Down-regulation of CYLD as a trigger for NF-κB activation and a mechanism of apoptotic resistance in hepatocellular carcinoma cells

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Received August 4, 2010; Accepted September 23, 2010

DOI: 10.3892/ijo_00000831

Abstract. The cylindromatosis gene (CYLD) was identified as a tumor suppressor gene, which is mutated in familial cylindromatosis (Brooke-Spiegler syndrome), an autosomal-dominant predisposition to multiple tumors of the skin appendages. CYLD is a deubiquitinating enzyme acting as a negative regulator of the nuclear factor κB (NF-κB) signaling pathway by removing lysine-63-linked polyubiquitin chains from NF-κB activating proteins. In order to investigate the role of CYLD in apoptotic signaling in human hepatocellular carcinoma (HCC) cells, we first studied the expression levels of CYLD in HCC tissues. CYLD expression was lower in HCC both at protein and mRNA levels compared to the surrounding non-malignant tissue. In order to further study the role of CYLD in the apoptotic sensitivity of HCC cells, CYLD was specifically down-regulated in HCC cell lines via RNA interference. The specific down-regulation of CYLD resulted in increased resistance towards treatment with doxorubicin, 5-fluorouracil and cisplatin. In addition, the down-regulation of CYLD in HCC cells decreased the sensitivity towards tumor necrosis factor-α-induced apoptosis. The CYLD knockdown also led to the degradation of the NF-κB inhibitor, IκB-α, resulting in enhanced NF-κB activity in HCC cells. Finally, we found that CYLD expression was triggered by the multikinase inhibitor, sorafenib, by the inhibition of Raf-1, as well as by the blockage of the pro-survival kinases, MEK (U0126) and the epidermal growth factor receptor (AG1478). In summary, we show that CYLD is down-regulated in human HCC and is involved in the apoptotic resistance of HCC cells. Our data indentify the reconstitution of CYLD expression as an attractive approach for overcoming resistance to treatment in HCC.

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Key words: cylindromatosis gene, hepatocellular carcinoma, nuclear factor κB, IκB-α, apoptosis, tumor necrosis factor-α, doxorubicin

Introduction

Hepatocellular carcinoma (HCC) is among the most common malignancies worldwide. It ranks third on the list of malignancies leading to death (1,2). HCC is clinically characterized by its invasiveness, poor prognosis and limited therapeutic opportunities, which are mostly due to the high resistance of HCC cells towards chemo- and radiotherapy. Today, surgery is considered to be the only curative treatment procedure for most HCC patients (3). Apoptosis is a genetically determined process of controlled cellular suicide (4). The dysregulation of apoptosis is involved in the pathophysiology of liver diseases including hepatocarcinogenesis (5). The resistance of HCC cells towards apoptosis is a crucial point in cancer treatment as it impairs the efficacy of different therapy regimens (6). Nuclear factor κB (NF-κB) is known as a key molecule controlling apoptosis (7-10). The NF-κB protein family consists of five NF-κB subunits, which are divided into two classes due to their structural homology: Members of class one include p50 and its precursor p105 (NF-κB1), as well as p52 and its precursor p100 (NF-κB2). p65 (RelA), RelB and c-Rel are class two NF-κB family proteins. Active NF-κB dimers are generated via homodimeric and heterodimeric interactions of the subunits. For example, p50/RelA generally activates genes involved in diverging processes, including the regulation of immune and inflammatory responses, apoptosis and proliferation (11). The NF-κB signaling pathway has been found to be aberrantly activated

in human HCC tissues and cell lines (12). Other studies have also shown that the activation of the transcription factor, NF-κB, is one of the early events involved in neoplastic progression in the liver (13). The anti-apoptotic effects of NF-κB depend on the induction and expression of anti-apoptotic genes including cellular inhibitors of apoptosis, c-FLIP, anti-apoptotic Bcl-2 family members A1 (Bfl1) and BCL-x_L, as well as regulatory adaptor proteins for c-Jun N-terminal kinase (JNK) activation and NF-κB itself (14). The activation of the NF-κB transcription factor is mediated via the 'classical' or the 'alternative' pathway (15). In the 'classical' pathway, the binding of tumor necrosis factor-α (TNF-α), interleukin-1 or lipopolysaccharide, includes the activation of TGF-ß activated kinase 1 as well as the recruitment and the lysine-63 (K-63)-linked auto-ubiquitination of TNF receptor associated factor (TRAF) 2. This triggers the binding and activation of the IκB kinase (IKK) (inhibitor of NF-κB kinase) complex as a central step in receptor-mediated NF-κB activation. The IKK complex includes IKKα, IKKß and IKKγ [NF-κB essential modulator (NEMO)]. IKK activation also involves the conjugation of the K-63-linked ubiquitin chains to the JNK and NF-κB regulatory signaling factor, TRAF6, and to the IKK regulatory subunit, NEMO (15-17). The activation of IKK leads to the proteasomal degradation of IκB (inhibitor of NF-κB) proteins, and thus the NF-κB subunits translocate into the nucleus to fulfill their transcriptional functions (18). In HCC, IKKα and IKKß have been found to be highly expressed, causing enhanced NF-κB activation, and thus contributing to the malignant properties of liver cancer (19). There is much evidence that K-63-linked ubiquitin chains facilitate the protein-protein interactions in the NF-κB signaling cascade (20-22).

The tumor suppressor cylindromatosis gene (CYLD) was identified as a gene mutated in familial cylindromatosis, an autosomal-dominant predisposition to multiple tumors of the skin appendages (23). CYLD encodes a protein containing an ubiquitin C-terminal hydrolase domain, allowing the protein to act as a deubiquitinating enzyme (20). CYLD removes K-63-linked polyubiquitin chains from proteins involved in the NF-κB signaling pathway (21). *In vitro* studies have identified CYLD as a protein associated with important cellular processes, such as cellular activation, apoptosis, inflammation, proliferation and tumorigenesis (16,22,24-26). CYLD acts as a negative regulator of the NF-κB signaling pathway and diminishes the expression of NF-κB-dependent genes due to its deubiquitinating activity on TRAF2 and 6 and NEMO (20,27). Another target of CYLD is the B-cell lymphoma 3-encoded protein (Bcl-3), which is associated with the NF-κB p50 or p52 subunits, thereby enhancing cell proliferation and oncogenesis (24,28). CYLD knockout mice do not spontaneously develop tumors, although they are more sensitive to chemically-induced skin tumors compared to wild-type mice. The loss of CYLD promotes the proliferation of tumors derived from epidermal keratinocytes due to the enhanced Bcl-3-dependent expression of cyclin D1. Functional CYLD deubiquitinates Bcl-3, thereby preventing its nuclear accumulation and its NF-κB co-activating function (24). In addition, another study has demonstrated that CYLD knockout mice are more susceptible to chemically-induced colon tumors (29). CYLD has been found to be down-regulated or lost in HCC and colorectal carcinoma cell lines. Moreover, HCC tissues have also revealed reduced CYLD expression levels (30). However, these studies present strong evidence that the loss of the tumor suppressive function of CYLD could be associated with different types of tumors in addition to human cylindromas (16). Regarding melanoma cell lines, it has been demonstrated that the CYLD expression is negatively regulated via the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway (31). The MAPK/ERK pathway regulates cell proliferation and differentiation. The activation of ERK rescues cancer cells from pro-apoptotic signals by modulating the balance of pro- and anti-apoptotic proteins (32,33).

In this study, we investigated the role of CYLD in the apoptotic sensitivity of HCC cells. We detected a significant down-regulation of CYLD in human HCC tissues compared to surrounding non-malignant liver tissues. Furthermore, our results show that the small interfering RNA (siRNA)-mediated CYLD knockdown, leads to enhanced NF-κB activity and increases the resistance of HCC cells towards chemotherapy- and TNF-α-induced apoptosis. Finally, we identified ways for the modulation of CYLD protein expression in HCC cells and found that the CYLD expression can be considerably reconstituted by the epidermal growth factor receptor (EGFR), MEK and Raf-1 targeting inhibitors.

Materials and methods

Reagents and cell lines. The HCC cell lines, Huh7 and Hep3B, were purchased from ECACC. Cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 1% Pen/Strep (PAA Laboratories, Pasching, Austria), 1% HEPES and 1% L-glutamine (Cambrex, Verviers, Belgium). Cells were cultivated at 37˚C with a concentration of 5% CO_2. Transfection experiments were performed in Opti-MEM (Invitrogen). Reagents were purchased from the following suppliers: TNF-α from Biomol (Hamburg, Germany) and cycloheximide from Carl Roth (Karlsruhe, Germany). 5-Fluorouracil (5-FU), doxorubicin from Sigma-Aldrich (Hamburg, Germany), cisplatin, AG1478, UO126, Raf-1 kinase inhibitor I (Raf-1 inh. I) from Calbiochem (Schwalbach, Germany) and sorafenib (BAY 43-9006) from Bayer (Leverkusen, Germany).

Immunohistochemistry. HCC cancer tissue samples from six patients [all with liver cirrhosis of different origin (chronic hepatitis B and C, non-alcoholic and alcoholic steatohepatitis and hemochromatosis)] undergoing elective surgery for HCC at the University Clinic of Mainz, were analyzed. Sample preparation was performed in accordance with the local ethics committee. Tissues were shock frozen and stored at -80˚C. Then, tissues were sectioned and immunohistochemically anlayzed. Epitope retrieval was performed by 20-min microwave pre-treatment of the slides in 10 mmol sodium citrate buffer, pH 6.0. After blocking of unspecific binding with TNB-buffer (1% BSA, 0.5% protein-blocking reagent in TBS) the slides were washed twice with TPBS (0.5% Tween in PBS). Incubation with CYLD primary antibody (Santa Cruz, Heidelberg, Germany) was performed in 1:50 dilution in TPBS overnight at 4˚C. For the negative controls, the

primary antibody was omitted. After washing in TPBS buffer twice, the slides were incubated with secondary anti-mouse Cy3-conjugated IgG antibody (Jackson Immuno Research, Newmarket, UK). After washing, the sections were counter-stained with 2 μg/ml Hoechst (DAPI) in PBS for 10 min at room temperature. The images were captured using a Zeiss Axiophot microscope (Zeiss, Gottingen, Germany) equipped with an Olympus Camedia camera (Olympus, Hamburg, Germany).

Real-time quantitative polymerase chain reaction (RT-QPCR). HCC cancer tissue samples as well as samples from surrounding non-malignant liver tissues from ten patients (all with liver cirrhosis of different origin undergoing elective surgery for HCC at the University Clinic of Mainz), were analyzed. Sample preparation was performed in accordance with the local ethics committee. Total RNA from HCC and non-malignant tissues of patients, as well as from the HCC cell lines, Huh7 and Hep3B, were isolated using TRI Reagent (Sigma-Aldrich). cDNA was synthesized from total RNA with oligo(dT) primers by using the Omniscript cDNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Specific mRNA transcripts were quantified using LightCycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany) and the following primers: CYLD forward, 5'-TTGACTCACAACCACAATCCA-3'; and CYLD reverse, 5'-TGTAAGAGATTTTGCAGGGT CTT-3'. The housekeeping gene ribosomal protein S6 (Rps6) was used as the reference: Rps6 forward, 5'-TCCGCAAAC TTTTCAATCTC-3'; and Rps6 reverse, 5'-GCTTCTTC AGAGCAATACGC-3'. Data acquisition and determination of gene expression was performed using the LightCycler software package (Roche). Each PCR reaction was run in duplicates. CYLD mRNA expression was compared to the expression of the housekeeping gene and was expressed as the x-fold expression relative to the non-malignant tissues or the control-treated cells, respectively.

RNAi transfection. In order to knock down protein expression, we administered specific siRNA targeting CYLD mRNA. As a control we used scrambled siRNA with unspecific sequences. The following siRNA sequences were applied (MWG Biotech, Ebersberg, Germany): CYLD, 5'-guaua ggacaguauauucaTT-3'(sense) and 5'-ugaauauacuguccu auacTT-3'(antisense); control, 5'-agacccacucggaugug aagagaua-3' (sense) and 5'-uaucucuucacauccgagugggucu-3' (antisense). Here, capitals represent desoxyribonucleotides and lower case letters represent ribonucleotides. Huh7 and Hep3B cells were seeded into 6- or 12-well plates and after 24 h were transiently transfected in Opti-MEM with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Expression levels were analyzed 24, 48 and 72 h after transfection via Western blot analysis.

Detection of apoptosis. HCC cells were seeded into 12-well plates and treated as indicated 24 h after transfection. After treatment (48 h), cells were washed with cold PBS, collected and re-suspended in a hypotonic buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/ml propidium iodide (Sigma-Aldrich). After 3-h incubation at 4˚C, nuclei from apoptotic cells were quantified for DNA content by fluorometric absorbance cell sorting (FACS) as previously described by Nicoletti *et al* (34).

NF-κB activity reporter gene assay. Huh7 cells were seeded into 6-well plates one day before transfection. Cells were transfected with firefly luciferase encoding plasmid under the control of a NF-κB-dependent promoter (NF-κB-Luc) (a kind gift from Gisa Tiegs, University Medical Center, Hamburg-Eppendorf, Germany) using Lipofectamine LTX and Plus Reagent (Invitrogen). The NF-κB-Luc plasmid was transfected either alone or co-transfected with either siRNA against CYLD or control siRNA. Cells were treated as indicated, then harvested and luciferase activity was measured using the luciferase reporter gene assay kit (Roche) in a multiwell plate reader (Appliskan, Thermo). Co-transfection of the GFP plasmid and siRNA were used as the control for equal transfection efficiency, anlayzed by FACS.

Caspase-3 activity assay. Cells were lysed in buffer containing 20 mM Tris/HCl pH 8.0, 5 mM EDTA, 0.5% Triton X-100 and 1X complete protease inhibitor cocktail (Roche). Protein concentration was equilibrated by Bradford assay (Bio-Rad, Munich, Germany). Lysates were incubated in reaction buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 10% glycerol, 0.05% CHAPS and 5 mM DTT) in the presence of 50 μM caspase-3 fluorogenic substrate (Ac-DEVD-AFC) (Biomol). Assays were performed in black MaxiSorp microtiter plates (Nunc, Langenselbold, Germany), and the generation of free AFC at 37˚C after 1 h incubation, was measured using a fluorometric plate reader (Appliskan, Thermo) set to an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Cell lysis and Western blotting. Cells were seeded into 6-well plates, cultured for 24 h and treated as indicated. Nuclear and cytosolic extracts were prepared as follows: Cells were washed once with ice cold PBS, scraped and lysed at 4˚C by gently pipetting in hypotonic buffer A [20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 0.5 mM PMSF, 1 mg/ml, 1:1000 protease inhibitor cocktail (Roche), 1 mM DTT and 0.2% NP-40]. After 10-sec centrifugation at 4˚C (13,000 rpm), the supernatants were collected as cytosolic extracts. Nuclear extracts were prepared by re-suspension of the crude nuclei in buffer B [buffer A modified to high-salt buffer (hypotonic buffer with 20% glycerol and 420 mM NaCl)] and freezing at -80˚C for 30 min. After thawing on ice, supernatants were collected after centrifugation at 4˚C (13,000 rpm) for 5 min. Whole cell lysis, SDS-PAGE and Western blotting were performed as described previously (35). Immunodetection of proteins was performed using the following antibodies: Anti-CYLD (Santa Cruz), anti-IκB-α, anti-p50, anti-p65/RelA, anti-HDAC1 (Cellsignaling, Frankfurt, Germany) and anti-α-tubulin (Sigma-Aldrich) as the loading control.

Statistical analysis. Data were analyzed using the Student's t-test (paired, two-sided) based on normal data distribution. A value of $P<0.05$ was considered to be significant.

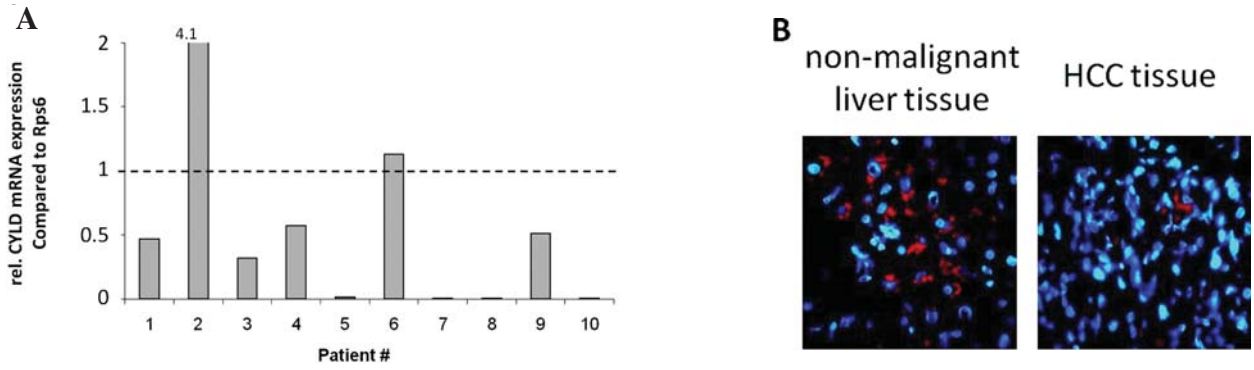

Figure 1. Reduced CYLD expression in human HCC. (A) Relative CYLD mRNA expression levels of ten HCC tissues compared to surrounding non-malignant liver tissues analyzed by RT-QPCR. CYLD expression in HCC tissues is shown relative to that in non-malignant tissues set to 1. Relative CYLD mRNA expression was calculated as described in Materials and methods. (B) Sections of cryopreserved HCC and adjacent non-malignant liver tissues were tested for CYLD expression by immunohistochemistry (red). Nuclei (blue) were counterstained using DAPI. Images are representative of at least six tumor and six non-malignant liver tissues.

Results

Reduced CYLD expression in human HCC. HCC cells show a relatively resistant phenotype towards apoptotic induction after chemotherapy (35). However, little is known about the alterations in the apoptotic pathways responsible for this resistance. As CYLD exerts pro-apoptotic functions, the loss of CYLD has been shown to contribute to apoptotic resistance in human cancer (16,20,22,24). Thus, we analyzed whether CYLD mRNA expression is altered in human HCC tissues. CYLD mRNA expression was considerably reduced in HCC tissues compared to the surrounding non-malignant liver tissues (set to 1) of the same patients (Fig. 1A). For the detection of CYLD expression at the protein level, HCC tissues were immunohistochemically analyzed compared to the non-malignant liver tissues. Heterogeneous CYLD protein expression levels were detected in both the HCC and non-malignant tissues. However, we detected reduced ratios of CYLD-positive hepatocytes in the HCC compared to the control tissues (Fig. 1B). These findings demonstrate that CYLD expression is frequently reduced in human HCC.

Specific down-regulation of CYLD enhances resistance of HCC cells to chemotherapy. It has already been demonstrated that HCC and colorectal carcinoma cell lines show reduced CYLD expression levels (30). We assumed that a further reduction in CYLD expression could enhance the resistance of HCC cells towards chemotherapeutic drug-induced apoptosis. Therefore, we applied either specific siRNA against CYLD mRNA or control siRNA (siControl) in the cell lines, Huh7 and Hep3B. The application of siRNA led to the effective reduction of CYLD protein expression levels after 24, 48 and 72 h (Fig. 2A, left panel). At the mRNA level, we detected a reduction in CYLD transcription of ~70% after 24 and 48 h. In the Hep3B cells, we detected a reduction of 75% after 24 h and 90% after 48 h compared to the CYLD mRNA expression of the siControl-transfected cells (Fig. 2A, right panel). siRNA transfection induced spontaneous apoptosis in ~6% of the Huh7 and Hep3B cells without significant differences between the CYLD siRNA (siCYLD) and siControl, indicating that the results were independent from the methodical toxicity of siRNA transfection. After transfection (24 h), cells were treated for 48 h with the chemotherapeutic agents, doxorubicin, 5-FU and cisplatin. Additionally, cells were treated with the multi-kinase inhibitor, sorafenib. Treatment of Huh7 cells with doxorubicin (1, 2, 4 μM for 48 h) resulted in significantly lower ratios of apoptotic cells after the suppression of CYLD. Doxorubicin (1 μM) induced apoptosis in 33% of the siCYLD-treated cells compared to 46% of the control-transfected cells. 5-FU treatment of CYLD-negative Huh7 cells also showed significant differences in apoptotic induction at concentrations of 50 μg/ml (siCYLD 29% vs. siControl 38%) and 100 μg/ml (siCYLD 36% vs. siControl 43%). For cisplatin, we observed highly significant differences at 2.5 μg/ml. Here, only 20% of the CYLD-negative cells were detected as apoptotic compared to 43% of the control-transfected cells. Sorafenib treatment showed no significant differences between the siCYLD- and siControl-transfected Huh7 cells (Fig. 2B). Treatment of CYLD-negative Hep3B cells with doxorubicin (4 μM, 48 h) resulted in an ~20% reduced rate of apoptosis relative to the control-transfected cells. Following the treatment with 5-FU, we also detected significant differences in apoptotic induction at concentrations of 25 μg/ml (siCYLD 34% vs. siControl 40%) and 50 μg/ml (siCYLD 36% vs. siControl 44%). The cisplatin (10 μg/ml, 48 h) treatment of CYLD-negative Hep3B cells led to 44% apoptotic cells compared to 61% in the control-transfected cells. In the sorafenib treatment, we did not detect significant differences between the siCYLD and siControl pre-treatment (Fig. 2C). Our findings support the hypothesis that reduced CYLD expression increases the apoptotic resistance of HCC cells, thereby contributing to protection towards chemotherapy.

Specific down-regulation of CYLD enhances resistance of HCC cells towards TNF-α-induced apoptosis. NF-κB controls cell survival in the TNF-R1 signaling pathway (7). As CYLD is known to be a negative regulator of NF-κB (27), we analyzed the effect of the CYLD knockdown on TNF-α-induced apoptosis in HCC cells. We treated Huh7 and Hep3B cells with TNF-α (0.1 and 0.2 μg/ml) in combination

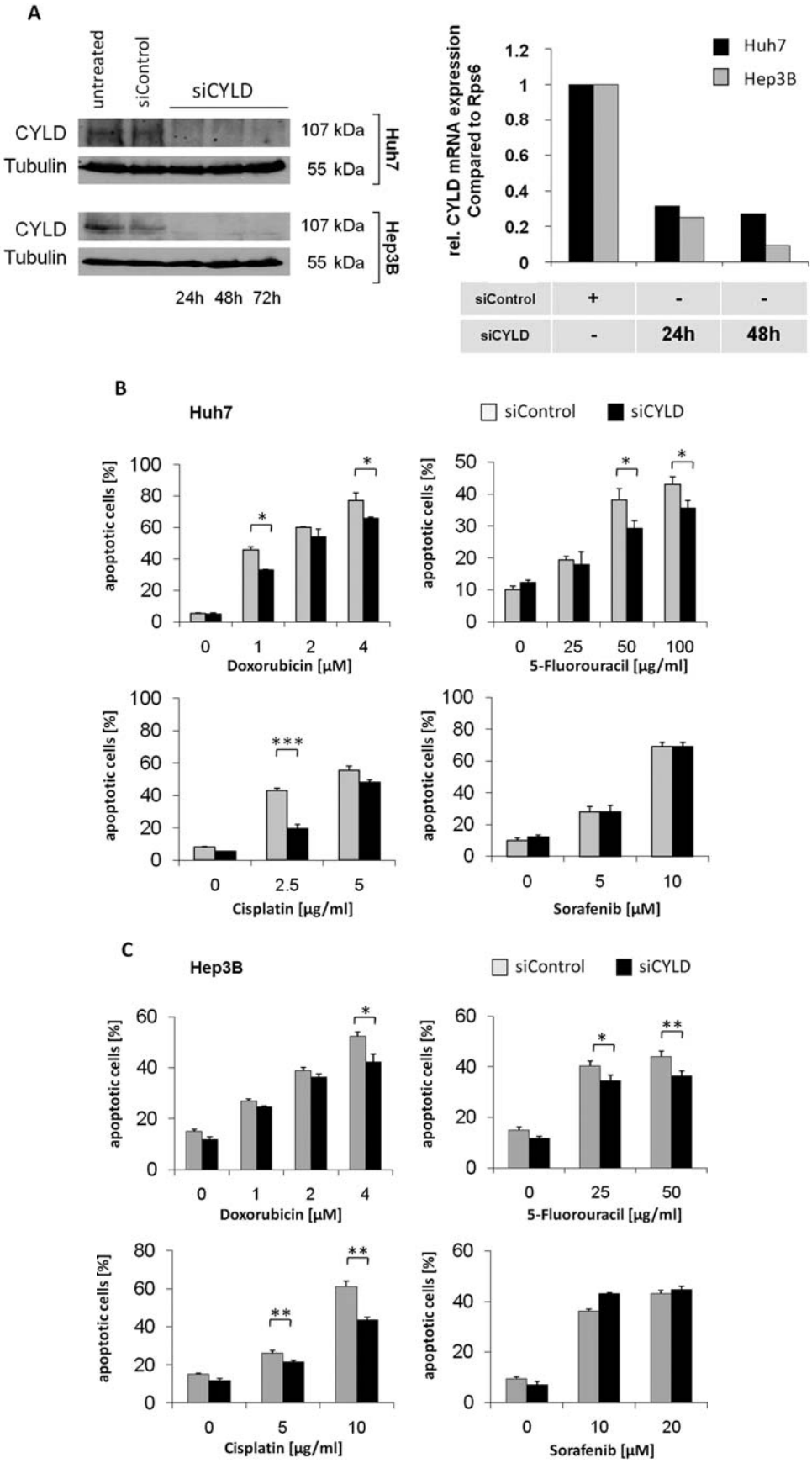

Figure 2. CYLD knockdown via RNA interference inhibits chemotherapy-induced apoptosis in HCC cells. Huh7 and Hep3B cells were transfected with 40 nM siRNA specific for CYLD (siCYLD) or unspecific control siRNA (siControl). Whole cell lysates were prepared 24, 48 and 72 h after transfection. Total RNA extracts were prepared 24 and 48 h after transfection. (A) Protein expression of CYLD and tubulin as the control for equal loading was analyzed by Western blotting (left panel). CYLD mRNA expression was analyzed by RT-QPCR relative to the expression levels of siControl-treated cells (right panel). (B) Huh7 and (C) Hep3B cells were transfected with siCYLD and siControl (40 nM). 24 h after transfection, cells were treated with doxorubicin, 5-FU, cisplatin and sorafenib for 48 h as indicated. Cells were harvested and analyzed for apoptotic induction by flow cytometry. Assays were performed in triplicates and are representative of at least three independent experiments. Values are the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

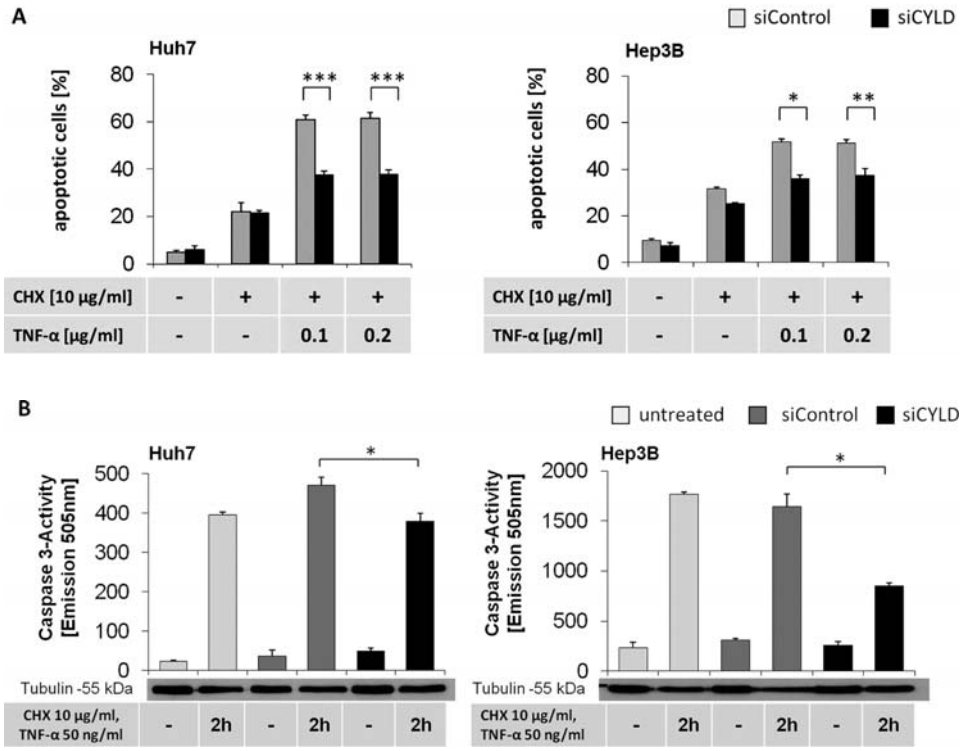

Figure 3. CYLD knockdown inhibits TNF-α-induced apoptosis of HCC cells. (A) Huh7 (left panel) and Hep3B (right panel) cells were transfected with 40 nM siRNA specific for CYLD (siCYLD) and unspecific siRNA (siControl). After transfection (24 h), cells were treated with TNF-α and 10 µg/ml CHX for 24 h as indicated. Cells were harvested and analyzed for apoptotic induction by flow cytometry. Assays were performed in triplicates and are representative of at least three independent experiments. (B) Caspase-3 activity assay of siCYLD- and siControl-transfected Huh7 (left panel) and Hep3B (right panel) cells. Cells were treated with TNF-α (50 ng/ml) and 10 µg/ml CHX for 2 h as indicated, lysed and caspase-3 activity was measured by fluorometric analysis. Application of equal protein amounts was validated by Western blot analysis using anti-tubulin antibody. Assays were performed in triplicates and are representative of at least three independent experiments. Values are the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

with 10 µg/ml cycloheximide (CHX) for 24 h. CHX is known to inhibit protein synthesis and to synergistically increase sensitivity to TNF-α-induced apoptosis (36). The CYLD knockdown in Huh7 cells decreased the ratio of apoptotic cells to 38% compared to 61% in the control-transfected cells after treatment with TNF-α (0.1 µg/ml) and CHX (Fig. 3A, left panel). In the Hep3B cells, the same treatment induced apoptosis in 36% of the siCYLD-transfected cells compared to 52% of the control-tranfected cells (Fig. 3A, right panel). In the treatment of HCC cells with 0.2 µg/ml TNF-α, we observed similar results (Fig. 3). Caspase-3 activity was significantly lower in the siCYLD-transfected Huh7 cells compared to the control-transfected cells after treatment with TNF-α (50 ng/ml) and CHX for 2 h (Fig. 3B, left panel). Caspase-3 activity was reduced by ~50% in the siCYLD-transfected Hep3B cells after 2 h of TNF-α/CHX treatment (Fig. 3B, right panel). These data illustrate the central role of CYLD in TNF-α-mediated apoptosis.

CYLD is a negative regulator of NF-κB activity in HCC cells. In order to analyze the impact of the CYLD knockdown on NF-κB activity in HCC cells, we transfected Huh7 cells with plasmid DNA encoding NF-κB-Luc in combination with siCYLD or siControl. siCYLD/NF-κB-Luc co-transfected Huh7 cells significantly showed higher basal NF-κB activity (~35%) and an ~53% higher NF-κB induction level after TNF-α treatment (50 ng/ml, 2 h), relative to the control co-transfected Huh7 cells. Efficient siRNA transfection and equal loading was confirmed via Western blot analysis (Fig. 4A, left panel). Equal NF-κB-Luc plasmid transfection efficiency was verified via FACS analysis of the GFP-positive cells in GFP-plasmid/siRNA co-transfection experiments (Fig. 4A, right panel). Western blot analysis of HCC cells treated with siCYLD for 24, 48 and 72 h showed lower expression levels of IκB-α, indicating increased NF-κB activity (Fig. 4B). In addition, Huh7 cells treated with siCYLD showed higher expression levels of p50. RelA was unaffected (Fig. 4C). In order to analyze the translocation of the p50 and RelA subunits, we performed Western blot analysis of the nuclear and cytosolic fractions of siCYLD- and siControl-transfected Huh7 cells after stimulation with TNF-α (for 10, 20, 60 min, respectively). We observed a more rapid RelA accumulation in the nucleus in the siCYLD, compared to the control-transfected cells. p50 was detected in the nuclei of siCYLD-treated cells, but not in the control cells, after 1 h of TNF-α stimulation. Cytosolic fractions showed higher basal levels of RelA and p50 in the siCYLD-treated Huh7 cells. Cytosolic IκB-α expression was reduced in the siCYLD-transfected and unstimulated Huh7 cells, compared to the unstimulated control cells. Incubation with TNF-α led to a rapid IκB-α degradation in both, siCYLD- and siControl-treated cells (Fig. 4D). Our results identify CYLD as a NF-κB regulating

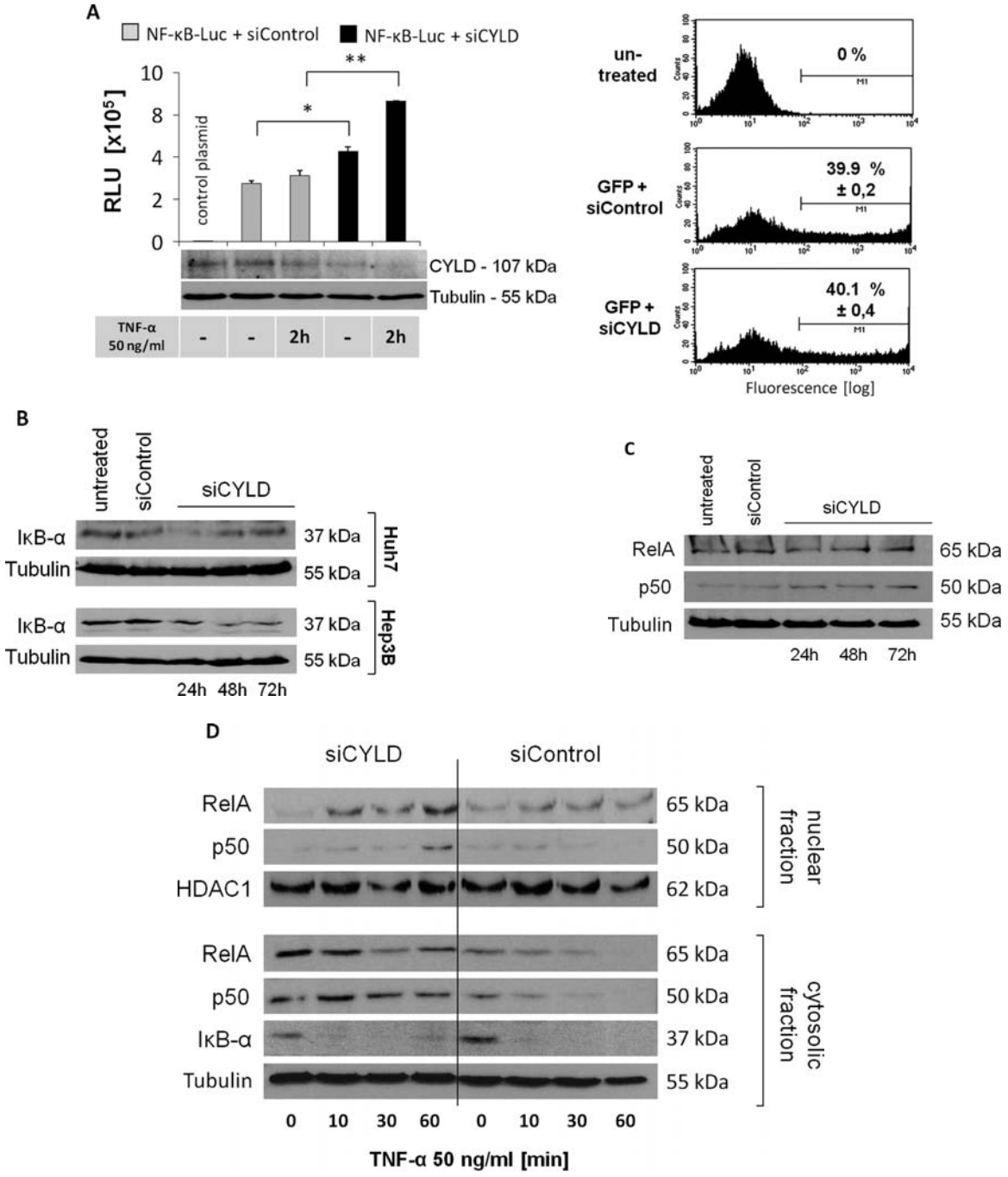

Figure 4. CYLD knockdown results in enhanced NF-κB activity in HCC cells. (A) Huh7 cells were co-transfected with 5 μg NF-κB-Luc plasmid and 40 nM unspecific (siControl) or CYLD siRNA (siCYLD), respectively. After transfection (24 h), cells were treated with TNF-α (50 ng/ml) for 2 h as indicated. Luciferase activity was analyzed by adding luminometric substrate to 20 μg protein in each sample. RLU, relative luminescence units. Values are the means ± SD. Assays were performed in triplicates and are representative of at least two independent experiments. CYLD expression for efficient knockdown and tubulin levels for equal loading were analyzed by Western blotting (left panel). Equal transfection efficiency was verified via GFP-plasmid/siRNA co-transfection, followed by FACS analysis (right panel). *p<0.05, **p<0.01. (B) Western blot analysis for IκB-α expression of whole lysates of CYLD siRNA and control siRNA treated Huh7 and Hep3B cells. (C) Western blot analysis for RelA and p50 expression of whole lysates of siCYLD- and siControl-treated Huh7 cells. (D) siControl- and siCYLD-transfected Huh7 cells were treated with TNF-α (50 ng/ml) for the indicated time. Nuclear protein levels of RelA, p50 and HDAC1 as the loading control were analyzed by Western blot analysis. Cytosolic fractions were analyzed for RelA, p50, IκB-α and tubulin as the loading control.

factor in HCC cells and indicate the importance of CYLD for the response to stimuli activating the 'classical' NF-κB pathway.

CYLD expression in HCC cells can be reconstituted by inhibition of the EGFR-Raf-MEK-ERK signaling cascade. The anti-apoptotic EGFR-Raf-MEK (MAPK/ERK) signaling pathway is a key signaling pathway involved in the regulation of proliferation, survival and differentiation, and is found to be frequently activated in human cancer, including HCC (32,37). A recent study on melanoma cell lines has proven the negative regulation of CYLD via this pathway

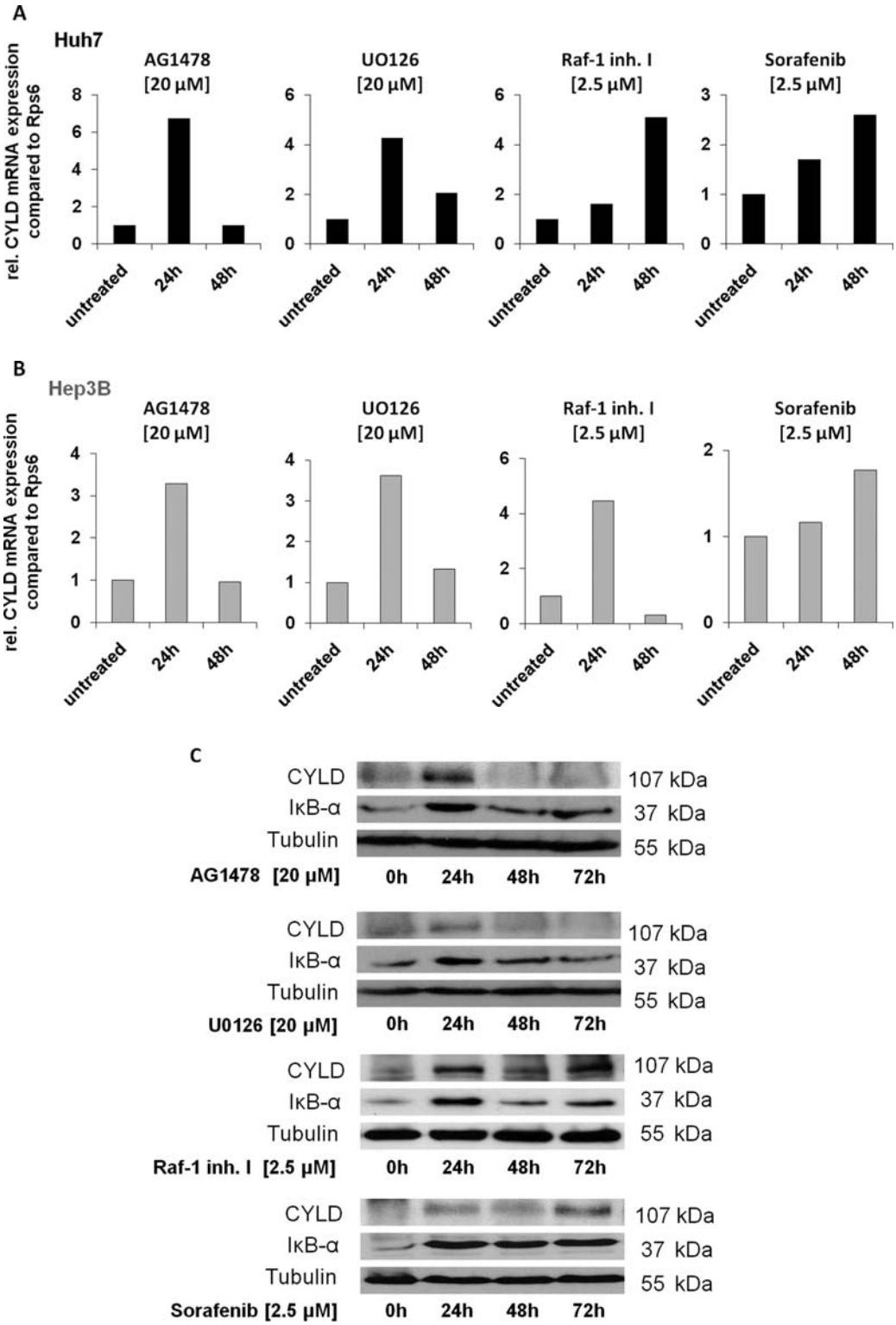

Figure 5. Reconstitution of CYLD expression in HCC cells by disruption of the EGFR-Raf-MEK-ERK signaling pathway. Huh7 (A) and Hep3B (B) cells were treated with the EGFR tyrosine kinase inhibitor, AG1478 (20 μM), the MEK inhibitor, U0126 (20 μM), Raf-1 inh. I (2.5μM) and sorafenib (2.5 μM) + 0.2% DMSO as the vehicle. Relative CYLD mRNA expression levels were detected by RT-QPCR relative to the CYLD expression of untreated HCC cells. (C) Western blot analysis of CYLD and IκB-α expression of Huh7 cells after 24, 48 and 72 h treatment with AG1478, U0126, Raf-1 inh. I and sorafenib. Tubulin was used as the control for equal loading.

(31). Thus, we wished to investigate whether the inhibition of key kinases in this pathway leads to altered CYLD expression in HCC cells. First, we treated HCC cells with a non-toxic concentration (20 μM) of the EGFR tyrosine kinase inhibitor, AG1478, for 24 and 48 h and analyzed CYLD mRNA expression levels. A 6.7-fold up-regulation of CYLD mRNA was detected in the Huh7 cells after 24 h. No up-regulation was observed after 48-h treatment. The inhibition of MEK with U0126 (20 μM), resulted in a 4.3-fold induction of CYLD expression relative to the CYLD mRNA

expression of the untreated Huh7 cells. After 48-h treatment with U0126, we observed 2-fold elevated CYLD mRNA expression levels. The Raf serine/threonine kinase isoform, Raf-1 (or C-Raf), belongs to the first kinases in the MAPK cascade activating MEK1/2. The treatment of Huh7 cells with 2.5 μM of Raf-1 inhibitor led to a 1.6-fold increase after 24 h and to a 5-fold increase of CYLD mRNA expression after 48-h incubation. Sorafenib has been approved by the FDA for the treatment of patients with advanced HCC. An *in vitro* inhibitory profile identified Raf-1 as the main target of sorafenib (37-39). Incubation of Huh7 cells with sorafenib (2.5 μM) increased CYLD mRNA expression levels 1.7-fold after 24 h and 2.6-fold after 48 h (Fig. 5A). The same treatment applied on Hep3B cells resulted in a 3.3-fold CYLD mRNA induction after 24 h AG1478, a 3.6-fold CYLD mRNA induction after 24 h U0126 and a 4.5-fold induction after 24 h Raf-1 inhibitor treatment. Similar to the results observed for the Huh7 cells, in the Hep3B cells, no CYLD mRNA up-regulation was detected after 48-h AG1478 treatment and only a 1.3-fold increase was detected after 48-h treatment with U0126.

In contrast to the Huh7 cells, 48-h inhibition of Raf-1 resulted in a reduction in CYLD mRNA expression levels in the Hep3B cells. Sorafenib treatment for 48 h increased CYLD mRNA expression 1.7-fold relative to the CYLD mRNA expression of the untreated Hep3B cells (Fig. 5B). In order to analyze the altered CYLD protein expression under the inhibition of the EGFR-Raf-MEK-ERK signaling pathway and to verify the data obtained from RT-QPCR experiments, we performed Western blot analysis of whole lysates from 24, 48 and 72 h inhibitor-treated Huh7 cells.

With the treatment of AG1478 (20 μM) and U0126 (20 μM), we observed an up-regulation of CYLD after 24 h. Longer incubation of Huh7 cells did not result in higher CYLD expression compared to the untreated cells (0 h). Inhibition of Raf-1 (2.5 μM Raf-1 inhibitor) led to a massive induction of CYLD expression. In addition, the treatment of Huh7 cells with sorafenib (2.5 μM) induced an enhanced CYLD expression in a time-dependent manner (Fig. 5C).

We then analyzed whether the reconstitution of the CYLD expression by inhibition of the EGFR-Raf-MEK-ERK signaling pathway leads to higher levels of the NF-κB inhibitor, IκB-α. As we had assumed, we detected an increase in IκB-α levels in Huh7 cells after 24-h treatment with AG1478 (20 μM) and U0126 (20 μM). Incubation of Huh7 cells with the Raf-1 inhibitor (2.5 μM) and sorafenib (2.5 μM), resulted in an elevation of IκB-α levels at all analyzed time-points (Fig. 5c). Our findings provide evidence that the CYLD expression in HCC cells is at least in part, negatively regulated by the pro-survival kinases of the EGFR-Raf-MEK-ERK signaling cascade. The recovery of CYLD expression is accompanied with higher expression levels of IκB-α, thus confirming the results of a negative NF-κB regulation via CYLD in HCC cells.

Discussion

The currently available therapies for advanced or metastasized HCC have limited effects on overall survival (40,41). Therefore, further investigation of the molecular mechanisms underlying HCC onset, progression and resistance are required, in order to identify targets in tumor signaling cascades and to find new potential strategies for HCC therapy. Current knowledge concerning the contribution of deubiquitinating enzymes to cancer biology as well as to the complex apoptotic machinery, is restricted. The aim of the present study was to investigate the role of the K-63 deubiquitinating enzyme, CYLD, in the apoptotic sensitivity of HCC cells. The results of this study show that CYLD expression is frequently reduced in human HCC tissues. In addition, reduced expression of CYLD inhibits chemotherapeutic drug- and TNF-α-induced apoptosis in HCC cells. Our data indicate that reduced CYLD expression or defects in the activity of CYLD, are important survival factors for HCC.

Apoptosis is a common occurence in multicellular organisms, eliminating unwanted and potentially harmful cells (42). Defects in apoptotic signaling, including an imbalance in death and survival signaling, contribute to tumor development (6). The transcription factor NF-κB family represents a key signaling complex in a network that includes a variety of kinases and targets a diverse range of genes. NF-κB signaling plays a critical role in cancer development and progression (43), which is underpinned by the observation that the expression of the NF-κB subunits in HCC tissues is increased compared to surrounding tissue (44). NF-κB is constitutively activated in various tumors and is thought to provide a critical survival signal that assists cancer cells in escaping apoptosis. Thus, NF-κB inhibition is considered to be a promising approach in anti-tumor therapy (45). Yeast two-hybrid screening models have identified CYLD as a protein that binds to NEMO, a regulatory subunit of IKK. Other studies have identified an association of CYLD with the IKK regulatory proteins, TRAF2 and 6 (20,22,27). Depending on the type of cell, a knockdown of the deubiquitinating enzyme, CYLD, promotes the ubiquitination of NF-κB-activating molecules (16). Mice lacking CYLD are highly susceptible to chemically-induced skin tumors. CYLD-negative skin tumors and keratinocytes show elevated anti-apoptotic NF-κB activity levels and an increased Bcl-3-dependent proliferation (24). Little is known about the role of CYLD in HCC, its influence on apoptotic signaling and its contribution to the resistance of HCC cells to chemotherapy and death receptor ligands. In this study, we demonstrate that CYLD expression is reduced in human HCC tissues compared to surrounding non-malignant tissues of the same patients both at protein and mRNA levels, thus pointing out the role of CYLD in malignant transformation in the liver.

Chemotherapeutic treatment in patients with HCC is often ineffective. In order to analyze whether reduced CYLD expression can contribute to this obstacle in therapeutic approaches, we performed CYLD knockdown experiments. HCC cells were transfected with siRNA against CYLD mRNA, and subsequently treated with chemotherapeutic agents, applied in local or systemic cancer therapies. CYLD knockdown by siRNA in Huh7 and Hep3B cells, led to a higher resistance to the chemotherapeutics, doxorubicin, 5-FU and cisplatin. These findings indicate the anti-apoptotic effect of reduced CYLD expression. The significance of these results is supported by the findings of Hellerbrand *et al*, where colorectal (CaCo2, HT29, SW480) and HCC (PLC, Hep3B)

cells showed a reduced CYLD mRNA and protein expression compared to non-malignant cells (30). We have previously shown that sorafenib induces apoptosis in Huh7 and HepG2 cells (46). However, the down-regulation of CYLD did not further sensitize HCC cells towards sorafenib-induced apoptosis. In contrast, we demonstrate that sorafenib is a strong inducer of CYLD expression in Huh7 and Hep3B cells (see below). Huh7 and Hep3B cells have been known to be susceptible to TNF-α-induced apoptosis in the presence of transcriptional or translational inhibitors, such as CHX, which inhibits the translation of NF-κB-dependent anti-apoptotic genes (47). In our study, we detected a reduced sensitivity of CYLD-negative HCC cells towards TNF-α-induced apoptosis. Additionally, the reduction of CYLD decreased IκB-α protein levels in both the Huh7 and Hep3B cells. Taking into consideration that IκB-α has been proposed to act as a tumor suppressor to control the oncogenic activity of NF-κB, it is possible that CYLD exerts its regulation on NF-κB at least in part, via the indirect stabilization of IκB-α. Nevertheless, an induction of RelA and p50 expression was observed in CYLD-negative Huh7 cells. Also, enhanced NF-κB activity in luciferase reporter-gene measurement after CYLD siRNA transfection, provides additional evidence regarding CYLD as a negative regulator of NF-κB signaling. The CYLD knockdown led to higher basal NF-κB activity. Moreover, the stimulation with TNF-α led to higher induction levels of NF-κB activity compared to the control-transfected cells, suggesting that CYLD potentiates TNF-α-induced apoptosis in an NF-κB-dependent manner.

We also observed a more rapid accumulation of p50/RelA subunits in siCYLD-treated HCC cells after TNF-α incubation in Western blot analysis. The results of the luciferase assay and Western blot analysis of nuclear p50/RelA accumulation concerning the basal/unstimulated NF-κB activity, are contradictory. However, the results substantiate the prior statement that CYLD is a negative regulator of NF-κB. More studies are required in order to clarify the impact of CYLD on other NF-κB subunits in the diversity of NF-κB signaling pathways.

The anti-apoptotic role of NF-κB in chemotherapy-induced apoptosis has been demonstrated by Wang *et al* in fibrosarcoma cells. Here, daunorubicin exposure resulted in a strong nuclear accumulation of p50/RelA subunits, indicating the protective effect of NF-κB activation on anthracycline-induced apoptosis. However, the inhibition of NF-κB by a super-repressor form of IκB-α dramatically increased the apoptotic response (48).

Our data indicate that reconstitution of CYLD expression could be a novel approach in sensitizing HCC cells towards apoptosis. In melanoma cells, CYLD has been shown to be negatively regulated via the ERK signaling pathway (31). In order to reconstitute CYLD in HCC cells, we included four inhibitors of the Ras-Raf-MEK-ERK signaling cascade. First, we focused upstream on the EGFR. It has been shown that the overexpression of EGFR represents a protective factor against apoptotic stimuli in HCC. EGFR receptor inhibitors have already entered clinical trials in patients with HCC (49). Our results show that treatment with the specific EGFR tyrosine kinase inhibitor, AG1478, caused an up-regulation of CYLD in Huh7 and Hep3B cells. Secondly, we applied the MAPK/ERK kinase (MEK) inhibitor, U0126. MEK is a key component downstream of Raf serine/threonine kinases and MEK inhibitors have been described to sensitize human cancer cells to apoptosis (50). U0126 also led to the reconstitution of CYLD, indicating that the high activity of the anti-apoptotic EGFR-MEK-ERK axis contributes to the reduced expression of CYLD in HCC. MEK is a downstream target of the Ras small GTPases, representing the most frequently mutated oncogene in human cancer. In addition, Ras is a central downstream effector of EGFR (32). Constitutively active Ras oncogenes are common in 30% of HCCs and are known to be responsible for the hyperactivated Raf kinase isoforms [A-Raf, B-Raf and Raf-1 (or C-Raf)] (51). Hwang *et al* detected hyperactivated Raf-1 in 100% of 30 HCC samples (52). In addition, Raf-1 can prolong cell survival, independently of the MAPK signaling pathway (53). We treated HCC cells with a Raf-1 inhibitor and detected a strong up-regulation of CYLD expression, both at mRNA and protein levels. We then treated Huh7 and Hep3B cells with the multikinase inhibitor, sorafenib, which is also known as a Raf-1 inhibitor and has been approved for the therapy of advanced HCC. Sorafenib acts by the inhibition of the RAF/MEK/ERK pathway, leading to a disruption of survival signals in HCC cells (37). Consistent with our findings regarding EGFR, MEK and Raf-1 inhibition, sorafenib induced CYLD expression in Huh7 and Hep3B cells. This effect could contribute to the known pro-apoptotic properties of sorafenib in HCC.

The present study shows for the first time that the down-regulation of the deubiquitinating enzyme, CYLD, contributes to the complex mechanisms of survival signaling in human HCC. CYLD expression is reduced in human HCC tissues. CYLD knockdown via siRNA reduces the sensitivity of HCC cells to chemotherapy- and TNF-α-mediated apoptosis and leads to a higher NF-κB activity by a reduction in IκB-α protein levels through the promotion of the ubiquitination of NF-κB regulating factors (16). CYLD expression in HCC cells can be recovered by the inhibition of the Ras-Raf-MEK-ERK signaling pathway, indicating the negative regulation of CYLD via this pathway. Thus, the reconstitution of CYLD could be a promising approach for HCC therapy.

Acknowledgements

This study was supported by research grants from Merck Serono GmbH, Darmstadt, Germany, to H.S-B. We are also grateful to Sandra Heine for her excellent technical assistance.

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