation and contact inhibition for 24 h and stimulation with 100 nM TPA for 24 h. (B) Cell-cycle distribution of EGFP or EGFP-CYLD-infected melanoma cells 16 h after synchronization with 30 μM nocodazole. (C) BrdU pulse labelling of keratinocytes synchronized by serum starvation and contact inhibition followed by BrdU exposure (10 μM) for 12 or 24 h after the release. (D) BrdU pulse labelling of EGFP and EGFP-CYLD lentivirus-infected melanoma cells after arrest with nocodazole (30 μM for 16 h) and exposure to BrdU (10 μM) for 12 or 24 h after the release. (E) Duration of mitosis in EGFP- or EGFP-CYLD-infected melanoma cells after synchronization by a double thymidine (2 mM) block. (F) The G1 cell-cycle phase of melanoma cells transiently transfected with EGFP (control), CYLD1–212, or/and Bcl-3 or HDAC6 siRNA and synchronized with nocodazole. (G) The G1 cell-cycle phase of melanoma cells transiently transfected with mock (control), CYLD, CYLDΔ2, or CYLDΔ222–956 and synchronized with nocodazole. *P<0.05, **P<0.01, ***P<0.001.

cells as compared with EGFP-expressing control cells (Figure 6B and Supplementary Figure S7). To further corroborate this finding, we performed BrdU pulse labelling of TPA-stimulated keratinocytes as well as melanoma cells. TPA-stimulated Cyld+/+ keratinocytes and EGFP–CYLD-expressing melanoma cells showed reduced levels of BrdU-positive nuclei as an indication of delayed S-phase progression in the presence of activated CYLD (Figure 6C and D). To exclude the possibility that the delay in the G1/S-phase transition in CYLD-expressing cells is due to differences in mitosis, we quantified mitotic intervals by live-cell imaging. The experiments revealed that EGFP–CYLD-expressing melanoma cells and EGFP-expressing control cells displayed similar rates of mitosis (Figure 6E).

We have reported earlier that CYLD decreases the proliferation of keratinocytes (Massoumi et al, 2006) and melanoma cells (Massoumi et al, 2009) by retaining Bcl-3 in the cytoplasm and thereby reducing the expression of cyclin-D1. Interestingly, depletion of Bcl-3 in EGFP-expressing or control melanoma cells increased the number of cells in G1-phase (Figure 6F), while expression of CYLD1–212 or siRNA-mediated depletion of HDAC6 had no significant effect (Figure 6F). To further assess whether the catalytic activity and/or HDAC6 and MT binding of CYLD are necessary to induce a delay in the cell cycle, we analysed the duration of the cell cycle in melanoma cells expressing full-length CYLD, catalytically inactive CYLDΔ2, or a deletion mutant that lacks the first CAP-Gly domains (CYLDΔ222–956) and does not bind HDAC6. Full-length CYLD induced a delay in G1/S transition, whereas the catalytically inactive CYLDΔ2 or CYLDΔ222–956 had no effect. Together, these results suggest that both deubiquitinase activity as well as HDAC6 binding are required for CYLD to regulate the cell cycle (Figure 6G).

It has been suggested earlier that the localization of HDAC6 in the midbody might regulate mitosis by affecting MT dynamics during cytokinesis (Zhang et al, 2003). This
correlates with the notion that the cytoplasmic bridge connecting the two daughter cells contains high levels of acetylated MTs (Piperno and Fuller, 1985; Schatten et al., 1988; Chu and Klymkowsky, 1989). We observed that CYLD localizes to the midbody during cytokinesis (Figure 7A, upper panels). The localization of CYLD to the midbody has also previously been reported for dividing HeLa cells overexpressing CYLD (Stegmeier et al., 2007). In addition, we observed that CYLD colocalized with HDAC6 in the midbody of both non-stimulated and TPA-stimulated primary Cyld+/+ keratinocytes (Figure 7A, lower panels).

To analyse whether the observed localization of CYLD in the midbody could affect cytokinesis, we determined the duration of cytokinesis using live-cell imaging. The experiments revealed no differences in the duration of cytokinesis in non-stimulated Cyld+/+ and Cyld−/− keratinocytes (Figure 7B), while treatment of cells with TPA induced a significant delay in cytokinetic rate of Cyld+/+ as compared

Figure 7 The N-terminal part of CYLD induces a delay in the cytokinesis rate. (A) The confocal plane of keratinocytes stained for acetylated α-tubulin, CYLD, or HDAC6 as indicated. The arrows indicate the localization of CYLD and HDAC6 in the midbody. (B) Duration of cytokinesis of Cyld+/+ and Cyld−/− cells after TPA (100 nM) treatment. (C) Duration of cytokinesis in melanoma cells (Juso) transiently transfected with mock (control), CYLD, CYLD1–212, CYLD587–956, CYLD587–956, or CYLD5–212–CAP1-Gly mutant. (D) The confocal plane of melanoma (Juso) transfected with expression vectors FLAG-CYLD1–212, or FLAG–CYLD587–956 and stained for acetylated α-tubulin and FLAG. The arrow indicates the localization of CYLD1–212 in the midbody. **P < 0.01.
with that of Cyld\(^{-/-}\) keratinocytes (Figure 7B). This phenotype could also be detected in EGFP–CYLD-expressing melanoma cells as compared with EGFP-expressing control cells (Figure 7C). Furthermore, the N-terminal but not the C-terminal domain of CYLD localized to the midbody of melanoma cells (Figure 7D) and induced a delay in cytokinesis (Figure 7C). However, expressing CYLD\(^{1-212}\), carrying mutations in the CAP-Gly1 domain (Supplementary Figure S2B), to disrupt MT binding (Feierbach et al., 1999), failed to induce a delay in cytokinesis, whereas the catalytically inactive mutant CYLD\(^{275/8}\) retained this ability (Figure 7C). Altogether these results indicate that inhibition of Bcl-3 by the deubiquitinase activity of CYLD induces a delay in the G\(_1\)S transition, while the N-terminal domain of CYLD, which binds MTs and inhibits HDAC6 activity, regulates the perinuclear localization of CYLD and reduces the rate of cytokinesis.

Discussion

CYLD is a deubiquitination enzyme, which acts primarily as a tumour-suppressor gene. Mutations in the CYLD gene were discovered in patients suffering from cylindromatosis, which is a benign tumour derived from the cells of the skin (Bignell et al., 2002). We have recently shown that CYLD expression is very low in melanoma cells due to transcriptional suppression by high levels of Snail (Massoumi et al., 2009). Interestingly, gene mutation studies on cylindroma patients showed that the known mutations can cause either loss of expression or expression of C-terminally truncated versions of CYLD. The C-terminal part of CYLD harbours the UCH domain, which is responsible for the deubiquitinase activity of CYLD (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Massoumi et al., 2006). So far, no cylindroma patients with mutations giving rise to N-terminal truncations of CYLD have been identified (Massoumi and Paus, 2007). It was speculated that this could be due to the ability of this region to delay tumour growth and thus be associated with lower disease penetrance. In the present study we investigated the role of the N-terminal CAP-Gly domains of CYLD in the growth and proliferation of both primary and transformed cells derived from the skin.

The N-terminal region of CYLD contains three CAP-Gly motifs. We found that two of them (CAP-Gly1 and CAP-Gly2) associated with tubulin and inhibited tubulin deacetylation. In addition, we found that TPA treatment of Cyld\(^{+/+}\) keratinocytes caused redistribution of CYLD and induced its accumulation in the perinuclear region, which has been previously shown to be critical in mediating the interaction with its downstream substrate Bcl-3 (Massoumi et al., 2006). In addition, TPA treatment lead to a marked increase in the levels of acetylated MTs in this area, and CYLD was found to extensively colocalize and associate with acetylated MTs. These observations suggest that CYLD colocalizes preferentially with acetylated MTs, and that the TPA-mediated increase of acetylated MTs in the perinuclear region might in fact regulate the translocation of CYLD to this area. This hypothesis is supported by the observation that inhibition of tubulin deacetylation by downregulation of HDAC6 or inhibition of its activity by TSA induced a similar translocation of CYLD to the perinuclear area where it colocalized with the abundant acetylated MTs, whereas depolymerisation of MTs inhibited the translocation.

HDAC6, which has been shown to operate as a \(\alpha\)-tubulin-specific deacetylase (Hubbert et al., 2002), contains two intact catalytic domains in the central region of the protein. Mutations in the catalytic domains or treatment of cells with the HDAC6 inhibitor TSA abrogates the catalytic activity (Zhang et al., 2006) and increases the pool of acetylated tubulin. Several lines of evidence suggest that CYLD regulates the levels of acetylated tubulin by acting as an endogenous inhibitor of HDAC6. First, inhibition of HDAC6 by TSA or tubacin treatment failed to induce an additive effect on acetylated tubulin in the presence of CYLD, suggesting that TSA, tubacin, and CYLD act within the same pathway. In addition, CYLD colocalized with HDAC6 in the perinuclear region, and the endogenous proteins were found in the same protein complex. Finally, we observed that CYLD binds to the catalytic domains of this enzyme and inhibits its tubulin deacetylase activity in vitro.

What are the functional consequences of CYLD-mediated inhibition of HDAC6 and the increased levels of acetylated MTs? We observed that CYLD induces a delay in the G\(_1\)S transition of the cell cycle, both in melanoma cells as well as in keratinocytes. This is in contrast to an earlier study where downregulation of CYLD by siRNA in HeLa cells induced a delay in G2/M transition of the cell cycle (Stegmeier et al., 2007). This discrepancy could be explained by different functions of CYLD depending on the tissue/cell type in combination with the requirement of a specific stimulus such as TPA and/or by siRNA-mediated downregulation of CYLD versus complete absence of the protein. We have previously shown that CYLD, through deubiquitination of Bcl-3, prevents its nuclear translocation. This in turn inhibits the transcriptional activity of NF-kB p50/p52 and reduces the expression of cyclin-D1 (Massoumi et al., 2006). Since expression of cyclin-D1 is critical for the G\(_1\)S transition of the cell cycle, it is very likely that CYLD regulates the G\(_1\)S transition by inhibiting the expression of cyclin-D1. Interestingly, depletion of HDAC6 or expression of the N-terminal domain of CYLD, which interacts and inhibits HDAC6, was unable to increase the number of G\(_1\)-phase cells, suggesting that the inactivation of HDAC6 and hyperacetylation of MTs alone is not the cause of the G\(_1\)/S cell-cycle delay. This is supported by the finding that depletion of HDAC6 was not sufficient to induce the interaction of CYLD with Bcl-3, but TPA-mediated activation of CYLD is additionally required. However, a deletion mutant of CYLD, which is unable to bind HDAC6, but contains an intact catalytic domain, did not induce a delay in cytokinesis. Thus, we suggest that the association of CYLD with acetylated MTs leads to its perinuclear accumulation, which when followed by an additional TPA-mediated signal prevents nuclear accumulation of Bcl-3, leading to decreased cyclin-D1 expression and delay in G\(_1\)/S transition. It is interesting to note that a direct interaction between HDAC6 and Bcl-3 has been reported earlier in other cell types (Viatour et al., 2004), suggesting that formation of these protein complexes could be spatiotemporally linked.

The finding that CYLD colocalizes with HDAC6 in the midbody between bundles of acetylated MTs in both keratinocytes and melanoma cells, prompted us to investigate the rate of cytokinesis in these cells. Although CYLD is present in
the midbody of primary keratinocytes both before and after TPA treatment, CYLD was found to induce a delay in cytokinesis only after TPA treatment. We could also detect a reduction in the rate of cytokinesis in melanoma cells expressing EGFP–CYLD, and in line with the effect of EGFP–CYLD on G1/S-phase transition of melanoma cells, also this effect did not require an additional stimulus. Interestingly, the N-terminal domain, which binds to tubulin and HDAC6, but not the UCH-containing C-terminal domain of CYLD, was able to localize to the midbody and induce a delay in cytokinesis. In addition, mutations in the CAP-Gly1 domain, which impair MT binding, abolished this effect, whereas a catalytically inactive CYLD had no effect. This suggests that localization of CYLD to the midbody is important for its effect on cytokinesis, whereas deubiquitinase activity of CYLD is not required for this function. The reason why CYLD does not have an effect on cytokinesis in the absence of TPA treatment in the primary keratinocytes, despite its presence in the midbody, is unclear. It is possible that TPA induces additional changes in CYLD itself or in unidentified inhibitory proteins, which are necessary for the function of CYLD. This hypothesis is supported by our finding that CYLD is unable to bind HDAC6 in the absence of TPA treatment. The molecular details of this process need to be addressed in future studies.

The precise role of acetylated MTs and tubulin acetylation in the regulation of the cell cycle and mitosis is not understood, although it is known that the cytoplasmic bridge connecting the two daughter cells during cytokinesis is highly enriched in acetylated tubulin in many cell types (Piperno and Fuller, 1985; Schatten et al., 1988; Chu and Klymkowsky, 1989). Previous studies have shown that the degree of tubulin acetylation reflects the dynamics of MTs (reviewed by Westermann and Weber, 2003), and that stable, nocodazole-resistant MTs are essential for recruitment of myosin-II and RhoA to allow contraction of the cleavage furrow during cytokinesis (Bement et al., 2005). In addition, tubulin acetylation itself regulates the recruitment and dynamics of the MT motor protein kinesin-1, which regulates long-distance intracellular delivery (Reed et al., 2006). Interestingly, HDAC6-mediated tubulin acetylation was recently shown to regulate intracellular transport in neurons (Dompiere et al., 2007). It is, therefore, possible that CYLD, through its effect on HDAC6 or a direct effect on the MTs, regulates the dynamics of MTs and/or recruitment of proteins required for successful execution of cytokinesis. Interestingly, other tumour suppressors have previously been shown to use tubulin acetylation as a mechanism to regulate the cell cycle. Loss of the tumour suppressor LAPSER1, which also localizes to the midbody, leads to a decrease in acetylated tubulin accompanied by an increase in the rate of cytokinesis and proliferation (Sudo and Maru, 2007). The tumour suppressor CHFR also localizes to the midbody and regulates the levels of acetylated α-tubulin in the mitotic spindle during cytokinesis (Privette et al., 2008).

Taken together, CYLD simultaneously employs two functional properties to negatively regulate the proliferation rates of keratinocytes and melanoma cells. The abnormal cell-cycle progression through G1 phases and cytokinesis are both hallmarks of cancer cell proliferation. Since many tumour types and cancer cell lines lack or have low CYLD expression (Hellerbrand et al., 2007; Massoumi et al., 2009), our findings provide a mechanistic explanation for their elevated proliferation rates.

Materials and methods

Cell culture and transfection

The melanoma cell lines Mel Im and Mel Juso were derived from metastases of malignant melanomas (Rothhammer et al., 2005). Isolation and culture of primary normal human epidermal melanocytes (NHEM) were performed as previously described by Rothhammer et al (2005). Primary keratinocytes were isolated from back skin of 8-week-old mice and cultured as described by Romero et al (1999).

Viral infections

Stable expression of EGFP, EGFP–CYLD, HA–CYLD, and HA–CYLD/C5 in melanoma cells was achieved by lentiviral gene transfer. In brief, CMV promoter-driven lentiviral constructs were obtained by cloning EGFP, EGFP–CYLD, HA–CYLD, and HA–CYLD/C5 into a lentiviral vector (p15ErInSPTCMV). The titre of the lentiviral particles was determined by measuring the amount of HIV p24 gag antigen by ELISA (Alliance; NEN). To calculate the amount of infectious units (IU), the p24 titre was correlated to the biological activity of a similar virus carrying a green-fluorescent protein (GFP) cassette by using serial dilutions of the GFP virus to transduce 293T cells (1 ng of p24 = 1 × 105 IU). Melanoma cells were infected with 1 × 105 IU for 24 h using the calcium phosphate method and the medium was replaced two times before the cells were used in different assays.

Transient transfections

CYLD truncation mutants were amplified by PCR and cloned into CMV promoter-driven pcS2 construct. The CAP-Gly1 mutant was generated by mutating the GFTDG sequence in the CYLD1–212 construct into AFADA. Transient transfection assays were performed in six-well plates at 80% confluence using the Polyfect transfection reagent (Qiagen) and 1.0 or 3.0 μg of DNA, and 10 or 30 μl of Polyfect were suspended in cell growth medium containing no serum or antibiotics. The mixture was incubated for 10 min at room temperature to allow complex formation. Cells were washed once with phosphate-buffered saline (PBS) before adding 1 ml of fresh cell growth medium containing serum and antibiotics, after which the transfection mixture was added to the cells. Experiments were performed 24 or 48 h after transfection. All transfection assays were repeated three times and performed in triplicates.

Depletion of Bcl-3 and HDAC6

SirRNAs (control, Bcl-3, and HDAC6) were purchased from Santa Cruz. SirRNA duplexes (0.2 μM) were mixed with Lipofectamine (Invitrogen) transfection reagent (10 μl) and incubated at room temperature for 15 min. For each transfection, 0.8 ml sirRNA transfection mixture was added to 80% confluent cells in six-well tissue culture plate two times within 24 h. Experiments were performed 24 h after transfection.

Immunoblots and immunoprecipitation

For immunoblotting, lysates from whole cells or cytosolic and nuclear extracts were resolved in SDS ± PAGE gels and transferred to PVDF membranes, followed by incubation with primary antibodies anti-total α-tubulin; anti-tyrosinated α-tubulin; anti-acetylated α-tubulin; anti HA and α-FLAG M2 agarose beads (all from Sigma); anti-rat monoclonal tubulin (from Abcam); anti-HDAC6; anti-lamin B1 and anti-Bcl-3 (from Santa Cruz). Rabbit polyclonal Cyld antibody has been described earlier (Massoumi et al., 2006). For immunoprecipitations lysates were obtained from untreated or TPA-treated (100 nM) primary keratinocytes, human primary melanocytes (NHEM), or human malignant melanoma cell lines. The samples were centrifuged at 10 000 g for 20 min. For anti-CYLD immunoprecipitation the lysates were pre-cleared for 30 min at 4°C. The protein content was determined and compensated for equal content in all supernatants and used for immunoprecipitation. FLAG immunoprecipitation was performed by using anti-FLAG M2 affinity gel and eluted using FLAG-peptide purchased from Sigma. HA immunoprecipitation was performed by using anti-HA antibody and eluted using HA-peptide purchased from Sigma. EGFP immunoprecipitation was performed by using anti-GFP microBeads purchased from Miltenyi Biotech and used according to the manufacturer’s instructions. Immunoblots were developed with...
the ECL-plus reagent (Amersham Biosciences) according to the manufacturer’s instructions.

**Immunofluorescence**

Cells were grown on glass coverslips and fixed with ice-cold 100% methanol. After rehydration with PBS, unspecific binding was blocked with PBS containing 3% bovine serum albumin (BSA) and 5% goat serum, followed by incubation with primary antibodies against CYLD (Massoumi et al., 2006), tyrosinated tubulin (Chemicon), FLAG (Sigma), acetylated tubulin (Sigma), and HDAC6 (Santa Cruz), and fluorescence labelled secondary antibodies (Alexa, Invitrogen). Finally, the coverslips were washed and mounted on glass slides using Vectashield (Vector Laboratories). The fluorescence images were collected by laser-scanning confocal microscopy (DMIRE2; Leica) using Leica Confocal Software version 2.5 Build 1227 with ×100 oil-immersion objectives. All images were collected at room temperature. Colocalization was measured by line scans from immunofluorescence images using the MetaMorph software.

**In vitro tubulin deacetylation assay**

Tubulin deacetylation assays were performed as described earlier (Hubbert et al., 2002). Briefly, MAP-enriched bovine tubulin (cytoskeleton) was polymerised into microtubules in the absence of paclitaxel or glycerol by incubation for 30 min at 35°C. The polymerised microtubules were incubated at 37°C for 2 h with HA–HDAC6 and/or FLAG–CYLD immunoprecipitates (after elution using HA respective FLAG peptide; Sigma) in the absence or presence of TSA (0.5 mM). Samples were then placed on ice for 15 min. The supernatant was subsequently collected by centrifugation and analysed by immunoblotting with antibodies against acetylated tubulin and total tubulin.

**Flow cytometry and BrdU labelling**

Malignant melanoma cells were synchronized by treatment with 100 ng/ml nocodazole for 16 h. Primary keratinocytes were synchronized by a combination of contact inhibition and serum starvation for 12 or 24 h. The cells were then treated with 2 mM thymidine for 19 h. After a 5-h release in complete culture medium, the cells were treated with thymidine for another 16 h. After release, images of live cells were recorded with a Zeiss Axiovert 200M (Zeiss, Germany) with a cooled CCD camera (Roper Scientific, Princeton, NJ, USA). Image acquisition was performed with the MetaMorph software (Molecular Devices, Downington, PA, USA). For quantification of mitotic intervals, the duration of mitotic phases were defined as follows: prometaphase, from disassembly of nuclear membrane and nucleoli to equatorial arrangement of chromatin; metaphase, from the beginning to the end of equatorial arrangement of chromatin; anaphase, from the start of sister chromatid separation to reformation of nuclear envelopes and nucleoli; telophase, from the start of nuclear envelope and nuclear reformation to formation of two daughter cells.

For quantification of cytokinesis cells were plated on plastic culture dishes in complete culture medium and stimulated with 100 nM TPA, when indicated. Images of live cells were recorded as described above. The duration of cytokinesis was quantified from the formation of two daughter cells to abscission.

**Statistical analysis**

Data are presented as mean ± s.e.m. Statistical comparisons were assessed with analysis of variance (ANOVA) or Student’s t-test after data were confirmed to fulfil the criteria of normal distribution and equal variance. If overall ANOVA tests were significant, we performed a post hoc Tukey test. P < 0.05 was considered significant.

**Supplementary data**

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Quantification of mitotic phases and cytokinesis**

Malignant melanoma cells were plated on plastic culture dishes and synchronized using a double thymidine block. Briefly, cells were treated with 2 mM thymidine for 19 h. After a 5-h release in complete culture medium, the cells were treated with thymidine for another 16 h. After release, images of live cells were recorded with a Zeiss Axiovert 200M (Zeiss, Germany) with a cooled CCD camera (Roper Scientific, Princeton, NJ, USA). Image acquisition was performed with the MetaMorph software (Molecular Devices, Downington, PA, USA). For quantification of mitotic intervals, the duration of mitotic phases were defined as follows: prometaphase, from disassembly of nuclear membrane and nucleoli to equatorial arrangement of chromatin; metaphase, from the beginning to the end of equatorial arrangement of chromatin; anaphase, from the start of sister chromatid separation to reformation of nuclear envelopes and nucleoli; telophase, from the start of nuclear envelope and nuclear reformation to formation of two daughter cells.

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**Supplementary data**

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