The role of the deubiquitinating enzyme CYLD and its substrate BCL-3 in solid tumors

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LUND UNIVERSITY
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Academic Dissertation

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Abstract

The tumor suppressor CYLD and the proto-oncogene BCL-3 are known to be deregulated in various cancer types. The molecular background of how these genes participate in carcinogenesis is not fully understood. CYLD is a deubiquitinating enzyme known to specifically target lysine 63 linked ubiquitin chains, which can negatively regulate the BCL-3, NF-κB and JNK signaling pathways, leading to a decrease of cell survival or proliferation. BCL-3 is an alternative IκB family member that is needed for activation (or repression) of target genes by homodimeric p50 and p52.

The aim of this thesis was to further investigate the molecular mechanisms behind CYLD and BCL-3 regulation and how they might contribute to carcinogenesis. In particular, the role of BCL-3 in prostate cancer (PCa), and the role of CYLD in hepatocellular carcinoma (HCC) were studied.

In PCa we found up-regulation of BCL-3 in human prostate cancers with abundant infiltration of inflammatory cells. Using PCa cell lines we found that interleukin-6 (IL-6) could activate STAT3 mediated up-regulation of BCL-3, which in turn could elevate Id-1 and Id-2 expression. Knockdown of BCL-3 increased the sensitivity for anticancer drug-induced apoptosis. PCa cells with reduced BCL-3 levels that were subcutaneously injected into NUDE mice formed smaller tumors due to a higher percentage of apoptotic cells.

In other tissues Bcl-3 has been shown to regulate proliferation through expression of its target gene CYCLIN D1, a process that is negatively regulated by CYLD. We found that CYLD knockout MEF cells have significantly increased proliferation rates and increased levels of CYCLIND1 in a serum dependent manner when compared with wild type MEF cells. The reduced proliferation in wild type cells was mediated through up-regulation of CYLD by transcription factor serum response factor (SRF) in a p38 mitogen-activated protein kinase (p38MAPK) dependent manner. Knockdown of SRF by siRNA or inhibition of p38MAPK reduced the expression of CYLD and increased cell proliferation rate. These results suggest that SRF is a positive regulator of CYLD expression, which in turn reduces the mitogenic activation of wild type MEF cells.

For further investigation of the molecular mechanisms of CYLD in cancer we performed a tissue microarray, comparing benign liver tissue with HCC. We found that CYLD is significantly down-regulated in human (HCC) and that CYLD expression was inversely correlated with the expression of proliferation marker Ki67. *In vivo* experiments showed that CYLD deficient mice were more susceptible to the chemical carcinogen DEN-induced HCC. Furthermore, HCC isolated from CYLD knockout mice had elevated cell proliferation compared to wild type mice. This effect was mediated via TRAF-2 ubiquitination, JNK activation and c-MYC expression. In correlation to this result, transient transfection of CYLD into a HCC cell line restricted cell proliferation and reduced the activation of JNK. Together these results suggest that CYLD down-regulation is a risk factor for development and progression of HCC mediated through activation of JNK1

Key words: CYLD, BCL-3, Solid tumors, Ubiquitin, NF-κB, SRF, Id, Prostate cancer, HCC, IL-6, JNK

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Abbreviations

AP-1	Activator Protein 1
AR	Androgen Receptor
BCL-3	B-Cell CLL/Lymphoma 3
bHLH	basic-Helix-Loop-Helix
C-terminal	Carboxy-terminal
CAP-Gly	Cytoskeletal-Associated Protein-glycine-conserved
CArG-box	DNA sequence CC(A/T)6GG
ChIP	Chromatin immunoprecipitation
CLL	Chronic Lymphocytic Leukemia
CRPC	Castration Resistant Prostate Cancer
CYLD	Cylindromatosis gene
DEN	Diethylnitrosamine
DMBA	7,12-Dimethylbenz(a)anthracene
DUB	Deubiquitinating enzyme
ELK1	ETS-like gene 1
EMT	Epithelial-Mesenchymal Transition
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine triphosphase
HBx	Hepatitis B virus X protein
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
Hdm2	Mdm2, p53 E3 ubiquitin protein ligase homolog,
HLH	Helix-Loop-Helix

HSC	Hepatic Stellate Cell
HUVEC	Human Umbilical Vein Endothelial Cell
HVB	Hepatitis Virus B
HVC	Hepatitis Virus C
Id	Inhibitor of DNA binding/differentiation
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IL-6R	Interleukin 6 Receptor
JNK	c-Jun N-terminal Kinase
K	Lysine
kDa	kiloDalton
MADS	MCM1, AGAMOUS, DEFICIENS, SRF
MAL	Myelin and Lymphocyte protein
MEF	Mouse Embryonic Fibroblast
MRFT	Myocardin-Related Transcription Factor
MRTF-B	MKL/Myocrardin-like 2
MT	Microtubule
NEMO	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase gamma (IKKγ)
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B-cells
NIK	NCK interacting kinase
NTHi	Nontypeable Haemophilus Influenza
OTU	Otubain proteases
P	Protein
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic intraepithelial Neoplasia
PSA	Prostate Specific Antigen
Rel	v-rel reticuloendotheliosis viaral oncogene homolog

RIP	Receptor (TNFRSF)-Interacting serine-threonine Kinase 1(RIPK1)
ROS	Reactive Oxygen Species
SAP	Serum response factor Accessory Protein
sh	short hairpin
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
Snail1	Snail homolog 1 (SNAI1)
SRF	Serum Response Factor
STAT	Signal transducer and activator of transcription
TAK1	Ttransforming growth factor-β (TGF-β) activated kinase 1
TCF	Ternary Complex Factor
TF	Transcription factor
TGF-β	Transforming Growth factor β
TNF-α	Tumor Necrosis Factor α
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRAF	TNF receptor-associated factor
UCH	Ubiquitin C-terminal Hydrolases
USP	Ubiquitin Specific Protease
USP	Ubiquitin-specific protease
VCAM	Vascular Cell Adhesion Molecule

Background

THE UBIQUITIN SYSTEM

Ubiquitin is a 9-kd protein that is highly conserved from yeast to mammals. It got its name from being ubiquitously expressed in the multiple tissues. Ubiquitin functions as a reversible post-translational modification that regulates a myriad of cellular processes. Ubiquitin can be attached to the amino groups on lysine residues or to the N-terminal of substrate proteins, which can be other ubiquitins. When ubiquitins are attached to each other, chains of different constellations can be formed. Ubiquitin protein contains 7 lysine residues (K6, K11 K27, K29, K33, K48 and K63) to which other ubiquitin can bind and form a large variation of chains. Such chains include linear chains, branched chains and chains with irregularly linked ubiquitin [1]. It is predominantly the K48 linked ubiquitination that targets ubiquitinated proteins for degradation in the proteasome [2].

Before ubiquitin can be covalently bound to substrate proteins, the ubiquitin has to be activated by the E1-activating enzyme, and conjugated by one of the approximately 30 different E2-conjugating enzymes. The final attachment to the substrate is then carried out by an E3 ligating enzyme (Figure 1) [3].

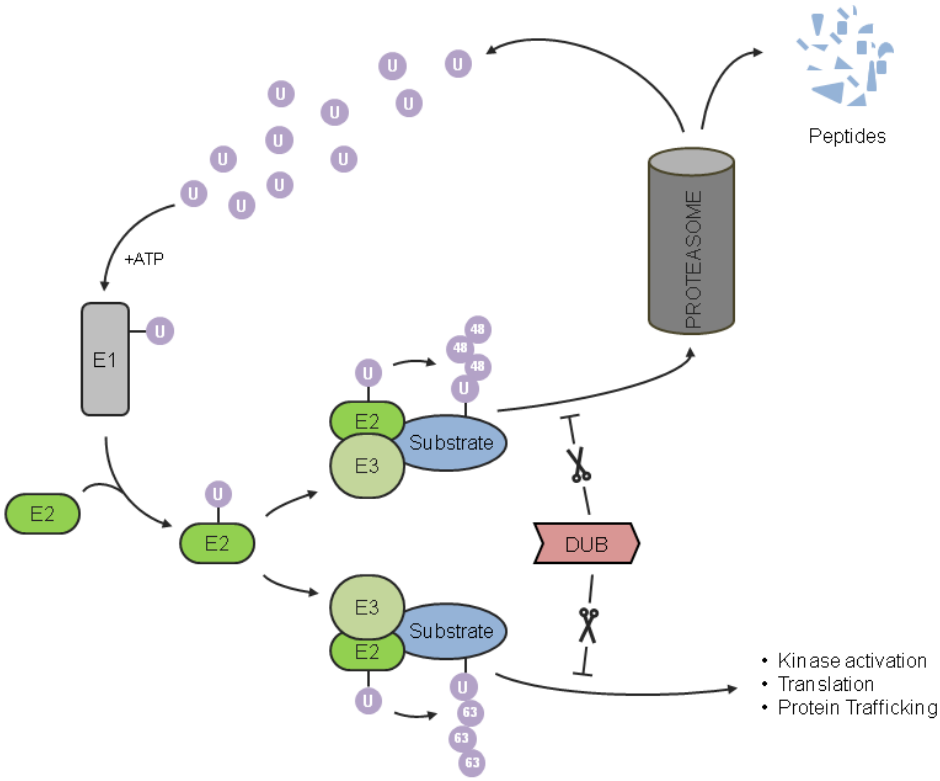

Figure 1. Schematic illustration of the ubiquitin pathway. *The unbound forms of ubiquitin are activated by the E1 enzyme in an ATP dependent manner. The activated ubiquitin is then transferred to an E2 conjugating enzyme that interacts with certain E3 ligases. The E3 ligase specifically binds to the substrate and mediates its ubiquitination. The most studied polyubiquitin chains are linked through the lysine 48 which leads to degradation in the proteasome, and chains linked through lysine 63 which does not target the substrates for degradation but may regulate protein trafficking or kinase activation instead. Ubiquitin can be removed from substrates by deubiquitinating enzymes (DUB).*

Until now, approximately 1000 different E3 ligases have been discovered. The E3 ligases can function either alone or in complexes. It has been postulated that the vast number of E2 and E3 enzymes are needed to ensure a high level of specificity for substrate ubiquitination [3].

Deregulation of members of the ubiquitin system has been suggested to promote carcinogenesis in various tissues. Cancer types with deregulated ubiquitination include hepatocellular carcinoma, colon cancer, breast cancer and prostate cancer [4-8]. Proteins can be mono-ubiquitinated with

a single ubiquitin molecule, or poly-ubiquitinated, where a chain of ubiquitin is attached to the protein. Mono-ubiquitination of substrates usually affects protein trafficking, endocytosis and gene expression or silencing [9]. There are several types of poly-ubiquitin chains depending on which amino group it binds the neighboring ubiquitin. The most studied ubiquitin chains are linked through the amino group on Lysine 48 (K48) or Lysine 63 (K63). As it was mentioned earlier, K48 destines the substrate for degradation by the proteasome, whereas K63 ubiquitination has been shown to be involved in kinase activation, translation and protein trafficking [10].

Like most posttranslational modifications, ubiquitination is reversible, and the deconjugation recquires one of the 95 known deubiquitinating enzymes (DUB) [11, 12]. DUBs are protease enzymes, however, unlike most other proteases, DUBs are generally produced as active enzymes. Most DUBs belong to the cysteine proteases superfamily and are further divided into four subclasses based on their ubiquitin protease domains; Machado-Joseph disease proteases (MJD), ubiquitin-specific proteases (USP), Otubain proteases (OTU) and ubiquitin C-terminal hydrolases (UCH). Structural analysis of the catalytical domain of UCH and UPS shows that they only have an active conformation while bound to ubiquitin [12].

Like most enzymes, the binding between the substrates and DUBs is usually transient and weak, however DUBs are also known to form a more stable complex with E3 ligases. E3 ligases are often able to autoubiquitinate themselves and, for instance, ring finger protein 41 (RNF41) mediates K48 linked self-ubiquitination. The deubiquitinating enzyme USP8 forms a complex with RNF41, resulting in its deubiquitination, preventing proteasomal degradation [13]. The E3-DUB complex can also cooperate to regulate or terminate a distinct signaling pathway [14].Many DUBs recognize and selectively deconjugates ubiquitin chains linked through specific lysine residues. The tumor suppressor cylindromatosis (CYLD) is a USP class DUB that selectively cleaves K63 or linear ubiquitin chains.

CYLD

Patients with familial cylindromatosis develops benign skin tumors predominantly in their head, neck, face and scalp regions [15]. The cause of cylindromatosis is mutation of the cylindromatosis (CYLD) gene [16, 17].

The tumor suppressor CYLD is a deubiquitinating enzyme (DUB) that regulates different signaling pathways by removing ubiquitin chains from substrate proteins. CYLD contains three cytoskeletal-associated protein glycine-rich (CAP-Gly) domains and an ubiquitin-specific protease (USP) domain [18].

The CAP-Gly has a conserved ability to bind tubulin and microtubules (MT) and can be found in a number of cytoskeleton-associated proteins [19].
The two first CAP-Gly domains in CYLD, are known to directly bind to tubulin, leading to an alteration in microtubule (MT) dynamics [20, 21]. Direct binding between α-tubulin and the two first CAP-Gly domains of CYLD is accompanied with increased acetylation of MT and translocation to the perinuclear compartment in TPA treated primary keratinocytes. The increased acetylation is achieved through direct binding of histone deacetylase 6 (HDAC6), thereby blocking α-tubulin deacetylation. In the perinuclear region, CYLD deubiquitinates B-cell CLL/Lymphoma 3 (BCL-3) which results in a delayed cell cycle progression due to decreased CYCLIND1 expression [21]. In human umbilical vein endothelial cells (HUVEC), decreased CYLD protein levels resulted in delayed cell migration [20]. It was later shown that in MEF cells expressing catalytically inactive CYLD also displayed delayed cell migration [22]. The third CAP-Gly domain cannot bind to tubulin, but instead binds to a proline rich sequence in NF-κB essential modifier (NEMO or IKKγ) [23]. This binding to NEMO enables CYLD to negatively regulate the IKK complex through deubiquitinating TNF-receptor-associated-facor 2 (TRAF2) or TRAF6, and thereby hindering the NF-κB signaling pathway [24, 25].

The USP domain contains the active site responsible for the deubiquitinating function of CYLD. Through crystallization of the USP domain in CYLD, a catalytic site composed of three residues, Cys601 His871 and Asp889 was identified [26]. Abolishment or substitution of Cys601 extinguishes the DUB function of CYLD [22, 24, 25, 27]. CYLD mediated deubiquitination has the ability to negatively regulate several members of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. A central step in NF-κB signaling pathway is the activation if IKK, and subsequently phosphorylation of inhibitor of κB (IκB). IKK activation triggered by different receptor signals, including tumor necrosis factor α (TNF-α), involves K63-linked ubiquitination of several NF-κB pathway members including TRAF2, TRAF6, NEMO and receptor interacting protein 1 (RIP1). CYLD has been shown to directly bind and de-conjugate K63-linked ubiquitin chains from these proteins and thereby negatively regulating NF-κB induced apoptosis [24, 25, 27, 28]. CYLD are known to deubiquitinate many substrates, not only proteins involved in NF-κB signaling (Figure 2) [18, 29].

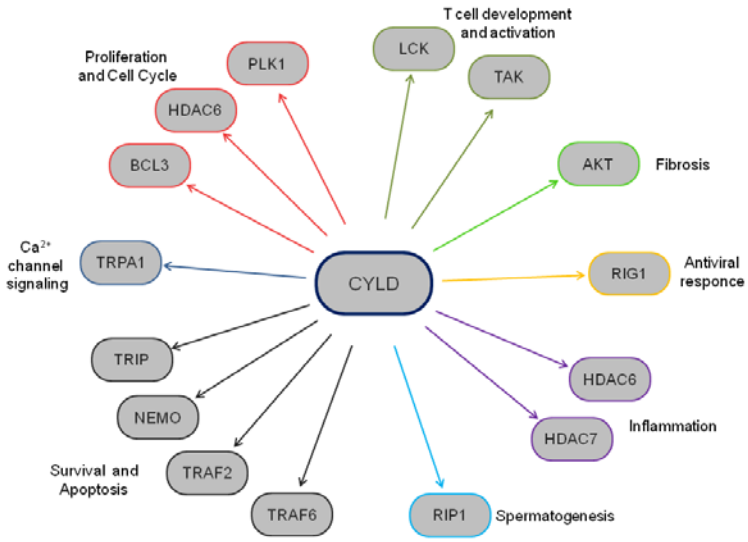

Figure 2. Representation of well known CYLD substrates. *Representation of all 14 known substrates for CYLD is presented in the illustration. The substrates are sorted into groups according to the function.*

Beside NF-kB, CYLD was shown to negatively regulate the c-Jun N-terminal kinase (JNK) signaling pathway in a TRAF2 dependent manner. CYLD knockdown leads to a hyper-activation of JNK due to increased K63-linked ubiquitination of TRAF2. As a result, there is further increased cell survival and potential for carcinogenesis [30, 31]. Recently, the paracaspase mucosa-associated lymphoid tissue 1 (MALT1) was shown to cleave CYLD between the second and third CAP-Gly domain upon T-cell receptor activation in a T-cell leukemia cell line. This cleavage leads to proteolytical inactivation of CYLD and a pronounced activation of JNK signaling [32].

Another substrate for CYLD mediated K63-linked deubiquitination, is the IκB family member B-Cell CLL/Lymphoma 3 (BCL-3). BCL-3 is a proto-oncogene that was first identified in patients with lymphoma with a common genomic translocation (t (14:19) resulting in BCL-3 overexpression [33, 34]. Unlike the classical IκB, BCL-3 does not sequester NF-κB in the cytoplasm, but rather regulates the expression activity of homodimeric NF-κB p50 or p52 in the nucleus. The nuclear import of BCL-3 is dependent on K63-linked ubiquitination, a process

regulated by CYLD [35]. Mice lacking CYLD were also shown to be more susceptible for skin papillomas after treatment with the chemical carcinogens 7,12-demethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol (TPA). A single dose of TPA was sufficient to drastically elevate the proliferation rate and CYCLIN D1 expression in the skin of CYLD knockout mice and CYLD deficient primary keratinocytes. This increased proliferation was mediated by BCL-3 transactivation of homodimeric p50 or p52 bound to the promoter of CYCLIN D1 in the nucleus. In TPA treated wild type cells, CYLD deubiquitinates K63-linked polyubiquitin chains from BCL-3, which prevents the translocation of BCL-3 from the cytoplasm to the nucleus [35]. Together this suggests that down-regulation of CYLD promotes skin cancer formation. The tumor suppressor CYLD has been reported to be down-regulated in several human cancer including melanoma, basal cell carcinoma, hepatocellular carcinoma and colon cancer (Table 1) [31, 36-39].

Cancer type/Tissue	**Reference**
Familial Cylindromatosis	[16, 40, 41]
Brooke-Spiegel Syndrom	[40-42]
Multiple Familial Trichoepthelioma	[41, 43, 44]
Melanoma	[38]
Saliva Gland Cancer	[45-47]
Head and Neck Cancer	[48]
Cervical Cancer	[49]
Lung Cancer	[50]
Prostate Cancer	[51]
Colon Cancer	[31, 36]
Hepatocellular Carcinoma	[36, 37]
Renal cell Carcinoma	[52]
Breast Cancer	[53, 54]

Table 1. *Complete list of tumor types in which CYLD is lost or down-regulated.*

NF-κB

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway can be activated is trough ligand mediated activation of several different receptors including the T-cell receptor (TCR) and the tumor necrosis factor receptor (TNFR). Activated NF-κB signaling changes the expression of target genes that can regulate a wide variety of cellular functions such as cell survival, migration and maturation of leukocytes [55, 56]. Hundreds of target genes are known to be regulated by NF-κB signaling pathway, including the cytokines IL-6, IL-1, IL-2 and TNF-α [57-62]. The NF-κB pathway is complex but essentially it regulates the five NF-κB transcription factor family members that belong to two classes. The first class includes RelA (p65), RelB and c-Rel, proteins that are synthesized as mature products and possess a transactivation domain. The second class consists of p50 and p52, which are synthesized as the large precursor proteins p105 and p100 respectively. The aforementioned transcription factors forms homo- or heterodimers that can activate (or repress) expression of target genes. The NF-κB pathway is divided into the classical pathway and the alternative pathway (Figure 3), which can have distinct target genes.

The classical NF-κB pathway can be initiated by various stimuli amongst which TNF-α is the most well characterized initiator. Ligand activation of the TNF receptor activates TRAF adaptor proteins through K63-linked ubiquitination. TRAF subsequently activates the IκB Kinase (IKK) complex. IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ (NEMO). The catalytic part of the IKK complex can phosphorylate IκB family members, leading to their K48 linked ubiquitination, and degradation in the proteasome. As a consequence, the NF-κB dimers, which were captured in the cytoplasm by direct interaction to IκB, are released and can translocate into the nucleus. In the nucleus NF-κB can bind to specific DNA sequences called κB sites located in target gene promoters, and activate transcription. [24, 63].

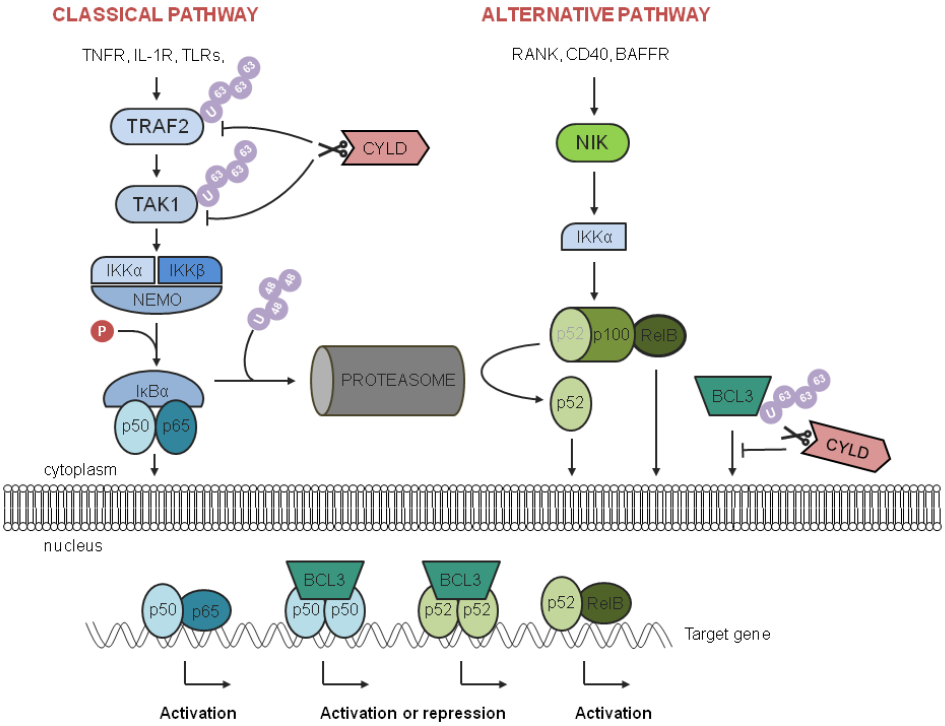

Figure 3. Classical and alternative NF-κB signaling pathways. *(Left) Activation of the classical pathway mediates polyubiquitination of TRAF2 leading to activation of TAK1 (transforming growth factor-β (TGF-β) activated kinase 1). This leads to ubiquitination of TAK1 which in turn activates the IKK complex, consisting of IKK-α, IKK-β and NEMO. Activated IKK complex phosphorylates IκBα which trigger its K48 linked polyubiquitination and subsequent degradation in the proteasome. This process enables p50 and p65 to enter the nucleus and activate transcription of target genes. (Right) The alternative pathway can be activation by various receptors including RANK, CD40 and BAFFR. NF-κB inducing kinase (NIK) is then activated and triggers proteasomal processing of immature p100 into p52, in IKKα dependent manner. Further, mature p52 can then enter the nucleus and may form a heterodimer with RelB and activate target genes. If p52 forms homodimers, Bcl-3 is needed for activation of gene expression. Bcl-3 can also bind to homodimeric p52 (or p50), without activating target genes. It has been shown that CYLD can regulate classical NF-κB pathway by deubiquitination of TRAF-2 and/or TAK1. CYLD can also block Bcl-3 from entering the nucleus, regulate the activity of homodimeric p50 and p52 dependent target gene expression.*

The alternative NF-κB pathway depends of activation of IKKα but not IKKβ or IKKγ [64-67]. NF-κB inducing kinase (NIK) is an upstream enzyme that can be activated by the B-cell activating factor receptor (BAFFR) [66] and

induce phosphorylation of IKKα, which then targets the p52 precursor p100 for phosphorylation and K48 linked ubiquitination. [68]. The c-terminal part of p100 which contains the Rel homology domain is then degraded in the proteasome. The remaining part of p100, now called p52 can then enter the nucleus and form hetro- or homodimers with other NF-κB family members. Homodimers of p52 or p50 can bind to promoter sequences but not initiate gene transcription without a co-activator, due to the lack of a transactivation domain. BCL-3 is an atypical IκB family member that does not sequester NF-κB in the cytoplasm, but has been shown to trans-activate homodimers of p52 or p50 and thereby initiate transcription of target genes [69, 70]. BCL-3 has also been shown to have an inhibitory effect on p50- and p52-homodimer regulated gene expression [71-75].

BCL-3

B-cell lymphoma 3-encoded protein (BCL-3) was first identified in patients with B-cell chronic lymphocytic leukemia with a (t (14;19) chromosomal translocation [34]. This proto-oncogene is an atypical inhibitor of κB (IκB) that does not sequester NF-κB in the cytoplasm. Instead, as it was mentioned earlier, BCL-3 binds to target genes by binding directly to promoter bound homodimers of p50 or p52 and subsequently induce either transactivation or repression of target genes [70, 73]. Target genes of BCL-3 have been shown to regulate numerous genes linked to proliferation, apoptosis and inflammation, including CYCLIND1, N-cadherin, GATA-3 and HDM2, [35, 38, 76-79]. BCL-3 knockout mice are viable and fertile, but have defects in their adaptive immune responses [80, 81]. BCL-3 deficient T-cells have defective Th1 and Th2 differentiation, which produce limited amount of Th2 typical cytokines such as IL-4, and IL-5. This low production of IL-4 and IL-5 was attributed to reduced levels of the BCL-3 target gene GATA-3 [79].

Several cytokines including TNF-α, IL-10 and IL-6 can induce BCL-3 expression, mediated through the NF-κB, AP-1 or JAK/STAT signaling pathways, respectively [82, 83]. The most well characterized pathway is activated by IL-6 leading to phosphorylation of STAT3 that dimerises and activates BCL-3 expression through directly binding to the HS4 enhancer in the BCL-3 promoter. This leads to an increase in apoptosis in a multiple myeloma cell line [84]. BCL-3 together with p50 can form an auto-regulatory loop and thereby repress its own transcription levels [85].

Post-transcriptional modifications of BCL-3 present another layer of regulation. Hyper-phosphorylation of the C-terminal part of BCL-3 has

been shown necessary for its activity and function [74, 86]. More recently, GSK3 has been shown to phosphorylate two specific serine residues in the C-terminal region of BCL-3 resulting in K48 linked poly-ubiquitination and subsequent proteasomal degradation of the protein [87]. In CYLD deficient keratinocytes, UV-light or TPA stimulation induced translocation of BCL-3 tagged with a K63-linked ubiquitin chain from the cytoplasm into the nucleus, leading to increased proliferation and expression of CYCLIN D1. In the presents of CYLD, the K63-linked ubiquitin chains are deconjugated from BCL-3 which prevents translocation into the nucleus [35].

Since the discovery that BCL-3 is an oncogene involved in leukemia, several groups have published that BCL-3 also functions as an oncogene in solid tumors. Cogswell *et al.* showed that breast tumor tissue contained significantly more nuclear BCL-3 compared with adjacent tissue [88]. Later it was shown that mice overexpressing c-Rel under the MMTV promoter, developed mammary gland tumors that had elevated levels of p50, p52, p65, RelB and BCL-3 in the nucleus [89]. Furthermore, xenograft experiments with a breast cancer cell line, MCF-7, overexpressing BCL-3 displayed its involvement in both tumor establishment and cell growth [90]. Other solid tumor types that BCL-3 has been reported to have oncogenic functions in include cylindromastosis, nasopharyngeal carcinomas, melanoma, endometrial tumors and colorectal carcinomas [38, 91-93]. For colorectal carcinoma, BCL-3 has been suggested to be used as both a prognostic and diagnostic marker [93].

In transgenic animal models, mice overexpressing BCL-3 or with depleted CYLD, have been shown to increase the proliferation of B-cells and keratinocytes, respectively [35, 94]. As it was mentioned earlier, in primary mouse keratinocytes the increased proliferation was due to elevated BCL-3-mediated CYCLIND1 expression, which accelerated the formation of papillomas in the well-established DMBA/TPA tumor model [35]. More evidence of BCL-3 induced proliferation has been shown *in vitro* using cancer cell lines from solid tumors. Rocha *et al.* showed that upon induction of p53 in non-small-cell lung carcinoma and osteosarcoma cell lines, BCL-3 expression was reduced, leading to decreased CYCLIND1 expression. This decrease in CYCLIND1 was due to a shift from p52-BCL-3 activating complexes to p52-HDAC1 repressing complexes resulting in decreased cell proliferation [95]. BCL-3 has also been shown to regulate CYCLIND1 expression in cancer cell lines originating from breast cancer, melanomas and hepatocellular carcinoma [38, 76, 96].

Another oncogenic property of BCL-3 is the ability to promote cell survival in cancer cells. The tumor suppressor p53 is an important regulator of cell survival and apoptosis. DNA damage can induce p53 activity and

subsequently trigger the apoptotic program. Under normal conditions the E3-ligase Hdm2 ubiquitinates p53, leading to degradation in the proteasome. The Hdm2 promoter contains a NF-κB binding site and BCL-3 is required for activating its expression. In breast cancer cell lines when BCL-3 is overexpressed, it can inhibit DNA damage induced p53 activity, resulting in an increased survival [77]. Another target gene of the BCL-3/p52 complex is the anti-apoptotic gene Bcl-2. Interestingly, in breast cancer and CLL, high p52/p100 expression was associated with elevated Bcl-2 levels [97].

TRANSCRIPTION FACTORS

Transcription factors (TF) are crucial for the regulation of gene activity. The main function of TF is to regulate the coordination of the complex that is needed for RNA polymerase attraction for specific genes. There are hundreds of different transcription factors, and they can be regulated through a myriad of signals and signaling pathways.

Serum Response Factor

Serum response factor (SRF) is ubiquitously expressed and belongs to the MADS-box family of transcription factors [98]. Dimeric SRF bind to a 10 base pair long DNA sequence called CArG box in the promoters of target genes. The CArG box is usually located in the proximal promoter or the first intron. There are approximately 200 experimentally validated target genes of SRF (Figure 4) [99-105].

Many of the target genes of SRF encode proteins important for actin cytoskeletal regulation and many phenotypes of SRF depletion relate to aberrant cell migration [100, 101, 104, 105]. In gene depletion studies, SRF has been shown to be essential for mouse gastrulation [106]. Other in vivo studies, with cell specific SRF depletion, indicate that SRF is important for liver regeneration and skin functions [107, 108]. In the later example, SRF was removed in keratinocytes, which further led to embryonic lethality, loss of barrier function and cell-cell contact due to abnormal cytoskeleton [107, 109]. Conditional depletion of SRF hepatocytes caused increased IGF-1, triglycerides and glucose levels in the liver. The hepatocytes also had an increased cell proliferation rate at the same time

as an increased occurrence of apoptosis. For male mice, this phenotype was postnataly lethal [108, 110].

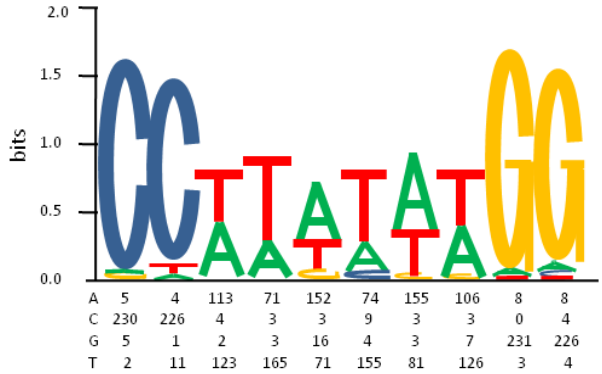

Figure 4. The CArG box. *The height of each stack reflects conservation at a specific position measured in bits. The height of each nucleotide within a stack indicates the relative frequency of that nucleotide at a specific position within the CArG box. The sequences from the 242 CArG boxes used in this figure originates from 182 experimentally validated SRF target genes.*

SRF alone has a relatively low transcriptional activity, but with over 60 different cofactors, strong SRF dependent expression can be regulated in a cell type, tissue and context specific manner [111]. Two principal regulatory pathways of SRF dependent gene expression have been characterized in non-muscle cells, each dependent on a certain family of cofactors (Figure 5)

The first pathway is dependent on signaling members of the ternary complex factor (TCF) family of Ets domain proteins ELK-1, SAP-1 and SAP-2. Activation is dependent on mitogen activated protein kinase (MAPK) phosphorylation. As the Etf domain can bind to CArG box in a phosphorylation-enhanced manner, this binding potentiates transcription of immediate-early genes [112, 113]. The second family of SRF cofactors is the myocardin-related transcription factors (MRTFs). The first member of MRTFs was myocardin, which contains two alternative splice variants expressed only in specific tissues. A shorter mRNA, expressed only in the smooth muscle tissue, and a longer mRNA expressed only in cardiac tissue [114-116]. The two other members of the MRTF family are the widely expressed MAL and MRTF-B [117-119]. The activity of MAL and MRTF-B is regulated by a pathway controlled by Rho-family GTPases and monomeric actin [114, 115, 120-122]. It is believed that MRTFs bind

directly to DNA flanking the CArG-box, however no sequence-specific interaction has yet been identified [123].

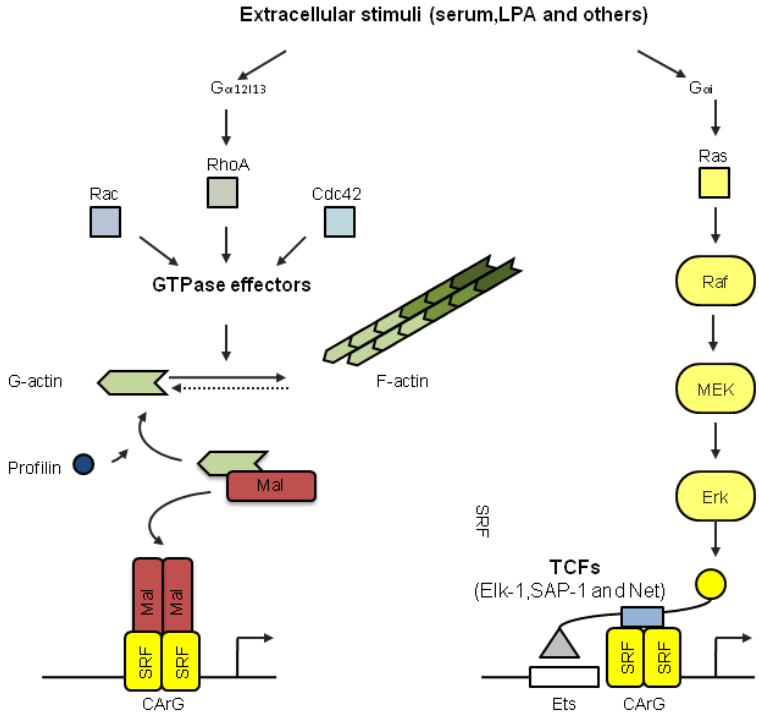

Figure 5. Model of two principal pathways that can regulate SRF activity. *(**Left**) Stimulation activates Rho dependent GTPases that mediate signaling through the actin treadmilling cycle. MAL can then be released from actin in a profilin mediated manner, allowing MAL to bind and activate SRF mediated target gene expression. (**Right**) Activated Ras-induced signaling through the MAP kinase pathway mediates Erk phosphorylation of TCFs. TCFs bind to both Ets and SRF, thereby inducing expression of target genes.*

Inhibitor of DNA binding

Basic helix-loop-helix (bHLH) transcription factors are a family with a conserved sequence motif consisting of basic residues that can bind to E-box DNA sequences. bHLH transcription factors form heterodimers which can activate or repress target gene transcription [124, 125]. A subfamily of bHLH transcription factors is the Id (Inhibitor of DNA binding) family, which lacks the DNA binding basic motif. To date, four members of the Id family have been identified (Id-1, Id-2, Id-3 and Id-4). All four members have a similar size and share a highly conserved helix-loop-helix (HLH) domain. Id proteins can form heterodimers with bHLH transcription factors and thereby preventing the DNA binding and gene transcription [126]. Although all members of the Id family are expressed in many organs and tissue types, they have a distinct expression pattern during embryogenesis. While, Id-1, Id-2 and Id-3 expression are overlapping, Id-4 has an individual expression pattern throughout embryogenesis [127, 128].

Increased expression of Id proteins has been found in numerous tumors types. Particularly, Id-1 and Id-2 have been associated with tumors including head and neck, colorectal, pancreatic and prostate cancers [129-135]. Id-1 and Id-2 have been suggested to regulate a multitude of cellular functions including proliferation, differentiation and cell survival [136-138]. In the prostate cancer cell line DU145, inhibition of Id-1 expression by antisense Id-1, leads to increased sensitivity to TNF-α induced apoptosis [135]

Several members of the transforming growth factor β (TGF-β) superfamily have been shown to induce expression of Id genes through SMAD activation [138-141]. Other factors including β-catenin/TCF, PI3K and IL-6 have also been shown to induce Id transcription [142-145]. In myeloid leukemia cells, IL-6 stimulation caused increased proliferation and induced expression of both Id-1 and Id-2 [145]. It is not known through which pathway IL-6 induces Id expression. However, inhibition of STAT3 which is known mediator of IL-6 signaling, leads to decreased production of Id-2 in colon cancer cells [146].

SOLID TUMORS

A solid tumor is a neoplastic lesion in a tissue that may be benign or malignant. Tumors can be formed in virtually all tissues in the body and are often classified according to their cellular origin. Hepatocellular carcinoma (HCC) is the leading cause of cancer-related death worldwide [147]. For men, the most common cancer type is prostate cancer, but the mortality rate is lower than for HCC [148]. Leukemia's rarely form solid tumors and are therefore by definition, not a solid tumor.

Prostate

The prostate gland is an exocrine gland located under the bladder surrounding the urethra. The function of the prostate is to produce a secretion that constitutes approximately one quarter of the seminal fluid. This prostatic fluid is important for the sperm viability and motility. It contains proteolytic enzymes that prevent clotting as well has high levels of the prostate specific antigen (PSA) [149]. The prostate reaches the size of a walnut soon after the onset of puberty due to the increased production of testosterone. Further enlargement of the prostate is very common for men older than 50 years or age, a process referred as benign prostatic hyperplasia (BPH) [150, 151]

Inflammation of the prostate

Inflammation of the prostate (prostatitis) is common amongst elderly men, both in acute and chronic forms. The prevalence of symptomatic prostatitis is estimated to be 16% in the US male population at some point of their life [152, 153]. In terms of asymptomatic prostatic inflammation it appears to be much higher. In a recent study, it was found that almost 50% of the patients had leukocytes in the expressed prostatic secretion, amongst men with PSA levels between 2,5 and 10 ng/ml. [154]. In the REDUCE (Reduction by DUtasteride of prostate Cancer Events) study, 80% of the prostate biopsies showed traces of inflammation [155].

There is an established connection between inflammation and prostate cancer [156-158]. In the strife to eradicate pathogens, infiltrated leukocytes in the prostate can produce reactive oxygen species (ROS) that potentially can lead to DNA damage and apoptosis in epithelial cells. Secreted peptidases can break down extracellular matrix and potentiate immune cell invasion. Infiltrated leukocytes are also known to produce cytokines and

growth factors that may cause increased proliferation and potentially dedifferentiation of the epithelium. The harmful environment together with increased proliferation can promote genomic instability and increased mutation rate. This state of increased proliferation and tissue destruction has been named proliferative inflammatory atrophy (PIA) [159-161]. When an inflamed prostate has developed PIA it is likely to evolve into prostate intraepithelial neoplasia (PIN), which in turn may further develop into prostate cancer (Figure 6) [156, 162].

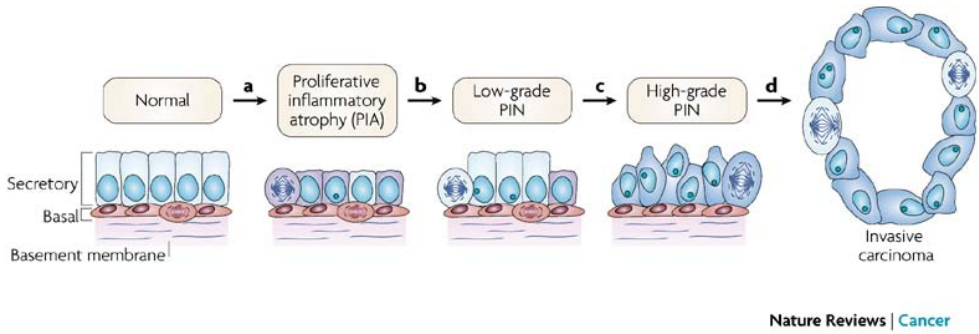

Figure 6. Model of early prostate neoplasia progression. *(a) Inflammatory cells are known to infiltrate prostate tissue and secrete factors that may induce DNA damage, tissue damage and proliferation. (b-c) Silencing of tumor suppressors and activation of oncogenes can contribute to genomic instability. (d) Genetically instable cells continue to proliferate. Reprinted with permission from Macmillian Publishers Ltd: Nature reviews Cancer, Apr;7(4), ©2007. License number: 2976971426836.*

IL-6 and prostate cancer

In inflammation, multiple cytokines have been identified as potential mediators in the interplay between prostatic inflammation and prostate carcinogenesis. One of the particularly interesting cytokines is interleukin 6 (IL-6), which is involved in numerous innate and adaptive inflammatory processes including B-cell activation and acute phase inflammatory response [163]. Endothelial cells, T lymphocytes and macrophages are well known sources for IL-6 production. [164, 165].

In the field of initiation and progression of prostate cancer, IL-6 has been shown to contribute in multiple ways. An elevated plasma level of IL-6 has been correlated with metastatic prostate cancer, hormone refractory prostate cancer and prostate cancer morbidity [166, 167]. When compared with adjacent benign prostate tissue, high expression of IL-6 and IL6-receptor has been found in malignant prostate epithelium and high grade PIN [168].

IL-6 has been suggested to affect androgen receptor (AR) activity [169]. Androgens are required for AR activation, which mediates development and maturation of the prostate. AR is a transcription factor belonging to the nuclear steroid-receptor family. Upon activation, AR signaling promotes proliferation and survival of epithelial cells. Primary prostate cancer cells are most often dependent on androgen for activating AR. As a result, depletion of androgens will initially prevent further proliferation and cause cell death, thereby reducing the tumor size [170]. However, most often the prostate cancer cells manage to circumvent the androgen blockade, and the tumor will progress. When a tumor has reached this stage it is referred to as a castration resistant prostate cancer (CRPC) [171]. How cancer cells can circumvent the need for androgen has not been completely elucidated. Recently evidence suggests that IL-6 induced activation of the AR leads to enhanced cancer cell growth *in vitro* and *in vivo*, indicating that IL-6 may participate in development of CRPC [172].

Liver

The liver is the largest internal organ in the body, and is the only organ that can regenerate lost tissue or induce compensatory growth. It has a wide range of functions, including digestion of chemicals, protein synthesis, glycogene storage and detoxification of the blood. Approximately 60 percent of all cells in the liver are hepatocytes that are responsible for many of the liver functions. Other cell types in the liver include the Kupffer cells and hepatic stellate cells (HSC). Kupffer cells are liver specific macrophages that are known to produce various cytokines and through extensive phagocytosis break down red blood cells. HSC contain lipid droplets important for storage of vitamin A and are the main producers of extracellular matrix (ECM) in the liver during fibrosis. Fibrosis is scarring of the liver tissue, which correlates with increased risk for developing hepatocellular carcinoma (HCC) [173, 174].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm worldwide and the third most frequent cause of death [175]. Interestingly, the incidence of HCC is 3-5 times higher in men than in females [176]. Of all known HCC cases, 70-90% of the patients had an already established chronic liver disease and HCC is the leading cause of death amongst cirrhosis patients [177, 178]. In eastern Asia and sub-Saharan Africa, 80% of HCC cases are caused by Hepatitis B virus (HBV), hepatitis C virus

(HCV) or exposure to the mycotoxin Alfatoxin B. In contrast, the main risk factors for developing HCC in North America, Japan and Europe are HCV and alcohol [179].

During chronic HBV infection, DNA of viral origin integrates in the genome of affected liver cells and can induce chromosomal instability, which can result in rearrangements or deletions [180]. Another mechanism by which HBV can induce hepatocarcinogenesis is the expression of the viral oncogene X protein (HBx) [181]. HBx has been shown to induce expression mediated through the JNK/AP-1 and the NF-κB signaling pathways [182, 183]. Due to the transactivation activity, HBx is known to regulate a variety of genes involved in the control of apoptosis and proliferation, including p53 and CYCLIND1 [184, 185].

Unfortunately, viruses that greatly increase the HCC risk, namely HBV and HCV, cannot be utilized in HCC mouse models. On the other hand, the chemical carcinogen, diethyl nitrosamine (DEN) has been shown to induce HCC in mice, whose gene expression profile is very similar to that of aggressive human HCC [186, 187]. DEN is metabolized by hepatocytes into a potent alkylating agent and one administration is enough to induce HCC in mice [188]. Furthermore, DEN induced carcinogenesis depends on inflammatory processes similar to those responsible for HCC induced by chronic viral hepatitis [176, 189].

Recent findings have implicated constitutive activation of NF-κB signaling as one of the key events involved in early progression of HCC [190]. In mice treated with DEN, increased necrosis of hepatocytes will activate NF-κB mediated expression of cytokines and growth factors including TNF-α, IL-6 and hepatic growth factor (HGF) in surrounding Kupffer cells. As a result there is an increase in survival and proliferation of remaining hepatocytes [186, 191].

Activation of the NF-κB signaling pathway is known to induce JNK activity [192]. Targets of JNK signaling include members of the activating protein 1 (AP-1) family of transcription factors. JNK1 was shown to be essential for human HCC cell proliferation in vitro and for xenografts into NUDE mice. Ablation of JNK1 in mice treated with DEN resulted in significantly smaller liver tumors when compared with wild type mice. The JNK1 deficient tumors cells had a decreased proliferation rate together with increased levels of the cell cycle inhibitor p21 and reduced c-Myc [193, 194]. c-Myc is a transcription factor that is up-regulated in approximately 70% of viral and alcohol related HCC [195].

The Present Investigation

AIM

The general objective of the work underlying this thesis was to examine the role of CYLD and BCL-3 regulation in solid tumors

The specific aims were as follows:

- To investigate the role of BCL-3 in prostate cancer progression.

- To characterize the upstream mechanisms of transcription regulation of CYLD in primary mouse embryonic fibroblasts (MEF).

- To examine the relevance of CYLD for development and progression of hepatocellular carcinoma (HCC)

RESULTS AND DISCUSSION

PAPER I

Expression of Id proteins is regulated by the BCL-3 proto-oncogene in prostate cancer

Summary

B-Cell CLL/Lymphoma 3 (BCL-3) is a member of the inhibitor of κB (IκB) family, which regulates a multitude of biological processes by regulating NFκB target gene expression. As high levels of BCL-3 expression and activity have been reported in different types of human cancers, BCL-3 has been designated as a proto-oncogene. In our study we found up-regulation of BCL-3 in human prostate cancers (PCa) with abundant infiltration of inflammatory cells. Elevated BCL-3 expression in PCa was dependent on STAT3 activation by the pro-inflammatory cytokine interleukin-6 (IL-6). Microarray analysis of PCa cells expressing antisense RNA targeting BCL-3, revealed decreased expression of the inhibitor of DNA-binding (Id) family as potential target genes of BCL-3. ChIP analysis confirmed direct binding between BCL-3 and promoter regions of both the Id-1 and Id-2 genes. Knockdown of BCL-3 reduced the expression of Id-1 and Id-2, which potentiated anticancer drug-induced apoptosis of PCa cells. Xenografts of PCa cells with reduced BCL-3 expression formed smaller tumors with a 5 fold increased number of apoptosis cells. Our data imply that inactivation of BCL-3 may lead to sensitization of cancer cells to chemotherapeutic drug-induced apoptosis, and suggests BCL-3 as a potential target gene in PCa treatment.

Results and Discussion

BCL-3 has been implemented to conduct oncogenic functions in several cancer types including epithelial breast cancer, endometrial and colorectal cancer [88, 92, 93]. In the present study we were interested in finding the role of BCL-3 in prostate carcinogenesis. To achieve this goal, we started with exploring the expression of BCL-3 in prostate cancer using the tissue microarray technique. We detected a correlation between up-regulated BCL-3 expression and high number of infiltrated inflammatory cells. Inflammatory cells are known to produce numerous cytokines including IL-6. A high serum level of IL-6 has also been suggested as a prognostic marker and has been correlated with poor prognosis for prostate cancer

patients [166]. Furthermore, IL-6 has previously been shown to up-regulate BCL-3 expression via STAT3 in multiple melanoma cells [84].

To further evaluate whether IL-6 can regulate the levels of BCL-3 in prostate cells, we initially compared the levels BCL-3 in 4 different prostate cancer cell lines including LNCaP, LNCaP-IL-6+, DU145 and PC-3 with a benign prostate cell line, PNT2. We found the highest expression of BCL-3 both at the mRNA and protein level in DU145 and LNCaP-IL-6+ cells. LNCaP-IL-6+ originates from the well-studied PCa cell line LNCaP that has been cultured in the presents of 5 ng/ml IL-6 for at least 50 passages and DU145 are known to produce high levels of endogenous IL-6 [196, 197]. These results suggested an IL-6 dependent induction of BCL-3 expression. We could further confirm this finding by culturing the prostate cell lines in the absence or presents of IL-6 for 24 hours. In all of the PCa cell lines tested IL-6 induced a robust induction of BCL-3. The only PCa cell line that we could not observe an up-regulation of BCL-3 was the PC-3. Interestingly, the differences between PC-3 cells and other PCa cell lines is that PC-3 cells exhibit very low levels of STAT3 [196, 198]. To further find whether STAT3 activation is involved in up-regulation of BCL-3 expression, we applied two different specific chemical inhibitors against STAT3. Inactivation of STAT3 reduced the expression levels of BCL-3 significantly in all PCa cell lines tested. We could further verify these results in DU145 cells where STAT3 expression was blocked using siRNA, in turn resulting in decreased BCL-3 protein levels and indicating that STAT3 is an up-stream mediator of BCL-3 expression.

Next, we sought to investigate the function of BCL-3 activity in PCa cell lines. Nuclear localization is essential for BCL-3 mediated regulation of target genes. Previous publications have shown how TPA or UV light can induce translocation of BCL-3 into the nucleus of keratinocytes [35]. First we explored if IL-6 stimulation affects nuclear translocation of BCL-3 by stimulation of PCa cell lines with IL-6 followed by fractionation of cell lysate into cytoplasm or nuclear compartments. We found that IL-6 stimulation indeed lead to a higher amount of nuclear BCL-3 compared with non-stimulated cells. This result was confirmed using confocal imaging of immunoflourescently stained BCL-3 in IL-6 stimulated LNCaP cells. These results suggest that in addition to up-regulation of BCL-3, IL-6 induces translocation of BCL-3 to the nucleus.

BCL-3 has been shown to regulate proliferation and cell survival in different cancer cells types [76, 77]. To further dissect the function of BCL-3 in PCa, we generated stable clones of DU145 cells expressing either shRNA against BCL-3 (DUshBCL-3) or scrambled shRNA (DUshControl).

As we could see a significant difference in chemically induced cell death comparing BCL-3 knockdown cells with control cells, we found no differences in cell proliferation, cell adhesion or cell migration.

In our search to identify the mechanism in which BCL-3 could protect cells from drug-induced apoptosis, we started by comparing the gene expression in DUshBCL-3 and DUshControl cells using microarray technology. We found that amongst the 20 most down-regulated genes in DUshBCL-3 cells were several members from the Inhibitor of DNA binding (Id) family present. The down-regulation of Id-1 and Id-2 in DUshBCL-3 cells compared with DUshControl cells was confirmed by quantitative RT-PCR and western blot analysis. We also observed the same effect in another pair of DUshBCL-3 and DUshControl clones, which suggest that this finding was not clone specific.

Both Id-1 and Id-2 were shown to be up-regulated in PCa, where they promoted invasiveness [134]. Id-1 and Id-2 have also been shown to induce cell survival through different mechanisms. In the PCa cell line LNCaP, Id-1 overexpression resulted in increased resistance against TNF-α induced apoptosis due to increased p50 and p65 levels in the nucleus. Knockdown of Id-1 in DU145 cells mediated increased sensitivity for TNF-α activated apoptosis due to reduced p50 and p65 in the nucleus [135]. Natural killer T-cells with depleted Id-2 undergo increased apoptosis in the liver and have increased expression of the anti-apoptotic Bcl-2 and Bcl-X_L [136]. To test the hypothesis that BCL-3 promotes cell survival and induces expression of Id-1 and Id-2, LNCaP cells overexpressing BCL-3 were used. Increased Id-1 and Id-2 expression levels, as well as decreased sensitivity to chemically induced cell death, was observed upon BCL-3 overexpression. In addition, staining of human prostate cancer tissue showed a significant correlation between high nuclear BCL-3 and expression of Id-1, indicating that BCL-3 regulates Id-1 expression in prostate cancer cells.

No previous reports have shown any direct binding of NF-κB to either of Id-1 or Id-2 promoter sequences. *In silico* analysis of the Id-1 and Id-2 promoter sequences predicted several NF-κB binding sites. To experimentally find proof that BCL-3 could bind directly to the predicted NF-κB binding sites we performed ChIP analysis and could show that BCL-3 binds directly to the promoter regions of both the Id-1 and Id-2 genes.

To further evaluate the role of BCL-3 in prostate cancerogenesis, DUshBCL-3 and DUshControl cells were subcutaneously xenografted into NUDE mice. Tumors developed from DUshControl cells were 4 times larger when compared with tumors from cells with reduced BCL-3. Ki67

and cleaved caspase 3 staining of tumors reveled that tumors with reduced BCL-3 had significantly more cells undergoing apoptosis, but no differences in proliferation could be detected. These results suggest that BCL-3 function as a pro survival gene through activation of Id gene expression in prostate cancer cells.

PAPER II

Serum Response Factor Controls CYLD Expression via MAPK Signaling Pathway

Summary

The tumor suppressor CYLD is a deubiquitinating enzyme that can negatively regulate several different signaling pathways through deconjugation of K63-linked polyubiquitin chains from various substrates. Loss of CYLD in different tumor types leads to increased cell survival or proliferations. In this study we found that CYLD knockout ($CYLD^{-/-}$) MEF cells have significantly increased proliferation rates in a serum dependent manner when compared with wild type ($CYLD^{+/+}$) MEF cells. The reduced proliferation in $CYLD^{+/+}$ cells in the presents of serum was mediated through up-regulation of CYLD by direct binding of serum response factor (SRF) to a CArG site located in the promoter of the CYLD gene. The serum-regulated recruitment of SRF to the CArG site was dependent on p38 mitogen-activated protein kinase (MAPK) activity. Inhibition of p38MAPK disrupted the recruitment of SRF to the CYLD promoter. Knockdown of SRF by siRNA or inhibition of p38MAPK reduced the expression of CYLD and increased the cell proliferation rate. These results suggests that SRF is a positive regulator of CYLD expression, which in turn reduces the mitogenic activation of $MEF^{+/+}$ cells

Results and Discussion

CYLD is a DUB enzyme that has been reported to have tumor suppressor functions in several tumor types, where it have been shown to regulate proliferation by reducing CYCLIND1 expression and induce apoptosis in a NF-κB dependent manner [28, 38]. In addition to NF-κB, CYLD can also interfere with JNK and p38 MAPK signaling [31, 199].

The aim of this study was to evaluate the phenotype of CYLD deficient MEF cells. No phenotype has so far been found in our previously

generated CYLD knockout mice [35]. CYLD is known to deubiquitinate K63-linked polyubiquitin chains from TRAF2 or TRAF6 in response to TNF-α stimulation and thereby negatively regulate classical NF-κB signaling and promote apoptosis in cell lines such as HeLa [24, 25, 27]. The investigation of apoptosis was performed by comparing gradual degradation of internucleosomal DNA after PI staining of $CYLD^{-/-}$ and $CYLD^{+/+}$ MEF cells. The cells were cultured in the presents or absent of serum, or stimulated with TNF-α, cyclohexamide or a combination of TNF-α and cyclohexamide. Surprisingly, no differences between CYLD deficient and wild type MEF cells could be detected, indicating that CYLD does not participate in TNF-α mediated apoptosis in primary MEF cells.

The finding that the proliferation rate of MEF cells was serum dependent intrigued us and we decided to investigate whether the CYLD expression is regulated by serum. We found that CYLD protein and mRNA levels were indeed down-regulated in the absence of serum and re-addition of serum elevated the CYLD expression levels in MEF cells. Serum withdrawal reduced the CYLD mRNA levels already after one hour, indicating that CYLD gene transcription if rapidly regulated in a serum dependent manner.

SRF is a transcription factor that binds to serum response elements (SRE) or CArPG box sequences in the promoter of target genes [111, 200]. *In silico* promoter analysis of the CYLD promoter sequence predicted two SRF binding sites located at -2284 and -1194 from the start of the 5´ UTR (untranslated region). Using ChIP, we detected a prominent recruitment of SRF to the CArPG box located at -1194 in the CYLD promoter only in the presents of serum. We investigated if SRF protein amounts were affected by serum, but no significant difference was detected after serum depravation. Knockdown of SRF using siRNA drastically reduced CYLD protein levels, indicating that SRF is an upstream regulator of CYLD expression. Reduced SFR protein levels also increased the proliferation rate of $MEF^{+/+}$ cells.

In the search for the upstream mechanism that could activate SRF in response to serum, we screened the participation of different MAPK signaling factors. Specific chemical inhibitors targeting ERK, p38 or JNK were added to serum deprived $MEF^{+/+}$ cells together with serum. CYLD protein levels were then used as a measurement of SRF activity. As mentioned previously, CYLD levels were up-regulated when serum is re-added. In cells stimulated with inhibitors against ERK or JNK, CYLD protein levels were indeed up-regulated. However, in cells treated with inhibitors against p38, CYLD protein levels remained low even after addition of serum. In concordance with this result, SRF ChIP in the

presents of serum and p38 inhibitor showed that SRF was unable to bind to the promoter of CYLD.

Several mechanisms have been shown to regulate SRF transcription activity, including phosphorylation and co-factors. Two principal families of cofactors of SRF has been described, members of the TCF family of Ets proteins, ELK1, SAP-1 and SAP-2 are activated by MAPK phosphorylation. The second family, MRTF, consists of myocardin, MRTF-B and MAL that are regulated by a pathway controlled by monomeric actin and Rho GTPases. In 2009, Descot *et al.* found that CYLD expression is unaltered following changes in actin-MAL signaling [201]. MAL belongs to the myocardin-related transcription factor (MRTF) family, which was not found to be directly activated by p38MAPK. In our study, we demonstrated that LPA, known to activate MAL-SRF dependent transcription, failed to affect proliferation in MEF cells. It has been shown that phosphorylation by Ca^{2+}/Calmodulin-dependent kinase (CaMK) and by p38MAPK through activation of MAPKAP kinase 2 (MK2) can regulate the DNA binding capability of SRF [202-204]. To investigate if the phosphorylated form of SRF bound to the CYLD promoter, we performed phospho-specific ChIP of SRF and could indeed determine that p-SRF bound to the -1194 CArPG site in the CYLD promoter, suggesting that SRF are regulated in a p38 and MK2 dependent manner.

Our results suggest that CYLD deficient MEF cells have an elevated proliferation rate in the presence of serum. SRF binds to the CYLD promoter in a serum dependent manner, and is a positive regulator of CYLD expression. Furthermore, knockdown of SRF results in decreased CYLD expression and an elevated proliferation rate. Inhibition of p38MAPK disrupted the specific interaction between SRF and the CYLD gene, and initiated increased proliferation suggesting that p38MAPK is essential for SRF activation.

Paper III

CYLD prevents development of hepatocellular carcinoma via inactivation of the JNK pathway

Summary

The tumor suppressor CYLD is a deubiquitinating enzyme able to cleave polyubiquitin chains from substrate and thereby regulate different signaling pathways. Mutations in or low expression of CYLD has been reported in different human cancer types. In this study we found that CYLD is significantly down-regulated in human hepatocellular carcinoma (HCC) and that CYLD expression was inversely correlated with the expression of Ki67. CYLD deficient mice were more susceptible to the chemical carcinogen DEN induced HCC, forming more and larger tumors. Tumors formed in CYLD knockout mice had elevated expression of Ki67 and CYCLIND1, indicating an increased proliferation rate. DEN exposure promoted TRAF2 ubiquitination and also activation of its downstream target JNK1 in CYLD deficient liver tissue. Activation of JNK1 after DEN stimulation was also detected in primary hepatocytes isolated from CYLD knockout mice. Transient transfection of CYLD into a HCC cell line restricted cell proliferation but had no effect on apoptosis, adhesion or migration. Together these results suggest that CYLD down-regulation is a risk factor for development and progression of HCC mediated through activation of JNK1

Results and Discussion

Hepatocarcinogenesis is a multiple step process that can include chronic inflammation and genetic alterations in proto-oncogenes or loss of tumor-suppression genes. In earlier studies, the tumor suppressor CYLD was shown to be down-regulated in hepatocellular carcinoma cell lines and tissues [36, 37]. In hepatitis C virus associated HCC, reduced CYLD expression was found in more than 30% of the tumors [205].

The aim of this study was to investigate whether CYLD has tumor suppressing functions in HCC, and if it does, which signaling pathways CYLD regulates. Firstly, male wild type and CYLD knockout mice were exposed to a single dose of the chemical carcinogen DEN. After 12 months duration, CYLD deficient animals had developed bigger and more numerous liver tumors compared with control mice. In previous studies,

CYLD deficiency has been correlated with increased migration and invasion of tumor cells in squamous carcinoma cells [206], however in our model system, no metastasized tumor cells could be detected in lymph nodes or other organs. The number of tumors, tumor weight, average tumor size and maximal tumor size was significantly higher in CYLD deficient mice compared with control mice. Immunohistochemical staining of the tumors against proliferation and apoptosis markers revealed a significant increase of Ki67 and CYCLIND1 expression in tumors from $CYLD^{-/-}$, mice but no difference in the number of TUNEL positive cells in tumors from $CYLD^{-/-}$ when compared with wild type mice could be detected. Furthermore, the short-term effect of DEN was evaluated after 48 to 72 hours post-injection. We could observe an increased number of BrdU positive cells, and western blot analysis showed higher expression of CYCLIND in the liver of $CYLD^{-/-}$ but not in control mice. In concordance with this result, in vitro experiments using over-expression of CYLD in the HCC cell line HepG2 resulted in a decreased proliferation rate whereas cell adhesion, migration and survival was unaltered. Our finding that low CYLD levels results in increased proliferation, both *in vivo* and *in vitro* experiments, fit with the inverse correlation between CYLD expression and observed proliferation in human HCC.

In the investigation of the underlying mechanism for CYLD mediated proliferation, different pathways were analyzed. JNK, NF-κB and BCL-3 are pathways that have been shown to regulate cell survival and proliferation in a CYLD dependent manner [207]. No difference in nuclear localized p65 or BCL-3 in tumors from CYLD knockout mice could be detected when compared with tumors from wild type mice, however a significant increase of phosphorylated JNK1 was observed in CYLD deficient tumors. Increased phospholylation of JNK1 could also be detected in liver samples already 48 hours after DEN administration. JNK1 has been associated with proliferation and carcinogenesis in both humans and in mouse models [193]. It was demonstrated that JNK1 knockout mice were less prone to develop DEN induced liver carcinomas when compared with wild type mice. [193, 194, 208]. It has also been shown that JNK1 signaling can affect cell proliferation, migration, differentiation and survival [209-211]. Ubiquitinated TRAF2 is a known activator of JNK1 mediated AP-1 transcription [25]

We found that in the absent of CYLD, ubiquitination of TRAF2 increased, causing an elevated phosphorylation of JNK1. This facilitated higher promoter activity of the AP-1, leading to transcriptional activation of genes important for cell proliferation such as CYCLIND1 and c-Myc. These results suggest that CYLD mediated inactivation of JNK signaling reduces

c-Myc and CYCLIND1 expression, thus preventing aberrant proliferation in hepatocytes and tumor cells. This finding was also in accordance with previous publications showing that JNK deletion reduces CYCLIND1 and HCC cell proliferation [194].

Conclusions

- IL-6 induces BCL-3 expression and nuclear translocation via STAT3 activation in prostate cancer cells.
- Activation of BCL-3 elevates the levels of Id-1 and Id-2 proteins through direct binding of their promoter regions in prostate cancer cell lines.
- High nuclear BCL-3 in human prostate cancer tissue correlated with Id-1 expression.
- Knockdown of BCL-3 in DU145 cells induces apoptosis in prostate cancer cell lines treated with chemotherapeutic drugs.
- Xenografted DU145 cells expressing low levels of BCL-3 formed significantly smaller tumors when compared with control, due to elevated apoptosis.

- CYLD deficient primary MEF cells have an elevated proliferation rate in the presents of serum when compared with wild type MEF cells.
- In primary MEF cells, CYLD is a negative regulator of CYCLIND1 protein levels.
- CYLD mRNA and protein levels in MEF cells decrease in the absence of serum.
- SRF directly binds to a CArG box in the CYLD promoter and initiates expression of CYLD in a p38 MAPK dependent manner.

- CYLD is down-regulated in human HCC tissue when compared with paired benign liver tissue.
- Low CYLD expression in human HCC correlates with high levels of proliferation.
- In CYLD knock out mouse liver, DEN induces a significant increase in number of tumors formed, tumor weight, average tumor size and maximal tumor size when compared with wild type mice.
- DEN treatment of CYLD knockout mice induces JNK1 phosphorylation and AP-1 activity through elevated TRAF2 ubiquitination.
- Tumors from CYLD knock out mouse liver have a higher expression of CYCLIND1 and c-Myc.

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Populärvetenskaplig sammanfattning

Bakgrund

I mitt avhandligsarbete har jag fokuserat på funktionen av generna BCL-3 och CYLD i cancer. Proteinerna som dessa gener kodar för har många olika funktioner, vissa varierar beroende på vilken vävnadstyp de befinner sig i. BCL-3 är en så kallad oncogen, vilket innebär att för mycket aktivitet av BCL-3 proteinet gynnar cancercellerna. CYLD, å andra sidan, är en tumörsuppressorgen som i vissa fall kan reglera aktiviteten av BCL-3 och på så sätt hindra att BCL-3 blir överaktivt, vilket missgynnar cancercellerna. I den ovanliga hudcancern familjär cylindomatosis har patienterna mutationer i CYLD genen, vilket leder till för mycket aktivt BCL-3, detta leder i sin tur till okontrollerad celldelning. BCL-3 är en så kallad transkriptionsfaktor vilket innebär att den kan binda till speciella DNA sekvenser i olika gener som är viktiga för att reglera produktionen av genen i fråga. I andra tumörtyper har det visat sig att för mycket BCL-3 bidragit till att tumörceller inte kan genomgå självinducerad celldöd (apoptos) som de bör, vilket leder till att det totala antalet tumörceller ökar.

Arbete I

Målet med det första arbetet var att undersöka om BCL-3 fungerade som en oncogen i prostatacancer, och i så fall hur. Vi började med att färga in BCL-3 i prostatatumörer och såg en correlation mellan en ökad mängd BCL-3 och många infiltrerade immunceller i tumörerna. Det är sedan tidigare känt att immunceller kan producera den inflammatoriska mediatorn IL-6. Höga nivåer av IL-6 i prostatacancer brukar innebära en dyster prognos för patienten.

För att kunna undersöka om IL-6 kan initiera BCL-3 produktion använde vi oss av etablerade prostatacancer celler som enkelt kan odlas i odlingmedium. Vid IL-6 tillsats så ökade produktionen av BCL-3 i cellerna. En av celltyperna, DU145, producerade redan eget IL-6 och hade höga BCL-3 nivåer, vilket gjorde dem ideala för att närmare undersöka funktionen av BCL-3. Vi modifierade dessa cellerna så att de nästan inte producerade något BCL-3 och undersökte vilken effekt detta resulterade i. Det visade sig att nedreglering av BCL-3 gjorde cellerna mer mottagliga för cellgifter som ibland används för cancerbehandling. Vi injicerade DU145 celler med eller utan BCL-3 under huden på möss och fann att celler utan Bcl-3 bildade tumörer som var en fjärdedel så stora, till följd av ökad apoptos. Efter detta intressanta resultat bestämde vi oss för att ta reda på mer om hur detta kunde komma sig. Därför jämförde vi den individuella produktionen för samtliga gener i normala DU145 celler och DU145 celler utan BCL-3. Bland de gener som hade störst skillnad i productionsnivå i de båda celltyperna hittade vi två gener tillhörande familjen ”Inhibitor of DNA binding” (Id). BCL-3 kunde binda direkt till specifika DNA sekvenser i båda dessa gener och därmed bidra till en ökad produktion av dem. Artificiell blockering av Id tillverkning visade sig också leda till ökad celldöd, till följd av behandling med cellgifter.

Tillsammans visar våra resultat att IL-6 ökar mängden BCL-3 som i sin tur ger en ökad Id produktion i prostata cancer. Dessutom motverkar BCL-3 en effektiv behandling av prostata cancer celler med cellgifter. Detta kan vara en bidragande faktor till att höga IL-6 värden kan ge en värre prognos för prostatacancer patienter.

Arbete II

I det andra arbetet undersökte vi funktionen av CYLD i celler isolerade från genmanupulerade CYLD ”knockout” möss eller normala ”vildtyp” möss. Tidigare har det visats i tumörtypen familjär cylindromatosis att brist på CYLD leder till ökad aktivitet av BCL-3 och att det i sin tur ger en ökad celldelninghastighet.

Vi observerade att celler utan CYLD hade en mycket högre celldelningshastighet jämfört med normala celler, men bara när de växte i odlingsmedium med blodplasma i. De snabbt växande cellerna hade mer aktivt BCL-3 jämfört med vildtyp celler. Vi mätte därför CYLD mängden i vildtyp celler som odlats med eller utan blodplasma. Det visade sig att CYLD nivåerna sjönk efter att plasma tagits bort från odlingsmediumet. För

att undersöka hur plasma kunde bidra till uppreglerad CYLD produktion, undersökte vi CYLD genens DNA sekvens och hittade en potentiell inbindningsplats för transcriptionsfaktorn SRF. Vi verifierade experimentellt att SRF fysiskt kunde binda till CYLD genen samt bidra till ökad produktion av CYLD, men bara när cellerna hade odlats i plasma. Artificiell blockering av SRF ledde till ökad celldelningshastighet. Dessa resultat föreslår att SRF reglerar cellers delningshastighet via reglering av CYLD, som i sin tur påverkar aktiviteten av BCL-3.

Arbete III

I det sista arbetet utforskade vi funktionen av CYLD i levercancern hepatocellular carcinoma (HCC), vilken är det typ av cancer som globalt sett orsakar flest cancerrelaterade dödsfall. De största riskfaktorerna för att drabbas av HCC är kronisk viral hepatitis infection samt alkoholism.

Först färgade vi in CYLD och en celldelningsmarkör i snitt från cancerfri lever samt HCC. Där såg vi att i hälften av HCC infärgningarna var CYLD kraftigt nedreglerat vilket även överensstämmde med de högsta infärgningarna av celldelningsmarkören. För att undersöka om brist på CYLD leder till ökad HCC bildning använde vi oss av vildtyp samt CYLD knockout möss. Mössen behandlades med DEN som är ett leverspecifikt carcinogent ämne. Efter ett år visade det sig att möss utan CYLD bildade fler samt större tumörer jämfört med vildtyp möss. Infärgningar av tumörer från möss visade ingen skillnad i aktiverat BCL-3 men tumörerceller från CYLD knockout möss hade ändå en signifikant ökad celldelningshastighet. Däremot fann vi en ökad infärgning för aktivit JNK som ingår i en signalväg som kan regleras av CYLD. Det är känt att ökad JNK aktivitet kan leda till högre celldelningshastighet genom att öka produktionen av c-Myc och CYCLIND1. I tumörerna utan CYLD kunde vi detektera högre nivåer av både c-Myc samt CYCLIND1. I HCC som bildats till följd av hepatitis infection eller alkoholism har 70% av fallen ökade nivåer av c-Myc.

För att stärka våra bevis för att CYLD har tumör supressor funktioner i HCC använde vi oss av humana HCC celler där vi artificiellt ökade nivåerna av CYLD. Dessa celler fick då en minskad celldelningshastighet samt en minskad mängd c-Myc, CYCLIND1 och aktivt JNK.

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