INVESTIGATION OF THE ANTIPROLIFERATIVE MECHANISMS OF THE CHEMOPREVENTIVE AGENT URSODEOXYCHOLIC ACID (UDCA) *IN VITRO*

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To my grandmothers: Yaya Pita i Àvia Maria

To the memory of my grandfathers: Yayo Josep i Avi Josep

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2. ABBREVIATIONS

5-ASA	5-aminosalicilic acid; Mesalazine
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AKT	Ser/Thr protein kinase; protein kinase B
AOM	Azoxymethane
AP-1	Activator protein 1
APO	Fas receptor
APS	Ammonium persulfate
ARF	ADP rybosilation factor
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related
BAX	Bcl-2 associated protein X
Bcl-2	B-cell lymphoma/leukaemia 2
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	Cyclosome
CA	Colic acid
CD95	Fas receptor
Cdc6	Cell division cycle phosphatase 6
Cdc25	Cell division cycle phsophatase 25
CDCA	Chenodeoxycholic acid
CDK	Cyclin dependent kinase
Chk	Chekpoint kinase
COX	Cyclooxigenase
CRC	Colorectal cancer
CSK	Cytoskeletal
Cy3	Cyanine dye 3
DAPI	4’,6-diamidino-2-phenylindole

DCA	Deoxycholic acid
DIABLO	Direct IAP-binding protein with low pI, also referred to as SMAC
DMEM	Dulbecco´s modified Eagle´s medium
Dsh	Dishevelled
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
E2F	E2 promoter binding factor
EDTA	Ethylenediaminetetraacetic acid
EDU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
EP2	Prostaglandin E2 receptor
ERK	Extracellular-signal-regulated kinase
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	Relative celtrifugal force (RCF) or grams
G0	Gap zero phase (period in which cells are in quiescent state)
G1	Gap 1 (rest phase of cell cycle before replication)
G2	Gap 2 (rest phase of cell cycle before mitosis)
GSK3β	Glycogen synthase kinase 3 beta
HA	Hemagglutinin
HIF	Hypoxia-inducible factor
hMLH1	Human MutL homolog 1
hMSH2	Human MutS homolog 2
HNPCC	Hereditary nonpolyposis colorectal cancer
IAP	Inhibitor of apoptosis
IFN	Interferon
IgG	Immunoglobulin G

IL	Interleukin
iNOS	Inducible nitric oxide synthase
K-ras	Kirsten rat sarcoma
LB	Luria broth
LCA	Lithocholic acid
LEF	Lymphoid enhancing factor
LRP	Lipoprotein receptor-related protein
Luc	Luciferase
M	Molarity or Mitosis
MAPK	Mitogen activated protein kinase
MCM	Mini-chromosome maintenance
MDM2	Murine double minute 2
MIP-2	Macrophage inflammatory protein-2
MMR	Mismatch repair
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF1	Nuclear factor 1
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
ORC	Origin recognition complex
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase
PI	Propidium iodide
PI3K	Phosphoatidylinositol-3-kinase
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PMSF	Phenylmethanesulfonylfluoride
PPAR	Peroxisome-proliferator-activated receptor
preRC	Prereplicative complex

PSC	Primary sclerosing cholangitis
PTEN	Phsophatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma
RPMI	Roswell Park Memorial Institute medium
RT	Reverse-transcription or room temperature
RT-PCR	Reverse-transcription polymerase chain reaction
S	Synthesis phase of the cell cycle
SDS	Sodium dodecyl sulphate
Ser	Serine
Smac	Second mitochondiral activator of caspases
SMAD4	Mothers against decapentaplegic homolog 4
TAE	Tris acetate-EDTA buffer
TBS	Tris buffered saline
TCF	T-cell factor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Thr	Threonine
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TXA2	Tromboxane A2
Tyr	Tyrosine
UC	Ulcerative colitis
UDCA	Ursodeoxycholic acid
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau tumor suppressor

3. SUMMARY

Introduction: Colorectal cancer is the second most common cancer and the third most common cause of cancer death in the western world. Ursodeoxycholic acid (UDCA), a bile acid used in the treatment of primary biliary cirrhosis, has been shown to prevent colon cancer in animal models. The mechanism of the chemopreventive action of UDCA is not understood.

Objectives: The objective of this project was to investigate the antiproliferative mechanisms of UDCA.

Materials and Methods: Four established $p53^{wt}$ human carcinoma cell lines were treated with UDCA (0-400 µM) for 3 days and proliferation was investigated by cell count, MTT test and BrdU incorporation. Apoptosis was determined by DAPI staining and detection of PARP cleavage by western blot. Senescence was determined by β-galactosidase staining. The cell cycle was studied by FACS using nocodazole-synchronized or non-synchronized cells. Expression of c-Myc and other cell cycle markers were analysed by western blot. Effect of UDCA on PCNA binding to chromatine was detected by inmunohistochemistry and E2F-1 transcriptional activity was investigated by luciferase reporter assay. c-Myc was suppressed by transfection by pSuper-c-Myc plasmid. Overexpression of c-Myc was induced by transient transfection of pCMV-c-Myc plasmid. Transcriptional regulation of c-Myc was investigated by luciferase reporter assay and OneStep RT-PCR. The influence on the *wnt* pathway was tested in an isogenic pair of *wnt*-proficient and *wnt*-deficient cell lines.

Results: UDCA inhibited the growth of colon cancer cells in a partially reversible manner, the extent of inhibition was independent from the speed of growth of the cell line. UDCA did not induce apoptosis and induced senescence only in LS513 cells. S-phase population was decreased after treatment in all cell lines investigated, in HCT116 and HCT8 cells UDCA slowed down the cell cycle and induced G1→S transition delay, and in LS513 it induced increase in G2/M population and S-phase arrest. UDCA treatment downregulated c-Myc, cyclin A, CDK2, CDK6 and Rb, upregulated p21 and induced Rb hypophosporylation. UDCA decreased E2F-1 transcriptional activity and inhibited PCNA binding to chromatine. The G1→S transition delay by UDCA was concomitant with effect

on c-Myc, p21, and cyclin A expression and Rb phosphorylation. c-Myc suppression was sufficient to inhibit proliferation, induce p21 upregulation, CDK2 and CDK6 downregulation and Rb hypophosphorylation. c-Myc downregulation was not transcriptional and it was concomitant with increase in phosphorylation at threonine 58 and a persistant ERK phosphorylation. Cells overexpressing c-Myc did not proliferate and the *wnt* pathway deficiency did not affect the inhibition of proliferation by UDCA.

Conclusion: UDCA decreased the proliferation of colon cancer cells by delaying the G1→S transition and slowing down the cell cycle. Suppression of c-Myc contributed to the inhibition of proliferation. Together with p21 overexpression, Rb hypophosphorylation and cyclin A downregulation, it could play a role in the delay of the G1→S transition and inhibition of proliferation.

ZUSAMMENFASSUNG

Einleitung: Das kolorektale Karzinom ist die zweithäufigste Krebserkrankung und die dritthäufigste Krebstodesursache in der westlichen Welt. Es wurde gezeigt, dass, Ursodeoxycholsäure (UDCA), eine Gallensäure die zur Behandlung der primär biliären Zirrhose verwendet wird, die Entstehung des Kolonkarzinoms im Tiermodell und der chronischen Entzündung verhindert. Der Mechanismus der chemopräventiven Wirkung von UDCA ist noch nicht verstanden.

Ziele: Das Ziel dieses Projektes war es, die antiproliferativen Mechanismen von UDCA zu untersuchen.

Material und Methodik: Vier etablierte humane $p53^{wt}$ Kolonkarzinom-Zelllinien wurden mit UDCA (0-400 µM) für 3 Tage behandelt und die Proliferation wurde durch Zellzählung, MTT-Test und Bromodesoxyuridin (BrdU)-Einbau untersucht. Apoptose wurde durch DAPI-Färbung und Detektion von PARP-Spaltung mittels Western Blot bestimmt. Seneszenz wurde durch β-Galaktosidase-Färbung bestimmt. Der Zellzyklus von Nocodazol-synchronisierten oder nicht-synchronisierten Zellen wurde mittels FACS untersucht. Die Expression von c-Myc und anderen Zellzyklus-Markern wurde mittels Western Blot analysiert. Die Wirkung von UDCA auf PCNA-Bindung an Chromatin wurde mittels Immunhistochemie untersucht, und der E2F-1 transkriptionelle Aktivität wurde mit Hilfe von Luciferase-Reportersonden ermittelt. c-Myc Expression wurde durch Transfektion mit einem pSuper-c-Myc Plasmid unterdrückt. Die Überexpression von c-Myc wurde durch Transfektion mit pCMV-c-Myc Plasmid induziert. Die Transkriptionelle Regulation von c-Myc wurde mit Luciferase-Reportersonden und OneStep RT-PCR untersucht. Der Einfluss auf den Wnt-Signalweg wurde in einem isogenen Paar Wnt-kompetenter und Wnt-defizienter Zelllinien getestet.

Ergebnisse: UDCA hemmte das Wachstum von Kolonkarzinomzellen teilweise reversibel. Das Ausmaß der Hemmung war von der Geschwindigkeit des Wachstums der Zelllinie unabhängig. UDCA induzierte keine Apoptose und Seneszenz wurde nur in LS513 Zellen induziert. Der S-Phase Anteil war nach der Behandlung in allen Zelllinien reduziert. UDCA verlangsamte in HCT116 und HCT8 Zellen den Zellzyklus und verzögerte den G1→ S Übergang. Sie erhöhte in LS513 Zellen den G2/M Anteil und induzierte einen S-

Phasen Arrest. UDCA Behandlung reduzierte die c-Myc, Cyclin A, CDK2, CDK6 und Rb Expression, erhöhte die p21 Expression und induzierte Rb Hypophosporylierung. UDCA inhibierte die transkriptionelle Aktivität von E2F-1 und die Bindung von PCNA an Chromatin. Die Verzögerung des G1 → S Übergangs durch UDCA war gleichzeitig mit der Veränderung der Expression von c-Myc, p21 und Cyclin A und mit der Rb Hypophosphorylierung verbunden. Die c-Myc Supression war ausreichend, um die Zellproliferation zu hemmen, die Expression von p21 zu erhöhen, die CDK2 und CDK6 zu erniedrigen und die Rb Hypophosphorylierung zu inhibieren. c-Myc wurde auf transkriptionellen Ebene nicht reguliert. Das geschah zeitgleich mit der Zunahme der Phosphorylierung an Threonin 58 und einer anhaltenden ERK-Phosphorylierung. Zellen in denen c-Myc Überexprimiert war proliferierten nicht und das Fehlen des Wnt-Signalwegs hatte keinen Einfluss auf die Hemmung der Proliferation durch UDCA.

Fazit: UDCA verringert die Proliferation von Kolonkarzinomzellen durch Verzögerung des G1 → S Übergangs und eine Verlangsamung des Zellzyklus. Die Unterdrückung von c-Myc trägt zur Hemmung der Proliferation bei. Zusammen mit der p21 Überexpression, der Rb Hypophosphorylierung und der Cyclin A Supression, könnte dies verantwortlich für die Verzögerung des G1 → S Übergangs und die Hemmung der Proliferation sein.

4. INTRODUCTION

4.1. Etiology of colorectal cancer

Colorectal cancer is the third most frequently diagnosed cancer in men and women, and most frequent cause of cancer deaths in the Western countries (1).

Carcinogenesis is a multistep process, in which the first step involves a mutation in DNA which alters the growth characteristics of the cell. This results in an abnormally proliferating focus of cells that can be identified as aberrant crypt foci. These cells are still "benign" but are predisposed to undergo further genetic changes that may result in cells with a malignant phenotype, also called carcinoma (2).

Fearon and Vogelstein proposed a genetic model of colorectal tumorigenesis. They proposed that a tumor develops through a series of genetic alterations which involve oncogenes and tumor suppressor genes that respectively induce or suppress cancerous phenotype (Figure 4.1) (3). In this model, initial mutations of the adenomatous polyposis coli (APC) gene allow adenoma formation and the development of dysplastic crypts. Tumor promotion and progression is achieved due to a sequence of further mutations in *K-ras*, SMAD4, and *p53* genes which enable further adenoma growth, the expansion of individual cell clones, and tumor invasion and metastasis (4).

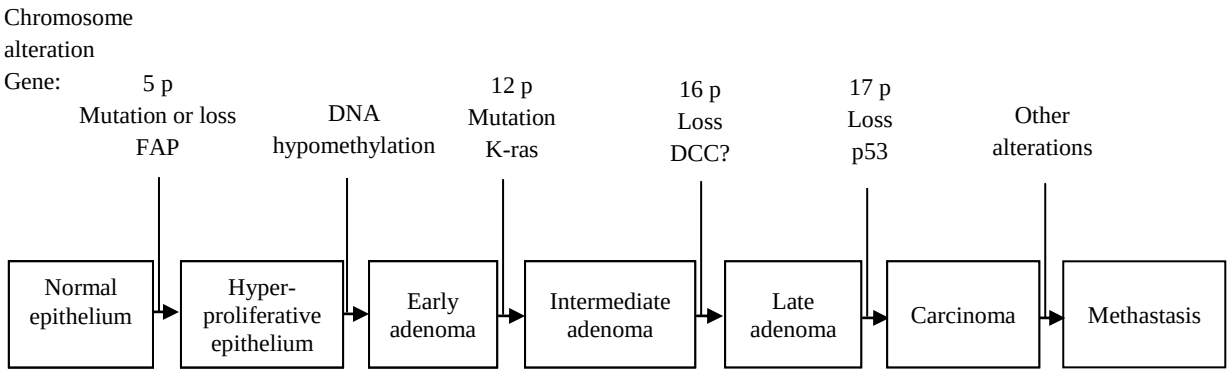

Figure 4.1. A genetic model for colorectal tumorigenesis. The chromosomes altered in colorectal cancer development are 5 p, 12 p, 16 p and 17 p, in which mutations take place. This model shows how normal epithelium develops into carcinoma, and the different phases in between, in which genomic alterations take place (FAP = Familial Adenomatous Polyposis) (3).

Colorectal cancer can arise sporadically, or results from a genetic predisposition or can be associated with colitis.

4.1.1. Sporadic colorectal cancer

Sporadic colorectal carcinoma develops as a result of chromosomal instability or microsatellite instability. This often results in mutation of p53 and APC. 85 % of sporadic and hereditary colorectal cancers show mutation of APC, which is considered an early event. APC has the function of destabilizing free β-catenin, one of the central members of the *wnt* pathway. In sporadic adenoma, other genetic changes can occur such as mutation of the oncogene *K-ras*. p53 loses its function at a later stage and this is considered to be the event that is associated with the adenoma to carcinoma transition (5).

4.1.2. Hereditary colorectal cancer

Colorectal cancer can develop due to a genetical predisposition. The most common hereditary colorectal cancer syndrome is the Lynch syndrome also called Hereditary Nonpolyposis Colorectal Cancer (HNPCC). 2 % of the colorectal cancers are of this type. Mutations in several mismatch repair (MMR) genes, as hMSH2 or hMLH1, can be involved, leading to microsatellite instability (6). Familial Adenomatous Polyposis (FAP), an autosomal dominant condition in which thousands of adenomatous polyps develop in the colon during childhood or adolescence, is another genetic predisposition to colorectal cancer. It is caused by the germ line mutations in the APC gene and it accounts for less than 1 % of the colorectal cancers (7).

4.1.3. Colitis-associated colorectal cancer

Patients who suffer from inflammatory bowel diseases, ulcerative colitis (UC) or Crohn´s disease, have a high risk of developing colorectal cancer. Colitis-associated cancer occurs when normal cell growth and tissue homeostasis is disrupted due to sequential mutations and epigenetic alterations of cancer-related genes, with DNA methylation, histone modification and mutations induced by reactive oxygen species (8).

Molecules that have been implicated in carcinogenesis include proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukins 1 and 6 (IL-1, IL-6) or interferon gamma (IFN-γ), which promote carcinogenesis, by inducing the downregulation of MMR proteins like MSH2 and MSH6 through a variety of mechanisms. One of those is due to displacement of c-Myc from MSH2/MSH6 promoters by HIF-1a, induced by IL-1 and IL-6, prostaglandin E2 (PGE2) and reactive oxygen and nitrogen species (9, 10). High quantities of the free radical nitric oxide (NO) are associated with colitis and are produced by the inducible nitric oxide synthase (iNOS). iNOS is found to be overexpressed in inflamed intestinal tissue (11). High levels of reactive oxygen/nitrogen species are generated during inflammation, resulting in oxidative and nitrosative stress. In this environment, NO can generate strong reactive nitrogen species which can cause DNA damage and form carcinogenic nitrosamines resulting in mutagenesis. Oxidative stress can cause mutations in p53, Bcl-2 and APC (8). Moreover, NO has been shown to induce expression of cyclooxygenase-2 (COX-2) and PGE-2 production (12), both contributing to colitis-associated cancer.

COX-2 is a cytoplasmic protein that catalyzes the synthesis of lipid inflammatory mediators (prostaglandins and prostacyclins) from arachidonic acid whose expression is increased in inflammation, as well as in 80% of CRCs and 40% of colorectal adenomas (8). COX-2 has a role in the invasiveness of colon cancer by activation of metalloproteinase, matrix metalloproteinase and enhancement of VEGF expression (9, 13). PGE-2, which is one of COX-2 products, promotes colon cancer through activating the *wnt* pathway (14).

TNF, IL-6 and IL-1 are known to influence the capacity of cancer cells to metastasize. In particular, in models of chemically induced tumors, IL-1β secreted by malignant cells or infiltrating leukocytes contributes to increased tumor invasion, angiogenesis and immune suppression (9).

Another factor involved in colitis associated carcinogenesis is the transcription factor NF-κB, which induces the expression of inflammatory cytokines, adhesion molecules, key enzymes in the prostaglandin synthase pathway (COX-2), nitric oxide synthase and angiogenic factors (15). In addition, by inducing antiapoptotic genes, such as Bcl-2, it promotes survival in tumor cells and in epithelial cells targeted by carcinogens (9). It

regulates cell cycle progression by regulating target genes such as cyclin D1, c-Myc or cyclin E/CDK2 complex (15).

The molecular mechanism of colitis associated cancer is described in point 4.6.4.

There are differences between sporadic colorectal cancer and colitis-associated cancer. In sporadic colorectal cancer dysplastic lesions arise in one or two focal areas of the colon, whereas in colitic mucosa, dysplasia or cancer can be multifocal. Many molecular alterations are responsible for both types of cancer development but there are differences in the timing and frequency of these alterations. For example, while in sporadic colorectal cancer, *APC* loss of function is a very common early event, in colitis-associated cancer it is less so frequent and it usually occurs late. Mutations in *APC* are very rarely present in inflamed colitic mucosa. Moreover, *p53* plays an important role in colits-associated cancer and mutations occur at an early stage, whereas in sporadic colorectal cancer loss of *p53* gene function occurs late and is believed to be the defining event that drives the adenoma to carcinoma. COX-2 and *k-ras* mutations also occur at a different stage of tumor development (Figure 4.2) (16).

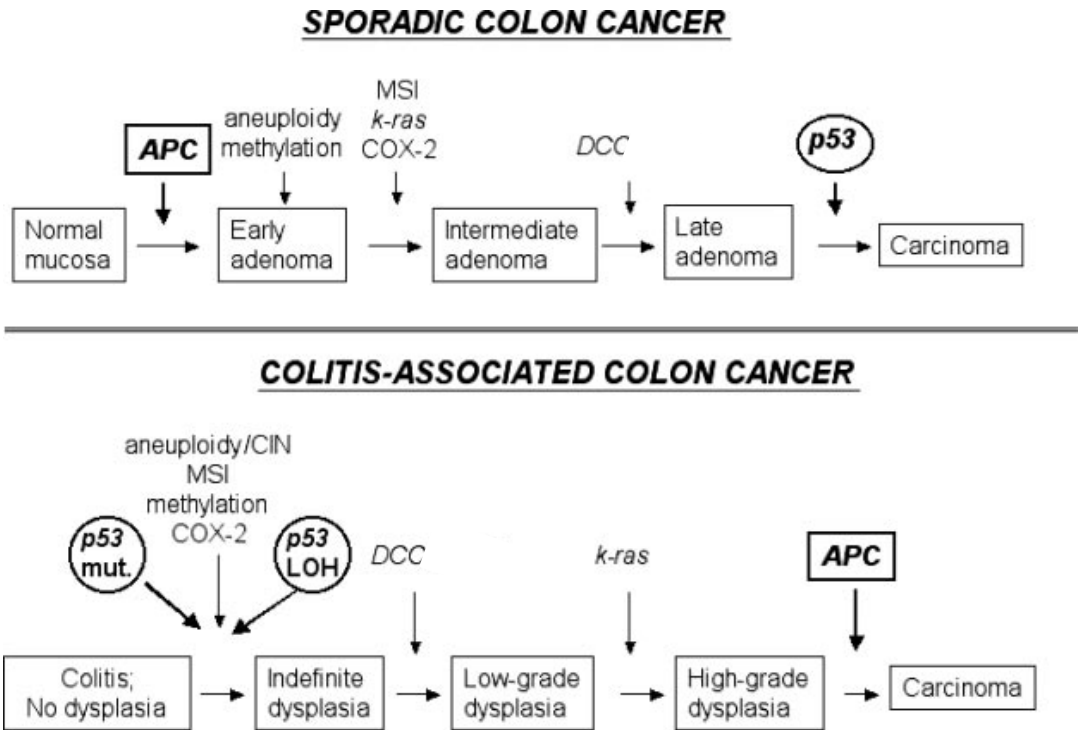

Figure 4.2. Molecular alterations in sporadic colorectal cancer and colitis-associated colon cancer. MSI= microsatellite instability, CIN= chromosome instability, LOH= loss of heterozygosis, mut= mutated, DCC= deletion in colon cancer (16).

4.2. The cell cycle and its regulation

Cell division consists of two consecutive processes: DNA replication and segregation of replicated chromosomes into two separate cells. Originally, cell division was divided into two stages: mitosis (M), the process of nuclear division, and interphase, the process between two M phases in which cells grow in size. The interphase includes G1, S and G2 phases. Replication of DNA occurs in the S-phase, which is preceded by a gap called G1 where the cell prepares for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis. G1, S, G2 and M phases are the traditional subdivisions of the cell cycle. Cells in G1, before entering DNA replication, can be in a quiescent state called G0, in which they are not proliferating (Figure 4.2) (17).

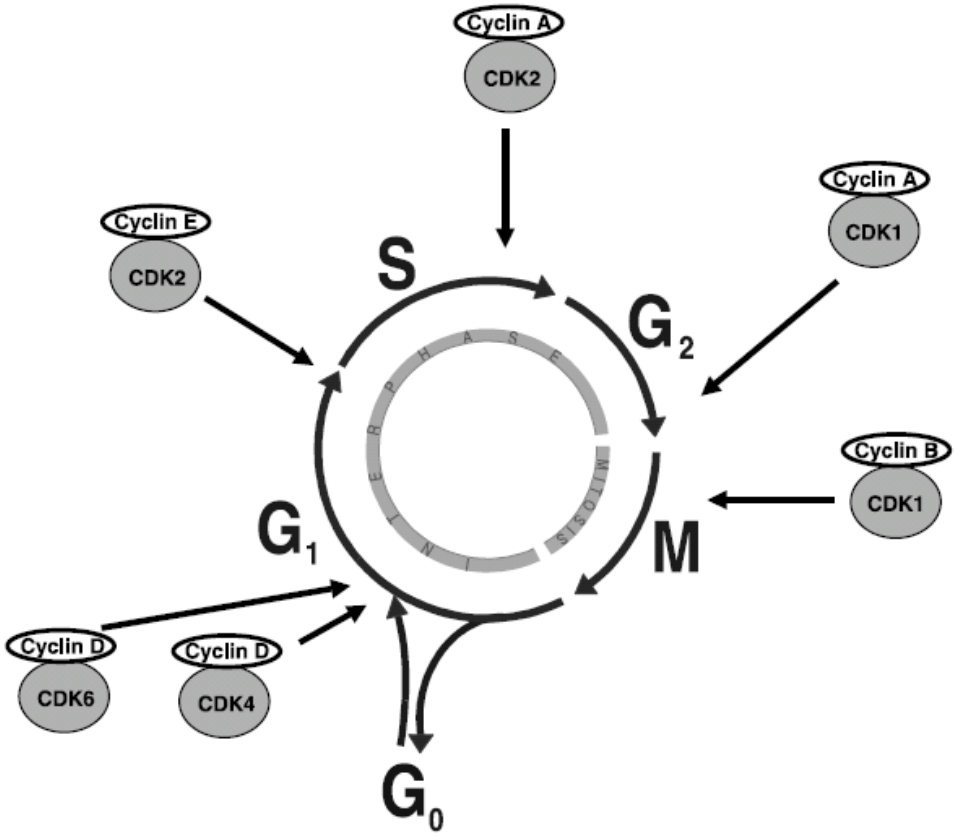

Figure 4.2. Regulation of the cell cycle by cyclin/CDK complexes. This figure shows the different phases of the cell cycle and the cyclin/CDK complexes which regulate each phase (17).

4.2.1. Regulation of the G1→S-phase transition and S-phase progression

Progression through G1 cell cycle phase is controlled by the activity of different cyclin-dependent kinases (CDKs) and their corresponding activating cyclins. Mainly, cyclin D/CDK4 and cyclin D/CDK6 complexes are essential for the progression through G1-phase, and cyclin E/CDK2 complex is important for the entry into S-phase. Cyclin/CDK complexes have the function of phosphorylating retinoblastoma protein (Rb), and Rb-like proteins (p130, p107) (18). Rb binds to E2F family of transcription factors and inactivates them. When Rb is phosphorylated, these factors are released and transcription of proteins involved in proliferation take place. When cells enter the cell cycle from quiescence, upon growth factor stimulation, D cyclins, CDK4 and CDK6 are induced. They are assembled and activated in the middle of G1-phase reaching a maximal of activity near the G1→S transition, where they phosphorylate Rb, whose phosphorylation is continued by cyclin E/CDK2 (Figure 4.3). The expression of cyclin E is maximal at the G1→S transition, where it binds to CDK2 (19-22).

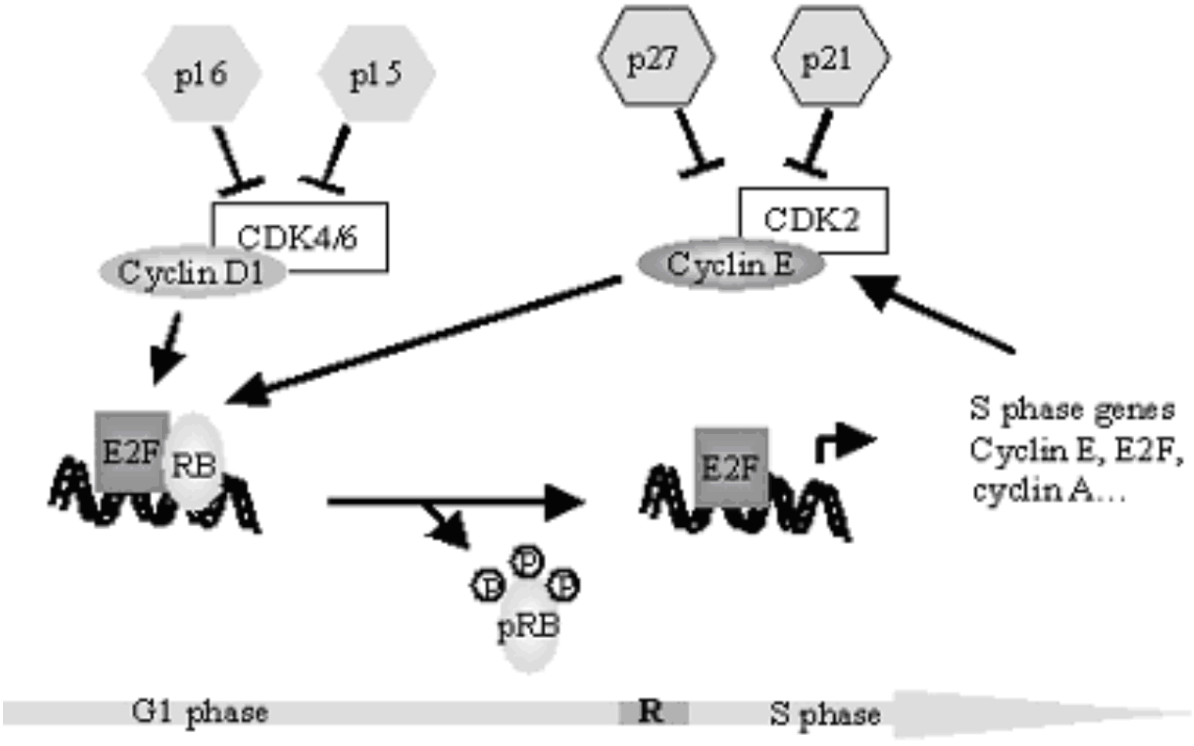

Figure 4.3. The G1→S transition of the cell cycle. cyclin D1/CDK4 and cyclin D1/CDK6 complexes induce phosphorylation of Rb and release of E2F transcription factor, to promote transcription of genes involved in G1→S transition. Rb is further phosphorylated by cyclin E/CDK2 complex in the G1→S transition checkpoint (R). E2F transcribes then genes involved in S-phase progression, such as cyclin A, as well as itself and cyclin E, creating a positive feedback loop. p16, p15, p27 and p21 are inhibitors of cyclin/CDK complexes (22).

In S-phase DNA replication takes place. As cells enter G1 from mitosis, cell division cycle 6 (Cdc6) and Cdt1 proteins are recruited to the origin recognition complex (ORC), a six subunit DNA binding complex. Then the helicase mini-chromosome maintenance protein (MCM) loads onto the origin, to form the so called the prereplicative complex (preRC). Shortly after the preRC assembly, Cdc6 is phosphorylated by CDKs and targeted for degradation, when MCM can start to unwind double stranded DNA, and DNA synthesis takes place, in S-phase, by DNA polymerases and associated factors like the proliferating cell nuclear antigen (PCNA). The coordination of these events is believed to be dependent on the activation of CDK complexes (Figure 4.4) (23, 24).

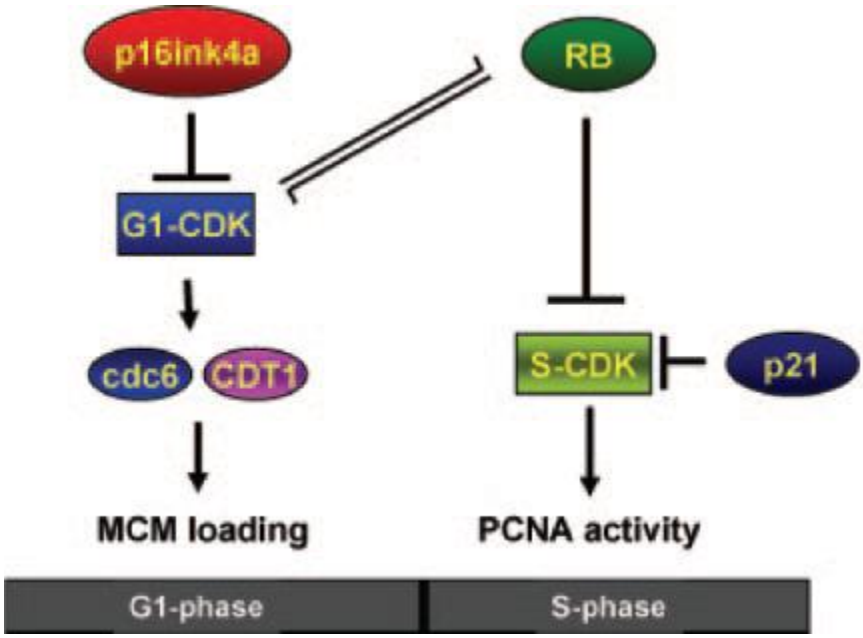

Figure 4.4 Regulation of DNA replication by Rb. Rb inhibits G1-cyclin /CDK complexes and S-cyclin/CDK complexes, preventing MCM loading to chromatin and PCNA activity (23).

4.2.2. Regulation of the S→G2/M transition

The transition between S to G2/M is also regulated by cyclin/CDK complexes. In this case, by cyclin A/CDK2, which continue phosphorylating Rb. Rb is continuously phosphorylated throughout the cell cycle, except when cells enter G0 phase. At S-phase entry cyclin A is transcritpionally induced by E2F. Many of cyclin A/CDK2 substrates are part of the DNA replication machinery, for example Cdc6 or MCM, which are inactivated

by phosphorylation, preventing rereplication of DNA. PCNA binding to chromatine is dependent on cyclin A/CDK2 activity, and cyclin A is required for elongation by DNA polymerase δ (21, 23, 25). Newly synthesized DNA is assembled by the action of histones, whose mRNA synthesis is regulated at the end of the S-phase by cyclin A/CDK1 complex (26).

In G2-phase the cell prepares itself for mitosis. There is cell growth and protein synthesis. Entrance into mitosis is triggered by cyclin B1/CDK1 complex. Cyclin B1/CDK1 complex, is expressed at late S-early G2 phase, and is maintained inactive by phosphorylation on Tyr15 and Thr14 residues of CDK1 by Wee/Myt protein kinases. Dephosphorylation is carried out by Cdc25 phosphatases which are activated by cyclin A/CDK2 complexes. The anaphase-promoting complex/cyclosome (APC/C) degrades cyclin A in prometaphase, the first stage of mitosis. Cyclin B1/CDK1 complex translocates to the nucleus at the end of G2-phase where it binds to the mitotic apparatus, spindle poles and main spindle fibres. It is associated with condensed chromatin at the beginning of the metaphase and degraded once cells enter the anaphase (27-29).

4.2.3. Inhibition of the cell cycle progress by CDK inhibitors

CDK inhibitors are proteins which bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity.

The protein p21, which is transcriptionally induced by the tumor suppressor protein p53, is one of them (17). It interacts and inhibits the cyclin-CDK family of kinases, forming complexes mainly with cyclin D1/CDK4 and cyclin E/CDK2 affecting G1→S progression, with cyclin A/CDK2 affecting S-phase progression, and it also affects G2/M-phase progression (30, 31). In addition, p21 binds to the proliferating cell nuclear antigen (PCNA), which is essential for DNA replication, and inactivates it (32, 33). (Figures 4.3 and 4.4).

p27 is another CDK inhibitor which binds and prevents the activation of cyclin D/CDK4 and cyclin E/CDK2 complexes, inhibiting the G1→S transition (Figure 4.3) (34).

Both p21 and p27 are also necessary cell cycle progression, in early G1, since the promote cyclin D-CDK assembly, and activate these complexes by directing them to the nucleus or increasing the stability of the D cyclins (35).

In addition, p16ink4a function is also restricted to G1 and involves the downregulation of Cdc6 and Cdt1. It inhibits cyclin D/CDK4 and cyclin D/CDK6 complexes (Figures 4.3 and 4.4) (23).

4.2.4. Cell cycle checkpoints

In response to replication stress or DNA damage, cells induce checkpoint pathways to inhibit DNA replication and cell cycle progression. Many control points exist within the cell cycle that play a major role in maintaining the integrity of the genome, by ensuring correct cell division. Checkpoints are signal transduction systems, and must receive a signal, then amplify and transmit that signal to other components that regulate the cell cycle (36).

4.2.4.1. The G1 (Restiction) checkpoint

The G1 (Restriction) checkpoint is governed by the ATM-Chk2-p53-p21 pathway. After damage, transcriptional activation of p21 by p53 results in inhibition of cyclin E/CDK2 activity, leading to a G1 delay. As the cell goes through the G1 checkpoint, the expression of ATR, Chk1 and cdc25A increases promoting the activation of CDK2. Then Chk1 phosphorylates cdc25A at several NH_2-terminal serine residues, resulting degradation of the phosphatase, and inactivation of cyclin E/CDK2 as the kinase remains phosphorylated and thus inhibited. After genotoxic stress, Chk1/Chk2 are activated and cdc25A is markedly destabilized, resulting in a p53-independent mechanism of G1-S delay following DNA damage (36, 37).

4.2.4.2. The intra-S-phase checkpoint

The intra-S-phase checkpoint affects the origins of replication in human cells. Replication origins which fire later are prevented from firing by activation of this checkpoint. The

intra-S-phase checkpoint is activated by phosphorylation of cdc25A on several NH_2 – terminal residues by Chk1/Chk2, resulting in enhanced proteolysis of the phosphatase, and CDK2 inactivation (36, 38, 39).

4.2.4.3. The G2/M transition checkpoint

The G2-M checkpoint prevents entrance into mitosis in the presence of DNA lesions introduced during G2 or acquired in S phase and carried through G2. Cyclin B/CDK1 represents the key downstream target of the G2/M checkpoint. Initiation of the G2/M checkpoint is mediated by the ATM/ATRChk1/Chk2-cdc25A/cdc25C pathway, and its maintenance requires p53 and its downstream effectors, such as p21 (36).

4.3. Transcription factors regulating proliferation

Transcription factors can be interesting targets in cancer therapy. The fundamental molecular requirements for human cancer might be the same in all tissues and these include loss of growth inhibition by ligands such as transforming growth factor-β (TGF-β**)**, dispensing with the need for growth stimulation (for example, by epidermal growth factor (EGF)), limitless replication potential (including telomere maintenance) and avoidance of apoptosis. All of these properties are hypothesized to depend on deregulated transcription. Signalling pathways begin with extracellular proteins which bind to specific cell-surface receptors. Activation of the receptor usually depends on serine or tyrosine kinases that are either intrinsic to the receptor or bound to the internal domain of the receptor. In the cytoplasm, activated transcription factors or proteins that activate transcription factors, relay the signal into the nucleus and change the transcription pattern of the cell. Transcription factors with overactivity act as oncogenes (40). E2F-1, c-Myc and p53 are examples of transcription factors which regulate proliferation and are involved in cancer.

4.3.1. E2F

The E2F family of transcription factors are central regulators of the cell cycle and are usually associated with Rb and thereby inactivated. When released from Rb binding due to its phosphoryation by cyclin/CDK complexes, E2F becomes active and induces the transcription of genes involved in DNA synthesis and cell cycle progression. Cyclin E and cyclin A, which activate CDK2 kinase induce phosphorylation of Rb, and release of E2F are downstream targets of this transcription factor (21, 41). This induces a positive loop, which ensures a linear and faster progression of the cell cycle at the G1→S transition (Figure 4.3). Moreover, E2F-1 autoregulates itself transcriptionally (42) and it induces transcription of histone 2A which plays an important role in DNA synthesis (43).

Deregulation of E2F occurs in most of the cancers. However, it is found to be rarely mutated. Forced expression of E2F-1, E2F-2 or E2F-3, in quiescent cells is sufficient to induce entry into DNA synthesis, and each of them can function as oncogenes in transforming assays (44, 45).

On the other hand, E2F-1 can act as a tumor suppressor by inducing apoptosis, through inhibiting the antiapoptotic pathway or by upregulating the expression of members of the caspase family (44). It has also been shown that it represses β-catenin/TCF-dependent transcription, which contributes to its pro-apoptotic role. This suppression of β-catenin activity reduces the expression of β-catenin downstream targets such as c-Myc (46).

4.3.2. c-Myc

c-Myc is another transcription factor which is related to cancer. It acts as an oncogene as its expression plays a role regulation of cell-cycle progression and survival. c-Myc is highly overexpressed in 5-10 % of colorectal cancers, and moderately overexpressed in 30 % of them (47, 48).

c-Myc is expressed at low levels in growing cells and is downregulated in quiescent and differentiating cells. It is one of the so called immediate early genes, participating in the cascade of events which follow growth factor stimulation after quiescence, and triggering of the G0→G1 transition (47).

4.3.2.1. Regulation of c-Myc

c-Myc gene has been identified as a downstream target of the canonical *wnt* signaling pathway (48-50). APC and β-catenin, which are often deregulated or mutated in colorectal cancer, are central targets of this pathway, and c-Myc plays a central role in the malignant phenotype due to APC loss (50).

The canonical *wnt* pathway is triggered by the binding of secreted *wnt* glycoproteins to the frizzled (Fz) seven-span transmembrane receptors together with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) single-span transmembrane co-receptors. In the absence of *wnt* signaling β-catenin is degraded after phosphorylation by the multiprotein complex containing glycogen synthase kinase 3β (GSK3β), the scaffold protein Axin and APC protein. In the presence of w*nt*, Dishevelled (Dsh) is activated and it inhibits β-catenin phosphorylation by displacing GSK3β from Axin, which is no longer able to perform its central role of scaffold protein, leading to stabilization of β-catenin. Stabilized β-catenin accumulates in the cytoplasm and is translocated into the nucleus, where it interacts with transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family which leads to the transcription of *wnt* target genes (Figure 4.5.) (51, 52).

In addition, c-Myc expression can be regulated at protein level by phosphorylation in Thr58 and Ser62 residues. Initially ERK protein kinase phosphorylates c-Myc in Ser62, which stabilizes the protein, and subsequently GSK-3β phosphorylates it on Thr58, which promotes degradation via the ubiquitin/proteasome pathway (53).

4.3.2.2. c-Myc downstream targets

c-Myc transcriptionally regulates many proteins involved in cell cycle progression and proliferation. These are E2F-1 (54, 55), D, E and A cyclins (56), CDK4 (57) and cdc25A (58). c-Myc represses transcription of and CDK inhibitors p21 (59) and p27 (60). Apoptosis can also be induced by c-Myc through several mechanisms, triggered by its target genes such as p53 or ARF (56, 61, 62).

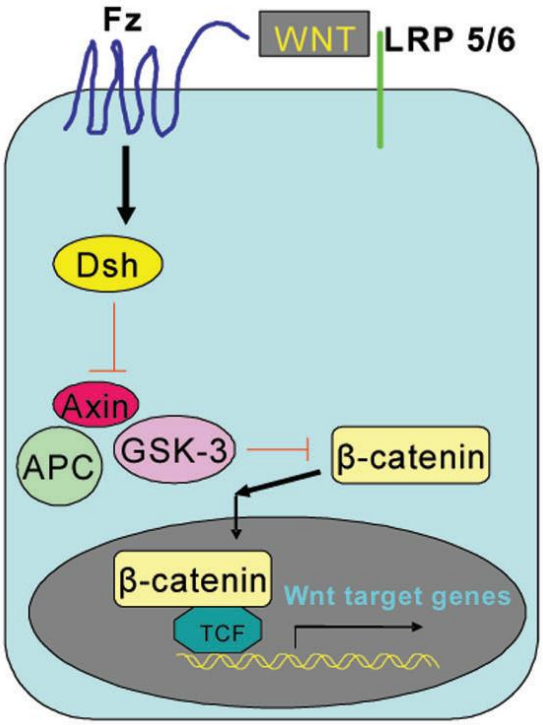

Figure 4.5. The canonical *wnt* pathway. Wnt signaling activates Dishevelled (Dsh), which inhibits the multiprotein complex containing Axin, APC and GSK-3β in charge of β-catenin degradation. This induces its accumulation, translocation into de nucleus, and together with TCF/LEF, transcription of the target genes (52).

4.3.3. p53

p53 is a transcriptional regulator of many genes that code for proteins which play a role in cell cycle regulation and apoptosis, such as p21, PUMA, BAX and MDM2 (63). Mutations in p53 are associated with tumorigenesis (48). c-Myc transcription factor contributes to the regulation of p53 expression (56).

4.4. Apoptosis

The term apoptosis is synonymous with the term programmed cell death which describes the pathway which dying cells follow, involving characteristic morphological and biochemical features.

Cells undergo a series of morphological changes during apoptosis (Figure 4.6). They become rounded, lose the contact with other cells and detach from the surface. The chromatin condenses and the nucleus is fragmented. The volume of the cytoplasm is

reduced due to loss of water and the proteins condense, although the majority of the cellular organelles remain intact. Finally the cell breaks into small vesicles surrounded by the plasma membrane called apoptotic bodies that will be phagocytosed by macrophages and nearby cells (64).

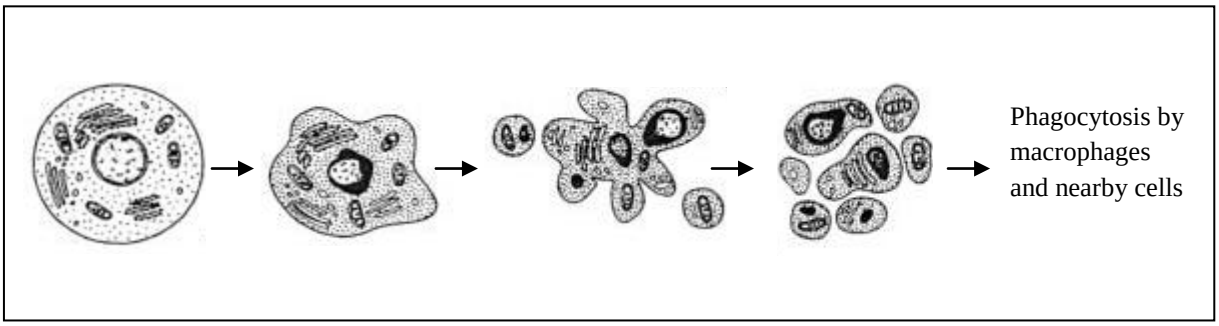

Figure 4.6. Illustration of Apoptosis. It shows how apoptosis takes place. A cell loses cytoplasmic volume, the proteins condense and it breaks into small vessels (apoptotic bodies) which finally are phagocytosed by macrophages and nearby cells (64).

Mechanistically, apoptosis can be triggered by two pathways, extrinsic (receptor pathway) or intrinsic (mitochondrial pathway). The extrinsic pathway is activated by the binding of ligands such as TNF-related apoptosis-inducing ligand (TRAIL) or CD95 (APO/Fas) to its respective receptors. This results in recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8, which becomes activated and initiates cleavage of downstream caspases. The mitochondrial pathway takes place through the release of cytochrome c, apoptosis inducing factor (AIF) or Smac/DIABLO from the mitochondria into the cytosol which triggers caspase-3 activation. Bid is a member of the Bcl-2 family that activates cytochrome-c-releasing when cleaved by caspase 8, and connects receptor and mitochondrial pathways. Caspase activation can be inhibited at receptor level by FLIP, or at the mitochondria by Bcl-2 family proteins and by the inhibitors of apoptosis (IAPs) (Figure 4.7.) (65).

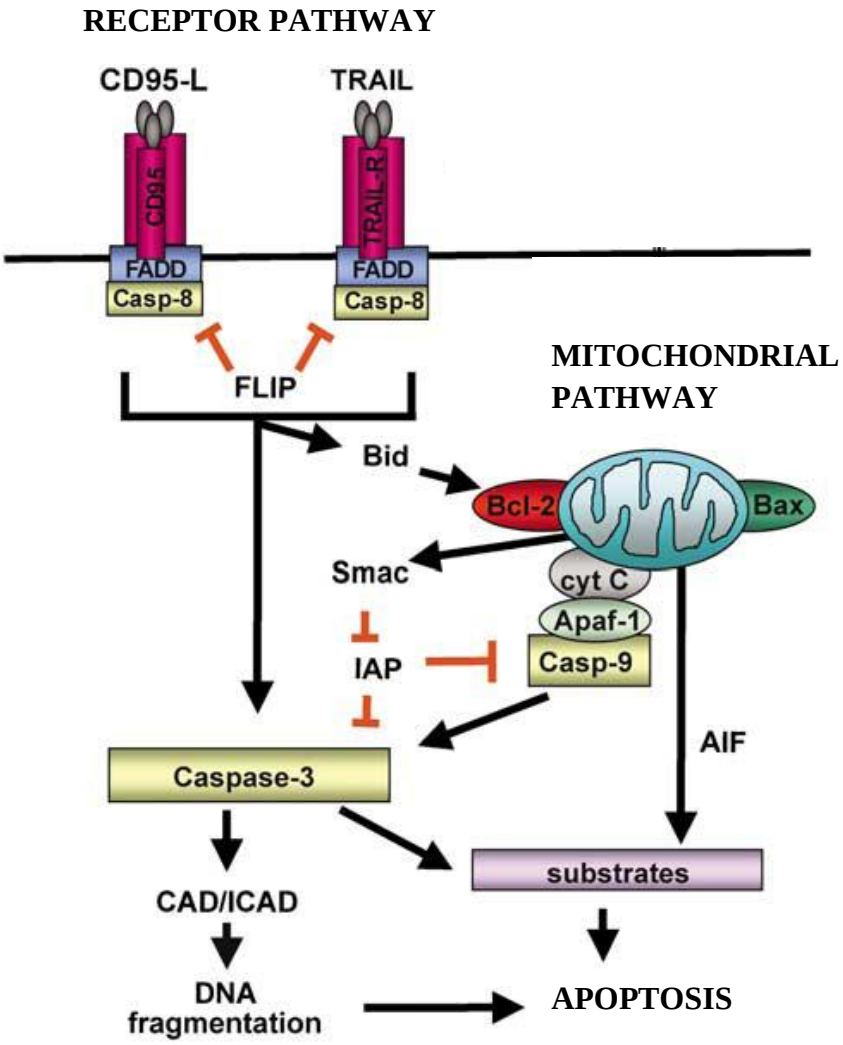

Figure 4.7. Apoptosis signaling pathways. Activation = black, inhibition = red. Adapted from Fulda and Debatin (65).

In certain tumors, including colorectal cancers, apoptosis can be decreased. Apoptotic resistance can be caused by mutation or suppression of p53, activation of the *wnt* pathway due to APC mutation, and Bcl-2 protein overexpression (66).

4.5. Cellular senescence

There are two types of senescence: replicative senescence and premature senescence. Senescent cells have a characteristic morphology in culture. They are enlarged and flattened, contain vacuoles and have an increased β-galactosidase activity (67).

4.5.1. Replicative senescence

Replicative senescence takes place after about fifty divisions in normal cells which are not able to divide any more. After each cell division, telomers are shortened and the genetic integrity is gradually lost, until they reach a critical minimal length and their protective structure is disrupted. When the total number of divisions is complete, the cells undergo cell cycle arrest at G1 phase (68, 69). Replicative senescence induces repression of genes involved in G1-progression, like the *E2F-1* transcription factor (70). Moreover, Rb remains hypophosphorylated probably due to the overexpression of p21 and p16, and p53 is also playing a role in the induction of replicative senescence. p53 deregulation or deregulation of other genes such as c-Myc, allow the cells to avoid replicative senescence (68).

4.5.2. Premature senescence

Premature senescence happens prior to telomere shortening and can be caused by stress, such as oxidative stress, which causes inactivation of p53 or ablation of Rb proteins. Oncogenes such as Ras, with the involvement of the p53 and $p16^{INK4A}$-Rb pathways (69), or suppression of c-Myc (69, 71), can also induce premature senescence. Finally, it can be induced by loss of tumor suppressor genes such as PTEN or NF1, accompanied by p53 induction or decrease in ERK and AKT activities, or VHL, whose loss can induce senescence in an Rb- and p400- dependent manner (69).

4.6. Prevention of colitis-associated colon cancer

4.6.1. Diet and lifestyle

Many factors that increase the risk of developing colorectal cancer can be controlled. In diet, high intake of red and processed meat, highly refined grains and starches, and sugars could be substituted by poultry, fish, and vegetables as the primary source of protein. Unsaturated fats taken as the primary source of fat, and unrefined grains, legumes and fruits the primary source of carbohydrates, would also help to prevent cancer. Moreover, calcium can reduce risk of colorectal cancer by reducing proliferation, stimulating differentiation, and inducing apoptosis in the colonic mucosa. Vitamin D intake is also a source of prevention, as vitamin D deficiency has been associated with inflammatory

bowel disease. Minimizing alcohol consumption and not smoking also decreases risk of colorectal cancer. While obesity has been linked to cancer, exercise is associated with prevention of cancer development (72).

4.6.2. Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) is a physiological component of normal human bile and has been used with therapeutic benefit in various cholestatic diseases of the liver including primary biliary cirrhosis and intrahepatic cholestasis in pregnancy (73).

Bile acids are the most abundant end products of cholesterol metabolism. The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized in the liver and secreted into the bile. More than 95% of the primary bile acids are reabsorbed in the ileum, but a small fraction passes into the colon, where they are metabolized by anaerobic bacteria to form and secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA). Certain secondary bile acids act as colon cancer promoters. For example, DCA, which in colorectal cancer is found in a higher concentration in fecal water, it is moderately mutagenic, associated with dysplastic changes and anti-apoptotic (74). On the other hand, UDCA, which is the most hydrophilic acid, as in its molecule contains two hydroxyl groups (Figure 4.7), is associated with prevention of colorectal cancer. Interestingly, this bile acid is not associated with any severe toxicity (73).

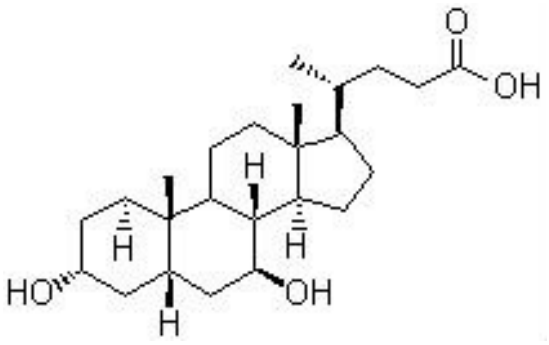

Figure 4.7. Molecuar structure of UDCA. UDCA is also called ursodiol, as it contains two hydroxyl groups.

As a therapeutic agent it has the benefit that at high doses it is well tolerated and it improves the prognosis of patients which suffer from primary sclerosing cholangitis (PSC), a chronic cholestatic liver disease which is strongly associated with ulcerative colitis (UC) (73). It has shown effectiveness as a cancer chemopreventive agent in preclinical studies. Patients with both PSC and UC have a higher risk of developing colorectal cancer and a shorter overall survival than the ones which suffer from UC alone (75-77). One study shows that UDCA treatment reduces the risk of dysplasia or cancer in these patients (78). Another study shows that UDCA does not affect the risk of dysplasia and cancer but it reduces the mortality in UC-PSC patients (79). Moreover, oral administration of UDCA prevented colitis-associated carcinogenesis in a DSS- induced mouse model of DSS-colitis (80).

The chemopreventive effect of UDCA is not well understood. It may be due to replacement of DCA or to its own molecular actions. UDCA suppressed induction of colorectal cancer by DCA, through disruption of proliferation, differentiation and apoptosis by reducing the fecal level of DCA (74). UDCA suppresses many of the signaling pathways activated by DCA, such as the MAPK pathway (81) or the activation of the transcription factor AP-1 (81, 82) or NF-κB (82), which lead to proliferation. In addition, it has been described that UDCA treatment reduced the number of tumors in AOM mouse model by inhibition of Ras mutations, wild type Ras activation and COX-2 expression (83). Moreover, inhibition of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-2 (MIP-2) expression (84) and IL-1 activity (82), and inhibition of iNOS expression which has also been observed after UDCA treatment (82, 84, 85), could explain the reduced risk of developing colitis-associated colorectal cancer in treated patients.

4.6.3. Other preventive agents

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) as well as specific COX-2 inhibitors, have been found to reduce the risk of colorectal cancer (72). Aspirin and sulindac are inhibitors of COX-2 which also inhibit COX-1. Their use is limited, due to their gastrointestinal toxicity. The NSAID celecoxib is a COX-2-specific inhibitor, and it is more effective in prevention of colorectal carcinogenesis (86). 5-Aminosalicylic acid (5-

ASA) is a NSAID that prevents colitis-associated colorectal cancer in a mouse colitis model (87). It also reduces growth of colon cancer cells through mitotic arrest and it also induces apoptosis (88, 89). Sulfasalazine is another common anti-inflammatory agent for IBD, which is associated with decrease in risk of developing colon cancer (90).

4.6.4. Inhibition of the molecular mechanisms of colitis-associated colon carcinogenesis

COX-2 is a key enzyme in the synthesis of prostaglandins. It is upregulated by inflammatory or oncogenic stimuli via interleukin-6 (IL-6) or other cytokines that induce nuclear factor–κB (NF-κB). COX-2 converts arachidonic acid to prostaglandins (PGH2, PGD_2, PGF2α, PGI_2, TXA_2 and PGE_2). PGE2 stimulates EP2, which up-regulates transcriptional activity of β-catenin and activates phosphatidylinositol-3-kinase (PI3K) and the protein kinase AKT. PGE_2 also stimulates EP4, which triggers phosphorylation of EGFR, activating PI3K, AKT, and the MAPK cascade. PGE_2 stimulation of PI3K signaling also activates the transcriptional activity of the peroxisome-proliferator–activated receptor–γ (PPAR-γ). These PGE_2-induced signaling pathways induce expression of a number of genes, including the angiogenic factor vascular endothelial growth factor (VEGF), the anti-apoptotic factor Bcl-2, and the pro-proliferative protein cyclin D1. In addition, many of the downstream targets of PGE_2 act in positive feedback loops to induce greater expression of COX-2. Interestingly, aspirin and other NSAIDs might also directly stimulate PPARs and block phosphorylation of AKT. 15-prostaglandin dehydrogenase (15-PGDH) is an inhibitor of PGE_2 whereas up-regulation of prostaglandin synthesis increases urinary PGE-M, the major metabolite of PGE_2 (72) (Figure 4.8), which can be used as a biomarker for the detection of advanced colorectal neoplasia and to measure effectiveness of chemopreventive treatment (91).

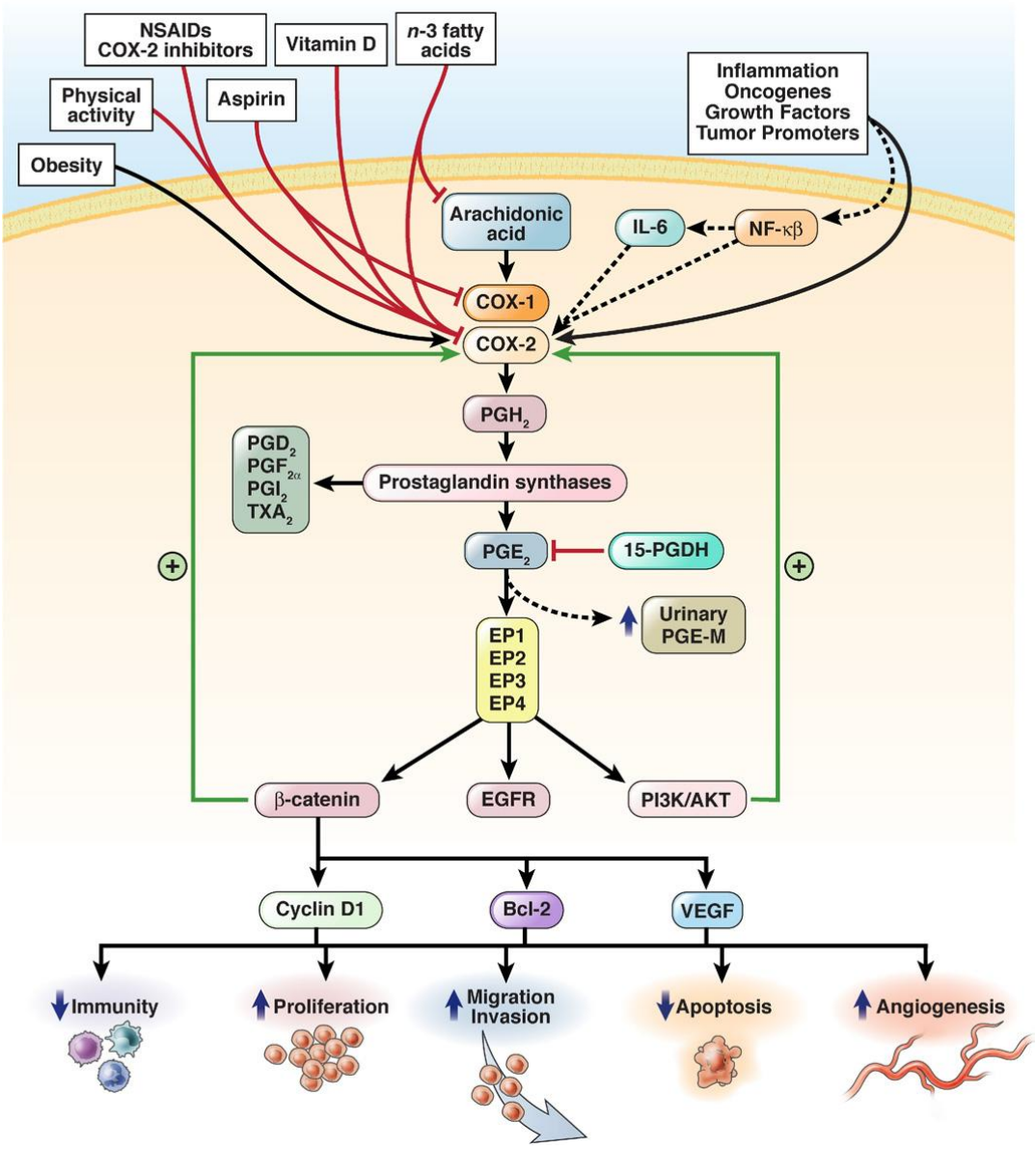

Figure 4.8. Proposed inflammatory mechanisms relating diet, lifestyle, and medication use to colorectal cancer. Red lines indicate anticancer effects, black lines indicate pro-cancerous effects. Green arrows show positive feed-back (72).

5. OBJECTIVES

UDCA is a chemopreventive agent whose mechanism of action is at the present unknown. The chemopreventive capacity could be due to its antiproliferative property.

The aim of this work was to investigate the antiproliferatory mechanisms of UDCA in colon cancer cells. The following questions were posed:

1. What is the effect of UDCA treatment on proliferation of colon cancer cells?
2. Does UDCA induce apoptosis?
3. Does UDCA induce senescence?
4. What is the effect of UDCA on the cell cycle?
5. Which molecules are targeted by UDCA?
6. How is the expression of these molecules regulated by UDCA?
7. What is the sequence of events after UDCA treatment?
8. Does the *wnt* pathway status play a role in UDCA-mediated inhibition of proliferation?

After answering these questions, the possible antiproliferative mechanisms of UDCA will be proposed and discussed.

6. MATERIALS AND METHODS

6.1. Materials

6.1.1. Common reagents and buffers

- 100 mM UDCA: 5 ml of solution contain 196 mg UDCA (Sigma-Aldrich GmbH, Taufkirchen, Germany) diluted in 100 mM NaOH.
- LB medium: LB Broth Base (Invitrogen GmbH, Darmstadt, Germany) was diluted as described by the manufacturer and autoclaved at 121 °C for 15 minutes. It was stored at room temperature.
- LB agar: LB agar (Invitrogen) was diluted as described by the manufacturer and autoclaved at 121 °C for 15 minutes. It was stored at room temperature.
- TAE 1 x buffer: 50 x stock contains 40 mM Tris base, 20 mM acetic acid, 1 mM EDTA in bidestilled water; diluted 1:50 to get TAE 1 x.
- TBS 1 x buffer: 10 x TBS buffer stock contains 9 g/l Tris-base, 68,5 g/l Tris-HCl and 88 g/l NaCl in H_2O, pH 7.4-7.6; diluted 1:10 to get TBS 1 x.
- 1 x TBS-T: 0,2 % Tween in 1 x TBS buffer.
- PBS: 8 g/l NaCl, 0,2 g/l KCl, 1,42 g/l $Na_2HPO_4 \cdot 2H_2O$, 0,2 g/l KH_2PO_4, pH 7.2–7.5.
- BSA (Thermo Scientific, Rockford, IL, USA): stock 2 mg/ml.
- Trypsin 20 x (Sigma).
- Trypsin buffer: 20 g/L EDTA in PBS, sterilized by atoclaving.
- Sato buffer: 10 % glycerol, 50 mM NaF, 137 mM NaCl, 12 mM β-glicerophosphate, 20 mM Tris-HCl pH 7.5, 0.2 % Nonidet P-40 (NP-40), 1.5 mM $MgCl_2$, 1 mM Na_3VO_4 (Sigma), 1 mM EDTA, 10 µg/ml Aprotinin, 1 mM PMSF (92).

- Sample buffer 5 x: 20 ml contain 1 g SDS, 5 g sucrose, 120 mg Tris Base, 5 mM EDTA, 0.025% (w/v) Bromophenolblue and 600 mg DTT in H_2O.
- Sample buffer 1 x: 1:5 dilution of sample buffer 5 x.
- Ampicillin: stock 100 mg/ml.

6.1.2. Antibodies

6.1.2.1. Primary antibodies

Antibody against	Company	Order number	Concentration used
c-Myc	Santa Cruz	sc-40	1 µg/ml in TBS-T
Phospho-c-Myc (Thr 58)	Cell Signaling	9401	0,132 µg/ml in TBS-T
CDK2	BD Transduction	610145	0,125 µg/ml in TBS-T
CDK4	Santa Cruz	sc-601	0,01 µg/ml in TBS-T
CDK6	Santa Cruz	sc-177	0,067 µg/ml in TBS-T
Cyclin A	Santa Cruz	sc-751	0,04 µg/ml in TBS-T
Cyclin D1	Santa Cruz	sc-718	0,1 µg/ml in TBS-T
Cyclin E	BD Pharmingen	51-1459GR	0,5 µg/ml in TBS-T 5 % BSA
ERK protein	Cell Signaling	9102	0,005 µg/ml in TBS-T
Phospho-ERK	Cell Signaling	9101S	0,032 µg/ml in TBS-T
p21	Santa Cruz	sc-397	0,04 µg/ml in TBS-T
p27	BD Transduction	610241	0,1 µg/ml in TBS-T
p53	Dako Cytomation	M7001	0,033 µg/ml in TBS-T
PARP	BD Pharmingen	556362	ascites stock solution diluted 1:4000 in TBS-T
PCNA	BD Pharmingen	555566	0,0625 µg/ml in TBS-T
Rb	BD Pharmingen	554136	0,167 µg/µl in TBS-T
γ-tubulin	Sigma-Aldrich	T-6557	ascites stock solution 1:100000 diluted in TBS-T
β-actin	Sigma-Aldrich	A-5441	ascites stock solution 1:200000 diluted in TBS-T
HA-tag	Cell Signalling	2367	0,032 µg/ml in PBS 1 % BSA, 0,3% Triton-X 100

Table 6.1. Primary antibodies

6.1.2.2. Secondary antibodies

Antibody	Conjugated with	Company	Order number	Concentration used
Goat anti-mouse	Peroxidase	Dianova	115-035-062	0,26 µg/ml in TBS-T
Goat anti-rabbit	Peroxidase	Dianova	111-035-003	0,53 µg/ml in TBS-T
Donkey anti-mouse	Cy 3	Dianova	711-165-152	1,5 µg/ml in TBS-T

Table 6.2. Secondary antibodies

6.1.3. Plasmids

- pCMV-Neo was provided by Dr. Lüder Wiebusch (Department of General Pediatrics, Charité Campus Mitte, Berlin, Germany)
- pCMV-c-Myc was provided by Dr. Daitoku Sakamuro (Department of Pathology, Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA) and is a human c-Myc-expression vector.
- HH67 was obtained from Prof. Dr. Eick (Institute for Clinical Molecular Biology and Tumour Genetics, Helmholtz-Zentrum-Muenchen, Munich, Germany) and it is the human c-Myc expression vector. It has an HA tag. The vector is CMV-T7 and it contains an insert of 64 kDa (57).
- pSuper empty vector was provided by Dr. Kenji Fukasawa (H. Lee Moffitt Cancer Center & Research Institute, FL, USA.)
- pSuper-c-Myc was provided by Dr. Juliane Lüscher-Firzlaff (Department of Biochemistry and Molecular Biology, Institut für Biochemie, Klinikum der RWTH, Aachen, Germany). It expresses siRNA against human c-Myc and it was constructed by cloning the sequence GCTCGTCTCAGAGAAGCTG into the Bgl II/Hind III sites of the pSuperDuper expression vector.
- PGL3-Basic luciferase reporter was provided by Dr. Paola Castagnino (Department of Pharmacology, University of Pennsylvania, Philadelphia, USA).

- The c-Myc luciferase reporter CG362 was provided by Prof. Dr. Eick. It contains the Hind III-Pvu II fragment of the human c-Myc promoter cloned in front of luciferase gene (93).

- (E2F)x4-E1bTATA-Luc reporter plasmid was provided by Dr. Walsh (Department of Molecular Cardiology/CVI, Boston University School of Medicine, Boston, Massachusetts, USA) and it was constructed by subcloning the PvuII/SacI fragment from the (E2F)x4-E1bTATA-CAT reporter into the SmaI/SacI site of PGL2-Basic plasmid as described (94).

- pCMV-E2F-1 was provided by Dr. Walsh and it is the mammalian expression plasmid that encodes amino acids 1 to 437 of the E2F-1 protein. It was obtained by cloning E2F-1 cDNA into the pCMVneoBam by use of BamHI linkers (95).

- pRL-TK *Renilla* luciferase reporter (Promega GmbH, Mannheim, Germany).

- pSV-β-Galactosidase reporter (Promega).

6.1.4. Restriction enzymes and buffers

All the restriction enzymes and buffers were obtained from Fermentas (St. Leon-Rot, Germany).

6.1.5. Bacterial strains

- E-coli Top 10´F competent cells (Invitrogen)

6.1.6. Cell culture

- HCT8 colon carcinoma cell line, obtained from Dr. Stefan J. Vermeulen (96) was cultured in RPMI 1640 medium, high glucose with stable glutamine (PAA Laboratories GmbH Graz, Austria) with 10 % FCS (PAA Laboratories).
- LS513 colon carcinoma cell line, obtained from Dr. Bernard Sordat (97), was cultured in DMEM, high glucose with stable glutamine (PAA Laboratories) and 10 % FCS.
- Co115 colon carcinoma cell line, obtained from Dr. Bernard Sordat (97), was cultured in DMEM, high glucose with stable glutamine and 10 % FCS.
- HCT116 colon carcinoma cell line, obtained from Dr. C. Richard Boland (Gastrointestinal Research Laboratory, Baylor University Medical Center, Dallas, Texas, USA), was cultured in DMEM, high glucose with stable glutamine and 10 % FCS.
- *Wnt-1* expressing cell line Rat2-wnt1 and the control cell line Rat2-mv7, deficient for *wnt*, were obtained from Dr. Jürgen Behrens (Nikolaus-Fiebriger-Center, University of Erlangen-Nürnberg, Erlangen, Germany), and cultured in DMEM, high glucose with stable glutamine and 10 % FCS.

6.1.7. Cell culture dishes and flasks

- Petri dishes: small surface (9,6 cm^2), middle surface (22,0 cm^2), big surface (56,7 cm^2) (BD Biosciences, Heidelberg, Germany)
- Cell culture flasks: 25 cm^2 and 75 cm^2 of surface (BD Biosciences)
- Nunclon™ 96-well plates, flat bottomed **(**Thermo Electron LED GmbH, Langenselbold, Germany**)**
- 12-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland)

6.1.8. Laboratory equipment

Equipment	Company	Model
Incubators	Heraeus (Hanau, Germany)	Heraeus 6000
	Heraeus	Hera Cell 150
	Memert (Schwabach, Germany)	
Shakers	LabSource (Southend-On-Sea, Essex, USA)	WB GFL 3005
Spectrophotometers	Pharmacia Biotech (Berlin, Germany)	Ultrospec 2000
	PEQLAB (Erlangen, Germany)	NanoDrop ND-1000
Microscopes	Olympus (Hamburg, Germany)	CK2
	Olympus	BX60
Camera	Soft Imaging System (Münster, Germany)	Colour View II
ELISA reader	Dynatech (Burlington, MA, USA)	MR 5000
Water baths	Julabo (Seelbach, Germany)	SW-20 C
	Daglef Patz (Wankendorf , Germany)	
	New Brunswick Scientific (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf)	Gyratory water bath shaker G76
Hoods	Faster S.r.I (Cornaredo, MI, Italy)	Bio 48
	Kojair Tech Oy (Nideggen, Germany)	Bio Wizard X-tra
Autoclave	Tuttnauer Europe B.V. (Breda, The Netherlands)	3870 ELV
Centrifuges	Eppendorf (Wesseling-Berzdorf, Germany)	Mini Spin
	Sigma Laborzentrifugen GmbH (Osterode am Harz, Germany)	2K15
	Beckman Coulter GmbH (Krefeld, Germany)	J2-21 (rotor JA-20)
	Heraeus	Sepatech Megafuge 1.0
	Shandon	Cytospin 3
Balances	Sartorius AG (Göttingen, Germany)	1419 (600,00/60,000 g)
	KERN (Balingen-Frommern, Germany)	470 (0,5-2000 g)
Power supplies	Bio Rad (München, Germany)	250/2.5
	Pharmacia Biotech	ECPS 3000/150
	Bio Rad	200/2.0
Luminometer	Berthold (Bad Wildbad, Germany)	LUMAT LB 9501
Electroporator	Bio Rad	Gene Pulser X cell electroporation system
Magnetic stirrer	Ika-Werke GmbH (Staufen, Germany)	Ikamag RCT
pH-meter	Knick Elektronische Messgeräte GmbH (Berlin, Germany)	
Vortex	Heidolph Elektro GmbH (Kelheim, Germany)	

Table 6.3. Laboratory Equipment

6.1.9. Kits

- Quicklyse Miniprep kit (QIAGEN, Hilden, Germany)
- QIAGEN plasmid Midi kit (QIAGEN)
- Click –IT EDU Alexa Fluor 488 Flow Cytometry Assay kit (Invitrogen)
- Galacto-light™ Chemiluminiscent Reporter Assay for β-Galactosidase (TROPIX Inc, Bedford, MA, U.S.A.)

6.2. Methods

6.2.1. Molecular Biology

6.2.1.1. Transformation of a plasmid into bacteria

Transformation of the plasmid into bacteria was performed by heat-shock technique. 2 µg of plasmid were mixed with 20 µl of competent cells, incubated 20 minutes on ice and then 45 seconds at 42 °C in a pre-warmed water bath. Then they were incubated 2 minutes on ice, 125 µl of LB medium were added and it was shaked at 500 rpm 1 h at 37 °C. 50 µl of the bacterial culture were spread on a plate containing LB agar and 100 µg/ml of ampicillin and incubated at 37 °C overnight. Several colonies were tested by restriction of the isolated DNA (point 6.2.1.3.). DNA isolation was perfomed by mini-prep (point 6.2.1.2.)

6.2.1.2. Plasmid Isolation

Bacteria containing the plasmid were inoculated in LB medium containing the corresponding antibiotic (ampicillin: 100 µg/ml). For mini-prep, the bacteria were inoculated in 5 ml of medium, and left overnight at 37 °C while shaking. For midi-prep, first 1 ml was inoculated and shaken at 37 °C for 4 h, it was then transferred to a sterile flask containing 200 µl LB medium + antibiotic and incubated in a shaker at 37 °C. The protocol followed to isolate the plasmid from the overnight bacterial culture was the one described by the manufacturer of the kit used. The concentration of DNA was determined by NanoDrop.

6.2.1.3. Restriction of DNA

0,5-1 µg of DNA in a volume of 2 µl were mixed with 1 µl of restriction buffer, 1 µl of each enzyme required to cut the plasmid and the corresponding volume of H_2O to obtain a total volume of 10 µl. The mixture was incubated for 2 h at 37°C in a pre-warmed water bath. The reaction mixture was stored at -20 °C if not analyzed immediately by agarose gel electrophoresis (point 6.2.1.4).

6.2.1.4. Agarose gel electrophoresis

DNA fragments were separated in an agarose gel containing 0.8-1.5 % (w/v) agarose and 10 µg/µl ethidium bromide (Sigma). To prepare the gel the agarose was dissolved in 1 x TAE buffer by boiling it in the microwave oven. When the homogeneous liquid was cooled down to a temperature of 60 °C the ethidium bromide was added, mixed and poured into the form with the corresponding well-comb. It was left to solidify at 4 °C. Samples were prepared by adding 6 x Loading Dye (Fermentas). GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as reference to determine the length of the fragment. The gel was run at 100 V for 30 min. in an electrophoresis apparatus (Bio-RAD) containing 1 x TAE buffer. Ethidium bromide-stained DNA bands were visualized on a UV-transilluminator (Biometra GmbH, Göttingen, Germany).

6.2.1.5. Preparation of glycerol stocks

To prepare glycerol stocks of the bacteria transformed with the plasmid, 1 ml of overnight bacterial culture containing the plasmid was diluted 1:1 with 50 % glycerol in LB medium (sterilized by autoclaving) and incubated 30 min. at room temperature. The glycerol stock was stored at -70°C.

6.2.1.6. Preparation of cell lysates

6.2.1.6.1. Lysis in Sato buffer

Cells were harvested by trypsinization and transferred into a falcon tube. If the floating cells were included, all the supernatants were also transferred. They were centrifuged 5 minutes at 1500 x g, the pellet was resuspended in PBS and they were counted using a Neubauer hemocytometer (Brand GmbH, Wertheim, Germany). They were centrifuged again, the pelleted cells were resuspended in Sato buffer (80 µl Sato buffer for each million of cells) and incubated on ice for 15 minutes. Lysates were precleared by centrifugation at 15000 x g for 20 minutes at 4°C and transferred to a new Eppendorf cup. They were stored at -70°C. The concentration of protein was determined by Bradford assay (point 6.2.1.7).

6.2.1.6.2. Lysis in sample buffer

Medium was discarded and cells were washed once with PBS. Sample buffer 1 x containing protease inhibitors (80 µl per million of cells) was added and the cells were scraped and transferred to a cup. 1 µl of benzonase was also added to digest DNA, and decrease viscosity. Samples were boiled 5 minutes and frozen at -20 °C. Number of cells and protein concentration was determined an additional dish lysed in Sato buffer as described in point 6.2.1.6.1, taking into consideration the volume difference from one sample to another.

6.2.1.7. Determination of the protein concentration

Protein concentration of the cell lysates was determined by Bradford assay, which is based on an increase of absorbance of the dye Coomassie Brilliant Blue G-250 under acid conditions when a redder form of the dye is converted into a bluer form while binding to protein. 1.5 ml Eppendorf tubes were prepared containing 795 µl H_2O, 5 µl of sample (1 µl or 2.5 µl of lysate in Sato buffer) and 200 µl of dye. Samples for a standard curve were prepared with 0, 2, 4, 6, 8 or 10 µg of BSA and 5 µl of Sato buffer in H_2O (volume of 800 µl), and 200 µl of the dye. The cups were vortexed, incubated at room temperature for 30 min. The absorbance was measured at a wave length of 595 nm. The optical density was plotted versus the micrograms of BSA to obtain the standard curve from which the amount of protein in each sample was calculated by interpolation. The protein concentration of the

lysate (µg/µl) was obtained by dividing the amount of protein by the volume used (1 µl or 2,5 µl).

6.2.1.8. SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their mobility, a function of their molecular weight, was carried out in a miniprotean apparatus from Bio-Rad. First the separation gel containing 7.5 % (for proteins with molecular weight more than 70 kDa), 10 % (70-25 kDa) or 12.5 % (< 25 kDa) acrylamide/bisacrylamide (Table 6.4.), was allowed to polymerize in a glass chamber at room temperature for 30 min. The stacking gel (3% acrylamide, 0.124 M Tris pH 6.8, 0.1 % SDS, 0.1 % TEMED, 0.1 % APS) was then prepared, poured and a 10 or 15-well comb was inserted. It was also left to polymerize for 30 min. The gel was then mounted into the electrophoresis chamber filled with electrophoresis buffer (0,25 M Tris Base, 1,92 M Glycin in and 1 % SDS in H_2O) , 20 µg of protein lysate diluted in sample buffer and boiled for 10 min to denature them, together with the standard marker for molecular weight (Gene Ruler Plus Prestained Protein (Fermentas)) were loaded and run at 100 V until the dye of the sample buffer was at the bottom.

	7,5 %	**10%**	**12,5 %**
Acrylamide/Bisacrylamide (30%)	2,5 ml	3,3 ml	4,2 ml
5 x separation buffer (*****)	2 ml	2 ml	2 ml
SDS (1 %)	1 ml	1 ml	1 ml
H2O	4,4 ml	3,6 ml	2,7 ml
APS (10%)	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl

Table 6.4. Composition of the separation gel. Total volume of 10 ml is enough for 2 small gels. (*****200 ml 5 x separation buffer contain 45,5 g Tris Base and 80 ml 1 M HCl in H_2O)

6.2.1.9. Western Blot

The proteins separated by SDS-PAGE electrophoresis (point 6.2.1.8) were transferred to a PVDF membrane (Immobilion-P transfer membrane (Millipore, Bedford MA)) using a Bio-Rad blotting apparatus filled with blot buffer (25 mM Tris, 192 mM Glycin, 20 % Methanol, pH 8-8,5), continuously stirred at 4 °C for 2 h at 200 mA. The membranes were then blocked with 5% nonfat milk in TBS-T buffer for 1 hour on a shaker at RT, and shaked with the corresponding primary antibody overnight at 4 °C. On the next day the secondary antibody was incubated for 1 hour at room temperature and the blot was developed with Super Signal West Pico Peroxidase and Luminol Enhancer solution (Thermo Scientific) and evaluated in a Fujifilm Chemiluminescence Documentation System (Fujifilm Europe GmbH, Düsseldorf, Germany).

6.2.1.10. Luciferase reporter assay

0,1 million cells were seeded in a 12-well plate, on the next day the reporter plasmid and the control plasmids (β-Gal or Renilla) were transfected and treated 5 hours after transfection. 24 or 48 h later, luciferase activity was measured in a luminometer and it was normalized to the β-galactosidase activity, measured with the chemiluminiscent assay Galacto-Light Plus, as recommended, or to Renilla-luciferase activity. Renilla activity was measured by adding the Stop-Glo solution of the Dual Luciferase System (Promega) to the same tube after measuring luciferase activity.

6.2.2. Cell biology

6.2.2.1. Passaging and maintenance of a cell line

Cells were maintained in 75 cm^2 flasks. Twice a week they were passaged in the same flask and every 6-8 weeks the flask was changed. Cells were harvested by trypsinization, well resuspended in fresh medium with 10 % FCS and a fraction of them was left in the flask; HCT8 and HCT116 1 ml out of 14 ml, Co115 and LS513 1 ml out of 10 ml. Fresh medium with 10 % FCS was given to obtain a final volume of 15 ml. They were incubated at 37 °C 5 % CO_2. The cells were maintained for 4-5 months and then they were discarded.

6.2.2.2. Treatment of the cells with UDCA

Cells were treated with 200 or 400 µM UDCA. For this a stock solution of 100 mM UDCA was made and the correct volume was added to obtain the desired final concentration of UDCA. In the case of MTT test, to obtain 200 µM or 400 µM UDCA, the stock solution was previously diluted 2:100 or 4:100 in medium, respectively, and 10 µl of medium was added to the 100 µl medium in each well.

6.2.2.3. Determination of the generation time

The generation time of a cell line is the time it takes for one cell to divide. To calculate this, 20.000 cells were seeded in middle dishes and counted after 4 and 7 days. The fold increase in cell number in respect to the number of seeded cells (0 days) was represented versus the time in days, in a logarithmic scale. The exponential function obtained was used to calculate the generation time, which is the time corresponding to the 2-fold increase in cell number.

6.2.2.4. Determination of cell proliferation

6.2.2.4.1 MTT assay

This test is a colorimetric assay based on the ability of reductase enzymes, only active in living cells, to reduce 3-(-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is yellow, to formazan which is purple whose absorbance can be measured at a wavelength between 500 and 600 nm. Cells were seeded in triplicates in a volume of 100 µl in a 96-well plate (HCT8 and HCT116 500 cells/well; LS513 1000 cells/well; Co115 2000 cells/well) and after 1 day they were treated with 200 or 400 µM UDCA. After 3 days of treatment 25 µl of MTT solution (Sigma) 5 mg/ml in PBS) were added and the plate was incubated for 2h at 37°C. 100 µl of extraction buffer (20 % SDS in 50% dimethylformamide in bidestillated H_2O, pH 4.7 adjusted with a solution containing 40% acetic acid and 0.5 M HCl in H_2O) were then added, mixed by pipeting up and down and the plate was incubated for another 2 h at 37°C. The absorbance was measured at 550 nm

in an ELISA reader and the percentage of growing cells was calculated by relating the absorbance to the one of the nontreated.

6.2.2.4.2. Cell count

Cells were seeded, on the next day treated with 400 µM UDCA for 3 days, harvested and counted using the trypan blue exclusion assay (see point 6.2.2.6.1). The percentage of growing cells after treatment is the number of viable cells (white cells) in the treated fraction divided by the number of viable cells in the nontreated fraction multiplied by 100.

6.2.2.4.3. Determination of reversibility of UDCA-effects

20000 cells were seeded and treated with 400 µM UDCA in triplicates for 3 days. Then they were washed once with PBS and grown for 3 days more in regular medium. Cells were harvested and counted. The number of growing cells after treatment was compared to the number of cells growing for 6 days without any treatment. A lower number of recovered cells after treatment than the number of nontreated cells means that the effect of UDCA is partially reversible.

6.2.2.4.4. Determination of BrdU incorporation

Bromodeoxyuridine (BrdU) is incorporated into newly synthesized DNA in cells entering and progressing through the S-phase of the cell cycle. Cells were seeded, treated for 3 days with 400 µM UDCA and labelled with BrdU (10 µM) for 30 min. Both adherent and floating cells were harvested, centrifuged for 5 min at 1500 x g, the pellet was washed once with cold PBS and 1 million cells were fixed in 1 ml of cold fixing solution (70 % Ethanol in PBS, -20 °C) and stored at -20°C for 2 h or longer. To stain them, fixed cells were centrifuged 5 min at 1500 x g, pellet was washed once with PBS, centrifuged again, washed once with 0.5 % BSA solution, centrifuged once more, resuspended in 100 µl 2 M HCl and incubated for 20 min at room temperature. 1 ml of 0.5 % BSA solution was added to the tube, the cells were centrifuged for 5 min at 1500 x g, the pellet was resuspended in 500 µl 0.1 M sodium tetraborate at pH 8.5 and incubated for 2 min at room temperature. 1 ml 0.5 % BSA in PBS was added, centrifuged again, the pellet was resuspended in 0.5 % Triton-X 100 in PBS and incubated for 10 min at room temperature. 1 ml 0.5 % BSA in PBS was added, centrifuged again, the pellet was resuspended in 60 µl anti BrdU-FITC (BD Pharmingen) or mouse IgG-FITC (BD Pharmingen) for the isotype control (neither

treated nor BrdU-labelled cells) 1:3 diluted in PBS containing 0.25 % Triton-X 100 and 0.5 % BSA and incubated for 1 h at room temperature in the dark. 1 ml of 0.5 % BSA in PBS was added, the cells were centrifuged and the pellet was then resuspended in 800 µl PI solution (0.1% Triton-X 100 in PBS, 200 µg/ml RNAse A (DNAse free), 10 µg/ml Propidium Iodide (Sigma)) and incubated for 30 min at room temperature in the dark. The percentage of BrdU positive cells was determined by FACS (point 6.2.2.8.1).

6.2.2.5. Detection of cell death

6.2.2.5.1. Trypan blue exclusion assay

Trypan blue stains cells with permeable membrane (necrotic). Cells were harvested and 50 µl of suspension culture was mixed with 50 µl of trypan blue solution (Trypan blue solution 0,4 % (Sigma) diluted 1:1 with PBS and sterile filtered for 5 min at room temperature. 10 µl of stained cells were placed in a Neubauer hemocytometer and the number of viable (white) and dead (blue) cells were counted. The average number of white cells per quadrant of the hemocytometer is calculated and multiplied by the factor $2x10^4$ to find the number of viable cells per ml of culture.

6.2.2.5.2. DAPI staining

Apoptotic cells show nuclear fragmentation and condensation of chromatine which appears as small dots which are extremely shiny when the chromatin is stained with DAPI. 0,2 million cells were seeded in a middle petri dish (22 cm^2), the next day they were treated with 400 µM UDCA for 3 days, all the cells were harvested (adherent + floating), centrifuged 5 min at 1500 x g and room temperature, the pellet was resuspended in PBS and cells were counted by the trypan blue exclusion assay (point 6.2.2.5.1). 0.2-0.4 million cells were transferred to a new Eppendorf cup, centrifuged at 1500 x g for 5 min at room temperature, the pellet was resuspended in 1 ml PBS, so that the cell density was 0,2-0,4 x 10^6 cells/ml and cytospins were prepared. 50 µl PBS were loaded into a cytospin chamber, containing the microscope glass slides, centrifuged for 3 min at 300 rpm, then 50 µl of the suspension culture (corresponding to 1-2 $x10^6$ cells) were loaded, centrifuged for 5 min at 500 rpm, the slides containing the cells were air-dried overnight and then fixed for 10 minutes with ice-cold methanol. They were then washed twice with PBS, incubated for 10

min at room temperature with DAPI solution (1 µg/ml in PBS), washed 3 times with PBS and a coverslip was mounted on top with Fluoromount G (Southern Biotech, Birmingham, Alabama, USA). When the Fluoromount G had solidified they were photographed under a fluorescence microscope.

6.2.2.6. Senescence-associated β-galactosidase staining

Acid β-galactosidase enzyme catalyses the hydrolysis of β-galactosides, including lactose. When cells are incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), acid β-galactosidase cleaves it and the cells are stained blue. Senescent cells show higher levels of acid β-galactosidase activity (98). 20.000 cells were seeded in a small petri dish, treated with 400 µM UDCA for 3 or 6 days, washed once with PBS, fixed in 1 ml fixing solution (2 % formamide, 0.2 % glutaraldehyde in PBS) for 5 min at room temperature, washed twice with PBS, and stained overnight at 37 °C, 5 % CO_2 in 1,5 ml stainig solution (40 mM citric acid buffer (17 ml 0.1 M citric acid mixed with 32 ml 0,2 M $Na_2HPO_4 \cdot 2H_2O$) pH 6, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/ml X-Gal). Cells were washed twice with PBS, a coverslip was mounted on them with Fluoromount G and the dish was fixed on a glass slide. Cells were evaluated for β-gal staining under the microscope and photographed.

6.2.2.7. Cell cycle analysis

6.2.2.7.1. Analysis of the cell cycle by Fluorescent Activated Cell Sorting (FACS)

Cells were seeded, treated with 400 µM UDCA the next day for 3 days and harvested. 1 million cells were fixed in 1 ml of cold fixing solution (70% ethanol in PBS, -20 °C) and stored at -20 °C for at least 2 h. Fixed cells were centrifuged for 5 min at 1500 x g and room temperature, washed once with PBS, resuspended in 800 µl of PI staining solution (0.1% Triton-X 100 in PBS, 200 µg/ml RNAse A (DNAse free), 10 µg/ml Propidium Iodide (PI) (Sigma)) and transferred into 5 ml polystyrene round-bottom tubes (Falcon). They were incubated for at least 30 minutes in the dark, and the cell cycle was measured by FACS not more than 3 hours later. PI stains the DNA by intercalating into double-stranded nucleic acids. Since PI also binds to double-stranded RNA the samples were

treated with RNAse A, in order to avoid contamination with PI bound to double-stranded RNA. Stained nuclei were analyzed using a Becton Dickinson FACScalibur, equipped with a doublet discrimination module. ModFit LT software was used to determine the cell cycle status. FACS measurements were performed by Marie-Luise Hanski.

6.2.2.7.2. Synchronization of cells in G1 by serum depletion

Cells were seeded and on the next day they were washed once with PBS to remove all nutrients and incubated for 24 h in medium without serum. Synchronized cells were treated as described and cell cycle was determined by FACS (point 6.2.2.7.1.).

6.2.2.7.3. Synchronization of cells in G2/M by nocodazole treatment

Cells were seeded and on the next day they treated with nocodazole (50 ng/ml) for 12 h. Then the synchronized cells were treated as described and cell cycle was determined by FACS (point 6.2.2.7.1.).

6.2.2.8. Transfection

6.2.2.8.1. Transfection by electroporation

2,4 million cells, resuspended in 0,5 ml RPMI without FCS, were mixed with 20 µg of plasmid and transferred to a 4 mm electroporation cuvette (Eurogentec). Electroporation was performed at 290 V and 1050 µF and cells were then immediately transferred to a middle dish containing medium with 10 % FCS, prewarmed at 37 °C. On the next day the cells were washed once with PBS and replated as desired.

6.2.2.8.2. Transfection with lipofectamine 2000

Lipofectamine 2000 (Invitrogen) is a cationic lipid formulation that offers the highest transfection efficiencies and protein expression levels on the widest variety of adherent and suspension cell lines. Transfection was performed as described by the manufacturer. It was done 1 day after seeding directly into the same dish. Normally this was performed in either 12-well plate or in small dishes.

6.2.2.8.3. Transfection with lipofectin

Lipofectin (Invitrogen), a lipid-based transfection reagent, was used to transfect plasmids for reporter assays. Transfection was done as recommended by the manufacturer.

6.2.2.8.4. Transfection with calcium chloride

Calcium chloride transfection was used when 3 plasmids had to be transfected at the same time. Cells were seeded in 12-well plate and transfected after one day. 3 h before transfection the medium of the cells was changed. The $CaHPO_4$ transfection kit (Nr. 2-463335, CP Laboratories, UK) was used. To prepare the transfection solution, 10 µl 2M $CaCl_2$ and 6 µg DNA in a volume of 90 µl H_2O were mixed drop wise into 90 µl 2 x DNA precipitation buffer. The mixture was incubated at RT 30 min, so that calcium-DNA precipitate is formed and it was added drop wise to the cells. On the next day cells were washed twice with PBS and fresh medium was given.

6.2.2.8.5. Stable transfection

$0{,}15x10^6$ cells were seeded in a 12-well plate and on the next day 0,5 µg of plasmid was transfected with lipofectamine 2000. A single-cell suspension was replated after one day of transfection in big dishes at a low density (20000 and 40000 cells/dish). G418 was added to the dish the next day (400 µg/ml was the cytotoxic concentration for HCT8 cells), and after 10 days at least 50 growing colonies (clones) were picked into 96 well-plate, and then expanded to a 24-well plate. Expression was determined by western blot and if any clone overexpressed the protein, it was grown further.

6.2.1.9. Detection of chromatin-bound PCNA by immunohistochemistry

Cells were seeded in small dishes (nontreated 0,1 x 10^6 cells/dish; treated 0,3 x 10^6 cells/dish), treated with 400 µM UDCA the next day for 3 days and then washed 3 times with PBS. Chromatin-free proteins (soluble in 0,1 % Triton X-100) were extracted by incubation for 20 min at 4 °C with 2 ml cold CSK buffer (10mM PIPES, pH 6.8, 100 mM NaCl, 300 mM MgCl, 1 mM EGTA, 1 mM DTT, 0,1 % Triton X-100) containing protein inhibitors (4 µl/ml PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 10 µg/ml Pepstatin) (99). Then the cells were washed twice with PBS and fixed with 3,7 % formaldehyde for

10 minutes, washed again twice with PBS, fixed with cold methanol (90% in PBS), washed with PBS 3 times, and blocked for 30 minutes with PBS + 3 % BSA (blocking solution). Cells were labelled with anti-PCNA antibody in blocking solution during 1 h, then washed 3 times with PBS and labelled for 1 h with the secondary antibody (donkey-anti-mouse Cy3 diluted 1:200 in blocking solution). Then they were washed thrice with PBS, stained with DAPI (0,5 µg/ml) for 10 minutes, washed twice with PBS, covered with a coverslip and photographed under a fluorescent microscope.

6.2.1.10. Detection of proliferation in c-Myc expressing cells by immunohistochemistry

Cells were transfected with c-Myc-HA, replated after 1 day in small petri dishes and on the next day labelled with 10 µM EDU (5-ethynyl-2´-deoxyuridine) for 30 min. Proliferation was determined by using Click-iT EDU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen). Cells were washed twice, fixed with Click iT fixative for 20 minutes at room temperature and washed 3 times with 0,5 % BSA in PBS. Cells were permeabilized with 100 µl Triton X-100 for 20 minutes at room temperature, washed again 3 times with 0,5 % BSA in PBS, blocked for 1 h at room temperature in blocking solution (5 % donkey serum (Dianova, Nr. 017000121) and 0,3 % Triton X-100 in PBS), which was then removed, and labelled overnight at 4 °C with anti HA-Tag antibody diluted 1:1500 in PBS containing 1% BSA and 0,3 % Triton X-100. Cells were then washed 3 times with 0,5 % BSA in PBS, labelled with the secondary antibody (donkey anti-mouse conjugated with Cy 3, diluted 1:200 in PBS containing 1% BSA and 0,3 % Triton X-100) at room temperature for 1 h, washed again 3 times with PBS 0,5 % BSA incubated with Click iT Reaction Cocktail (250 µl contain 216 µl 1x Reaction Buffer, 5 µl $CuSO_4$, 4 µl Fluorescent dye azide (Alexa 488) and 25 µl 1x Reaction Buffer Additive) at room temperature in the dark for 30 min. Cells were washed again 3 times with PBS 0,5 % BSA, stained with DAPI (0,5 µg/ml in PBS) 10 min at room temperature, washed twice with PBS, covered with a coverslip and they were analysed under the microscope.

6.2.1.11. One step RT-PCR

RNA was isolated with trizol and purified with RNeasy kit (Qiagen). The determination of c-myc transcript concentration was followed in a one step real time RT-PCR using StepOnePlus Real Time PCR System (Applied Biosystems, CA, USA) and OneStep RT-PCR kit (Qiagen). This experiment was performed by Santosh Krishna Subramanian.

7. RESULTS

7.1. Effect of UDCA treatment on proliferation of colon cancer cells

7.1.1. UDCA inhibits cell growth

In order to be able to study the mechanism of action of UDCA on colon cancer-cell proliferation, a test system comprising several established cell lines had to be established. For this purpose the effect of UDCA on the growth of four human colon cancer cell lines (HCT8, LS513, HCT116 and Co115) was investigated. Only $p53^{wt}$ cell lines were chosen because it was of interest to determine the effect of UDCA at an early stage, when the tumor begins to develop, while p53 mutation occurs at a later stage of tumor formation. UDCA treatment (400 µM, 3 days) inhibited the growth of all the cell lines by 50-80 %. This effect was partially reversible, as treated cells were able to grow after removal of UDCA although the growth was less than in the nontreated group (Figure 7.1).

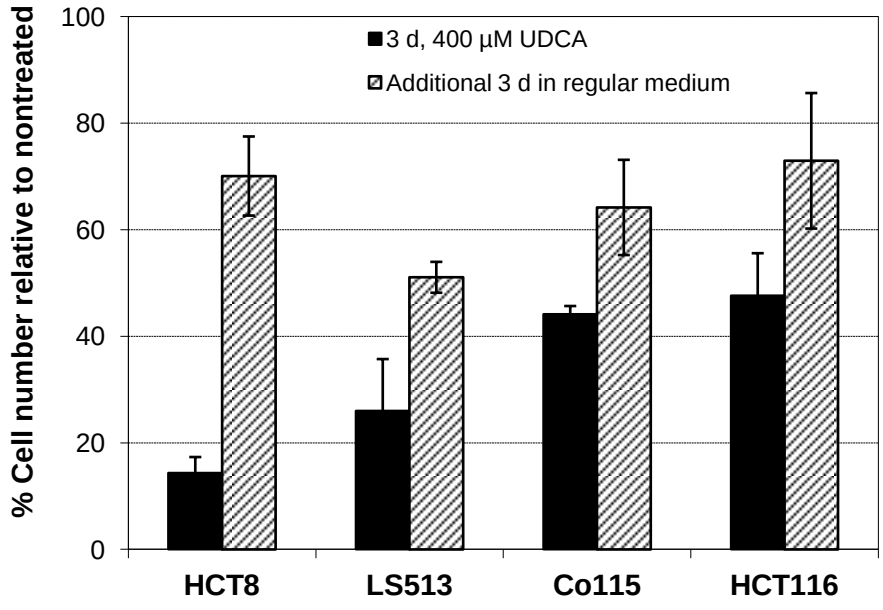

Figure 7.1. UDCA inhibits proliferation of colon cancer cells in a partially reversible manner. HCT8, LS513, Co115 and HCT116 cells were seeded and on the next day treated with 400 µM UDCA. After 3 days of treatment cells were counted (black bars). Another group of cells was treated with UDCA for 3 days and then allowed to grow for another 3 days without UDCA and counted (stripped bars). The cell number related to the number of nontreated cells was plotted. Means ± SD of 3 experiments.

7.1.2. UDCA inhibits the incorporation of bromodeoxyuridine

The incorporation of bromodeoxyuridine (BrdU) into the DNA of the cells is a measure of cell proliferation. The percentage of cells incorporating BrdU in the studied colon cancer cell lines was of 30-50% of the total population. This corresponds to the percentage of cells that are in the S-phase of the cell cycle. After treatment with UDCA, in HCT8, LS513 and HCT116 cells the incorporation of BrdU was about half of the BrdU incorporation by the nontreated cells, while in Co115 cells the incorporation was reduced by one quarter (Figure 7.2).

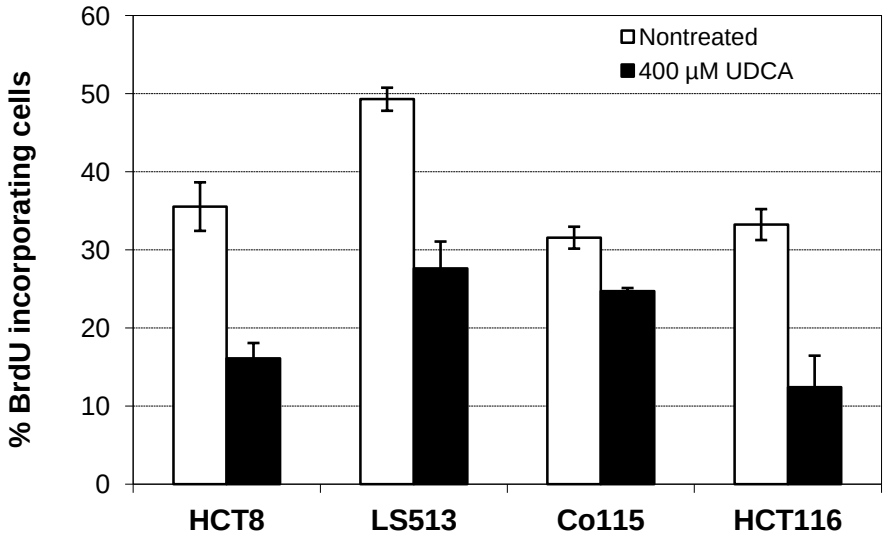

Figure 7.2. UDCA inhibits BrdU incorporation. Cells were treated with 400 µM UDCA for 3 days and were then labeled with BrdU for 30 min. The percentage of cells incorporating BrdU was determined by FACS. Mean values ± SD of 3 experiments. FACS measurements were performed by Marie-Luise Hanski.

The experiments show that UDCA inhibits proliferation of colon cancer cells to a different degree. The mechanism of this inhibition is still not known.

7.2. Investigation of the mechanism of proliferation inhibition in colon cancer cells

7.2.1 Inhibition of proliferation by UDCA does not correlate with the speed of growth of the different cell lines

Taking into consideration that cancer cells have usually the property of growing faster than normal cells, and since the investigated colon cancer cell lines show a different inhibition of proliferation after treatment, a correlation between susceptibility of the cell to UDCA treatment and its speed of growth could exist. To check this, the generation time of each cell line was determined as described in Materials and Methods (point 6.6.2.3) and compared with the inhibition of proliferation by UDCA (Table 7.1).

Cell line	Generation time (hours)	Proliferation inhibition (%)
HCT8	21 ± 2	86 ± 3
LS513	28 ± 3	74 ± 10
Co115	39 ± 6	56 ± 2
HCT116	23 ± 3	52 ± 8

Table 7.1. Comparison of the generation time and the extent of proliferation inhibition by UDCA. The generation time was determined as described in Materials and Methods. The proliferation inhibition after UDCA treatment for 3 days (400 µM UDCA) was determined by cell count.

HCT8 and HCT116 are the fastest growing cells. The percentage of inhibition was the highest in HCT8 cells but, by contrast, HCT116 cells had the least inhibition. Co115 and HCT116 cell lines showed similar inhibition of proliferation after UDCA treatment, but the generation time was approximately two-fold longer in Co115 than in HCT116 cells. These results showed that that the inhibition of proliferation by UDCA was not dependent on the proliferation rate of the cells.

7.2.2. UDCA does not induce apoptosis in colon cancer cells

The question was posed if UDCA induces apoptosis in colon cancer cells. DAPI staining did not reveal the presence of cells with condensed chromatin (Figure 7.3. A) and PARP cleavage was not observed after treatment (Figure 7.3.B). Cells treated with 400 µM UDCA for 3 days showed practically no sub-G1 population of the cell cycle (Table 7.2). In

addition, there was no increase in the percentage of trypan-blue stained cells after treatment compared to the percentage in the nontreated cells (data not shown).

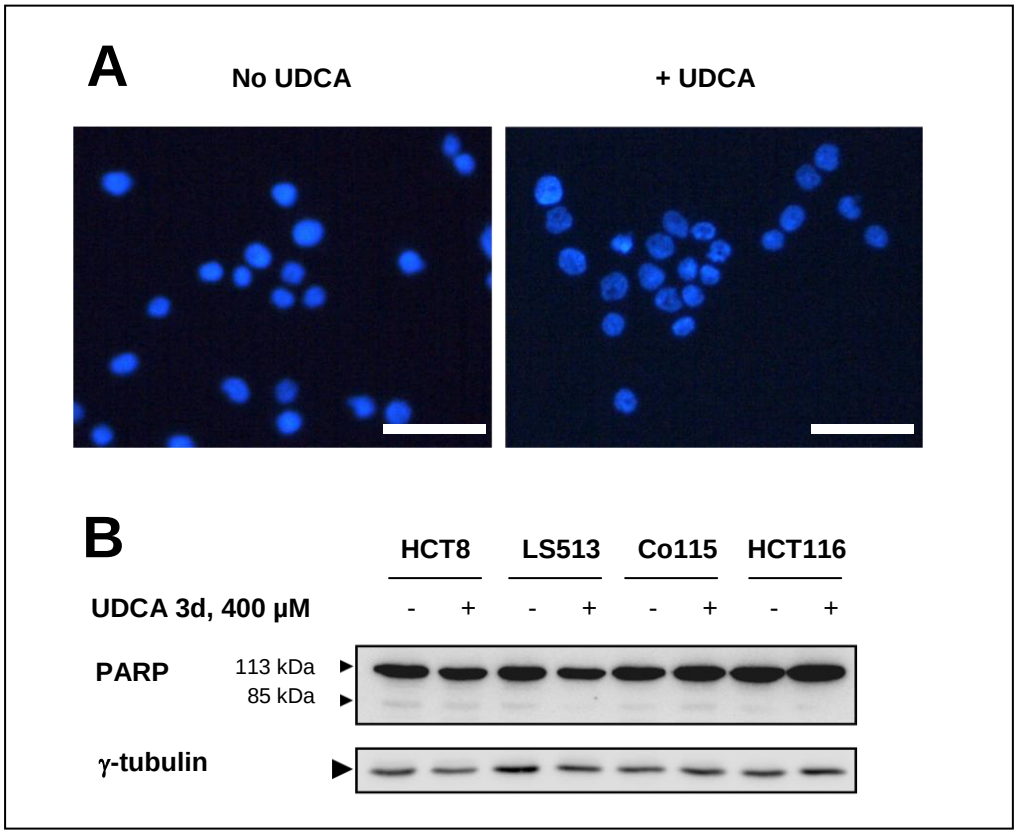

Figure 7.3. Chromatin condensation and PARP cleavage after treatment with UDCA. A: HCT116 cells were treated or not for 3 days with 400 µM UDCA, harvested and all the cells (adherent and floating) were transferred to coverslips by cytospin. The nuclei were stained with DAPI. Representative photomicrographs are shown. Bars: 50 µm. B: Cells were treated for 3 days at 400 µM UDCA and lysed in Sato buffer. PARP was detected by western blot.

These data indicated that apoptosis was not the reason for inhibition of cell proliferation by UDCA.

7.2.3. UDCA induces senescence in LS513 cells

To check if UDCA inhibits proliferation by inducing senescence, colon cancer cells were treated for 3 days with 400 µM UDCA and the induction of senescence was detected by determination of β-galactosidase (β-Gal) activity and evaluation of morphology. Only LS513 cells showed slight increase of β-galactosidase activity after 3 days of treatment, but

there was no induction of β-Gal by UDCA in any of the other 3 cell lines investigated (Figure 7.4). After 6 days of treatment senescence was more pronounced in LS513, and there was an increase in β-galactosidase activity in HCT116 cells. In this case, the vacuolated morphology in both cell lines was evident. The HCT8 and Co115 cell lines, showed sign of senescence after treatment (Figure 7.5). These results indicated that senescence was not the main cause of inhibition of proliferation after 3 days of treatment. In addition, it is again observed that different colon carcinoma cell lines respond differently to UDCA treatment.

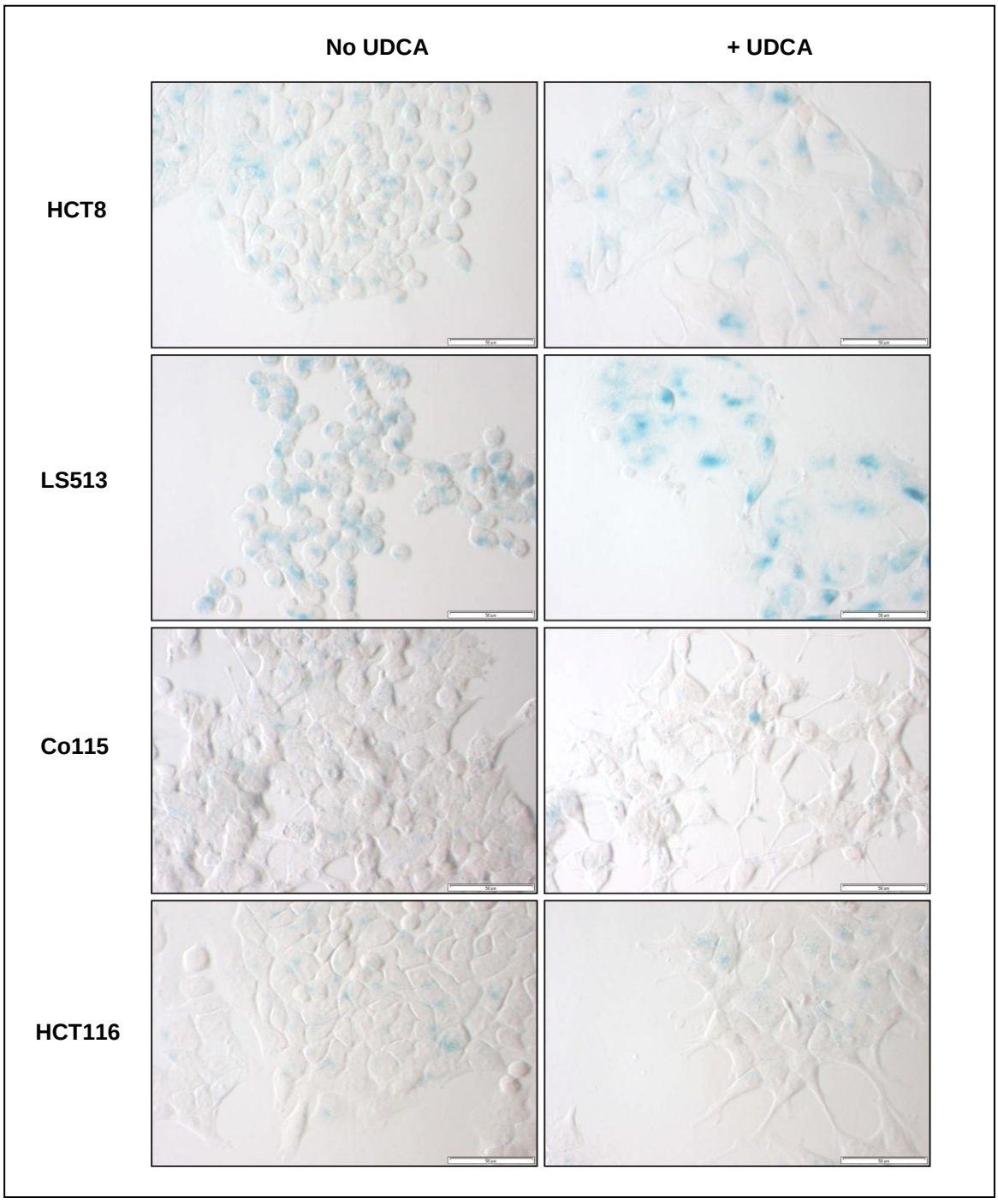

Figure 7.4. Test of senescence after 3 day UDCA treatment. Cells were treated with UDCA (400 µM) for 3 days and β-galactosidase activity was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The figure shows slight increase in β-Gal staining after UDCA treatment in LS513 cells, and no change in the other cell lines. Representative photomicrographs are shown. Bar= 50 µm.

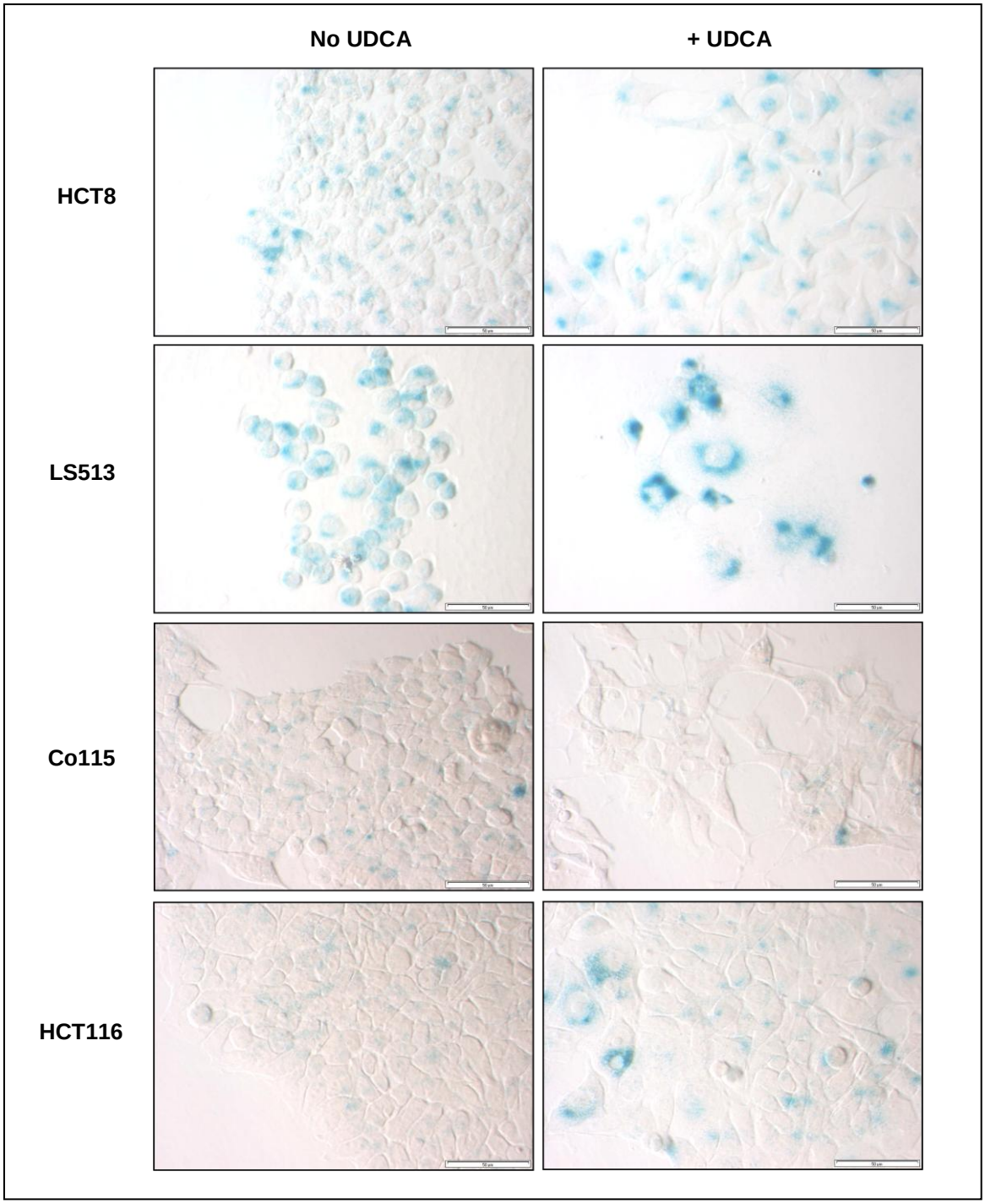

Figure 7.5. Test of senescence after 6 day UDCA treatment. Cells were treated with UDCA (400 µM) for 6 days and β-galactosidase activity was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Increase in β-Gal staining is visible in LS513 and HCT116 cells after treatment, and no change in HCT8 and Co115 cells. Representative photomicrographs are shown. Bar= 50 µm.

7.2.4. UDCA affects the cell cycle of colon cancer cells

Since neither apoptosis nor senescence were detected in HCT8 nor in Co115 cells the effect of UDCA on the cell cycle was investigated by FACS.

7.2.4.1. UDCA decreases S-phase population in colon cancer cells and slows down the cell cycle

In all cell lines the S-phase population was decreased significantly after treatment (Figure 7.6 and Table 7.2). Moreover, an increase in the percentage of cells in G1 phase was observed in HCT8 and HCT116 cells after treatment. However, in the case of LS513 no G1-phase population increase was observed after treatment but there was a higher percentage of cells in G2/M-phase, and in Co115 there was no other change rather than the decrease in S-phase (Table 7.2).

Cell line	HCT8			LS513			Co115			HCT116		
Phase	-UDCA	+UDCA	P	-UDCA	+UDCA	p	-UDCA	+UDCA	P	-UDCA	+UDCA	p
Sub G1	4 ± 4	3 ± 2	n.s.	2 ± 1	5 ± 2	0,024	5 ± 2	5 ± 2	n.s.	1 ± 0	2 ± 1	0,026
G1	47 ± 4	64 ± 3	0,003	42 ± 2	46 ± 4	n.s.	48 ± 4	50 ± 0	n.s.	43 ± 1	68 ± 4	<0,001
S	32 ± 2	17 ± 1	0,001	45 ± 2	33 ± 2	0,001	30 ± 2	24 ± 2	0,019	39 ± 4	17 ± 4	0,002
G2/M	16 ± 1	16 ± 1	n.s.	11 ± 1	16 ± 2	0,033	17 ± 2	21 ± 3	n.s.	16 ± 3	12 ± 3	n.s.

Table 7.2. Effect of UDCA treatment on the cell cycle. HCT8, LS513, Co115 and HCT116 cells were treated with UDCA (400 µM, 3 days) and cell cycle was determined by FACS. Mean of 3 experiments ± SD. (p determined in t-test, n.s. = not significant). FACS measurements were performed by Marie-Luise Hanski.

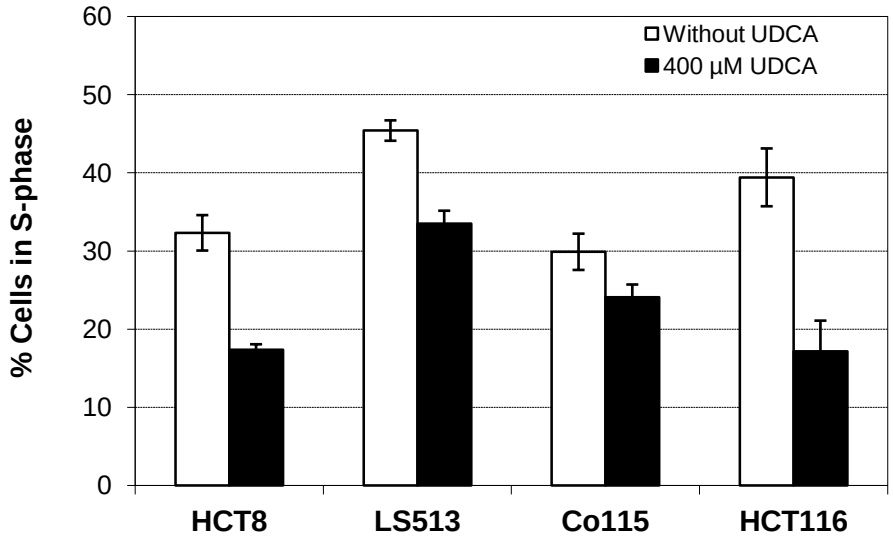

Figure 7.6. UDCA decreases the percentage of cells in the S-phase. Mean values of 3 experiments ± SD.

The effect of UDCA on the cell cycle progress was further studied in HCT116 cells synchronized cells synchronized in G2/M phase by nocodazole treatment for 12 hours. After nocodazole removal cells were allowed to progress in the presence or absence of UDCA.

After nocodazole treatment 90 % of the cells were in G2/M (Figure 7.7).

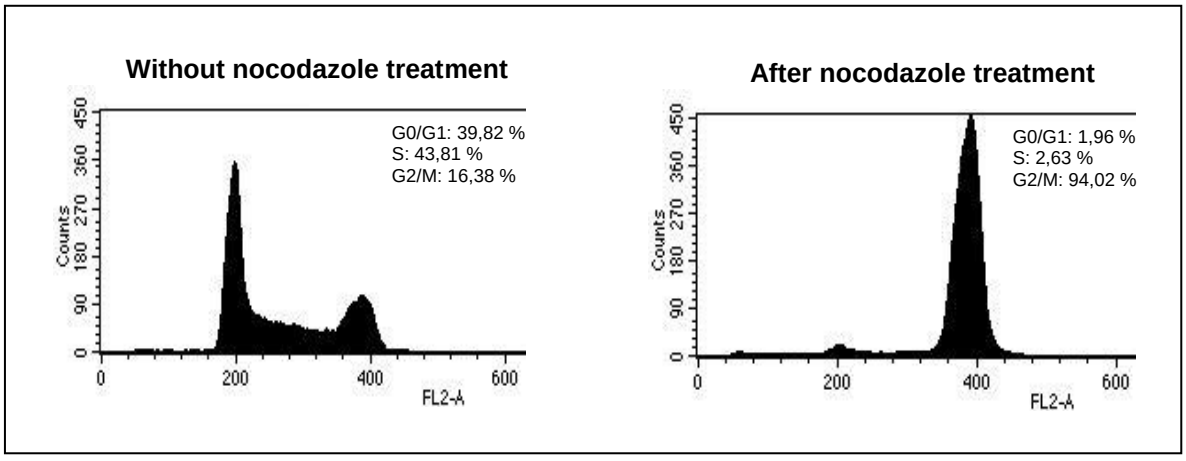

Figure 7.7. Synchronization of HCT116 cells in G2/M phase with nocodazole. FACS analysis of HCT8 cells (A) and HCT116 cells (B) treated or not for 12 h with nocodazole (50 ng/ml). FACS measurements were performed by Marie-Luise Hanski.

Figure 7.8 shows the percentage of cells in S-phase at different time points after removal of nocodazole and UDCA addition. The nontreated cells completed one cell cycle in 15 hours (red arrow) whereas in the presence of UDCA the cells progressed slower and completed one cell cycle in 21 hours (green arrow). The delay of the cell cycle progress due to the treatment was therefore 6 hours.

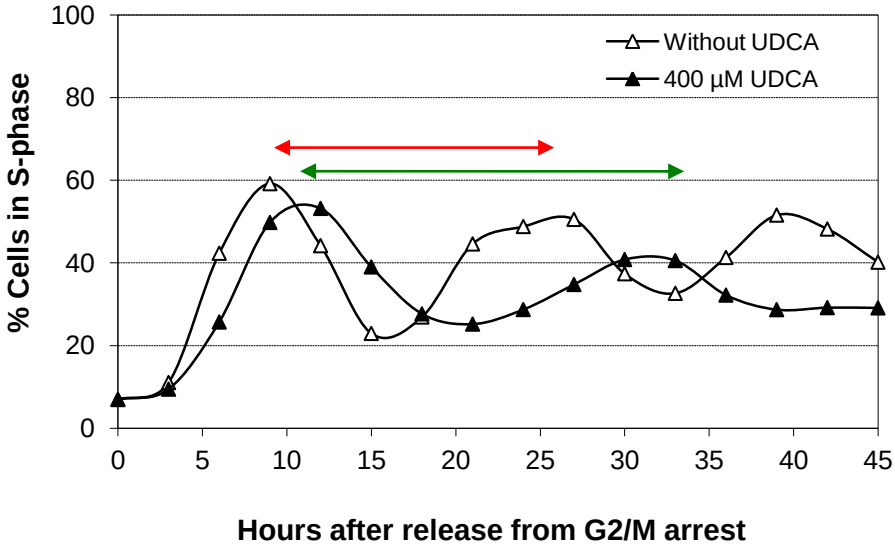

Figure 7.8. UDCA slows down the cell cycle. HCT116 cells were synchronized in G2/M by nocodazole treatment (50 ng/ml) (Figure 7.7) and then allowed to progress in the absence of nocodazole but in the presence or absence of UDCA treatment (400 µM). Cells were harvested every 3 hours and cell cycle was analysed by FACS. The percentage of cells in S-phase is plotted. The experiment was performed by Santosh Subramanian and Marie-Luise Hanski.

These data indicated that UDCA inhibited proliferation by slowing down the cell cycle. To clarify which cell cycle phase was affected after treatment, the effect of UDCA on the transitions G1 to S cell cycle phases and G2/M to G1 cell cycle phases was investigated.

7.2.4.2. UDCA delays the G1→S transition

In order to study the effect of UDCA on the G1→S transition cells were synchronized in G0/G1 phase by serum depletion and then allowed to progress in the cell cycle in the regular medium, in the presence or absence of UDCA. Starvation induced an increase in the G1 population population (Figure 7.9).

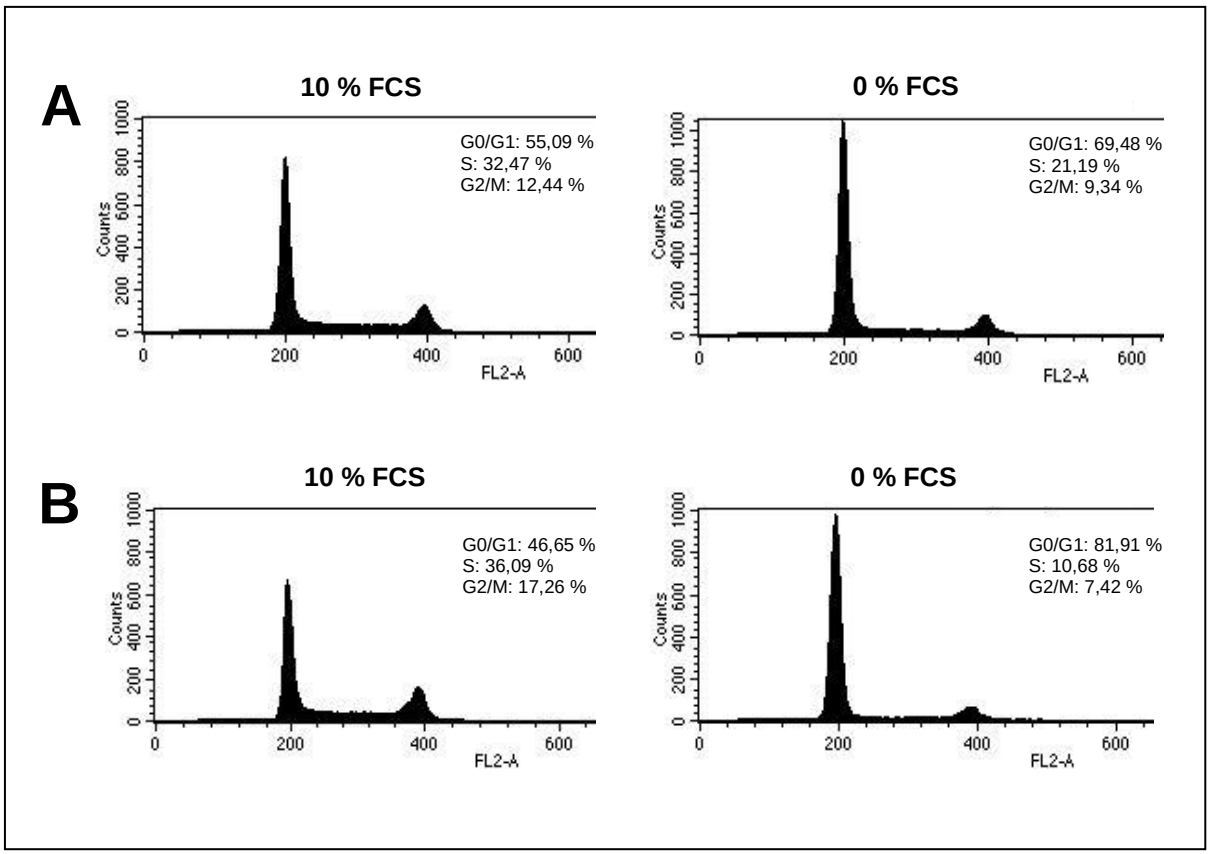

Figure 7.9. G1 arrest is induced by FCS depletion. FACS analysis of HCT8 cells (A) and HCT116 cells (B) grown for 24 h in medium with 10 % FCS or 0 % FCS. FACS measurements were performed by Marie-Luise Hanski.

In the absence of UDCA HCT8 cells entered S-phase after 4 hours of serum stimulation, whereas in the presence of UDCA, they entered S-phase after 12 hours. Thus, UDCA delayed the G1→S transition by 8 hours (Figure 7.10 A). In the case of HCT116 cells, the exit of the cells from G1 phase occurred after 8 hours of serum stimulation, and 2 hours later in the presence of UDCA (Figure 7.10.B).

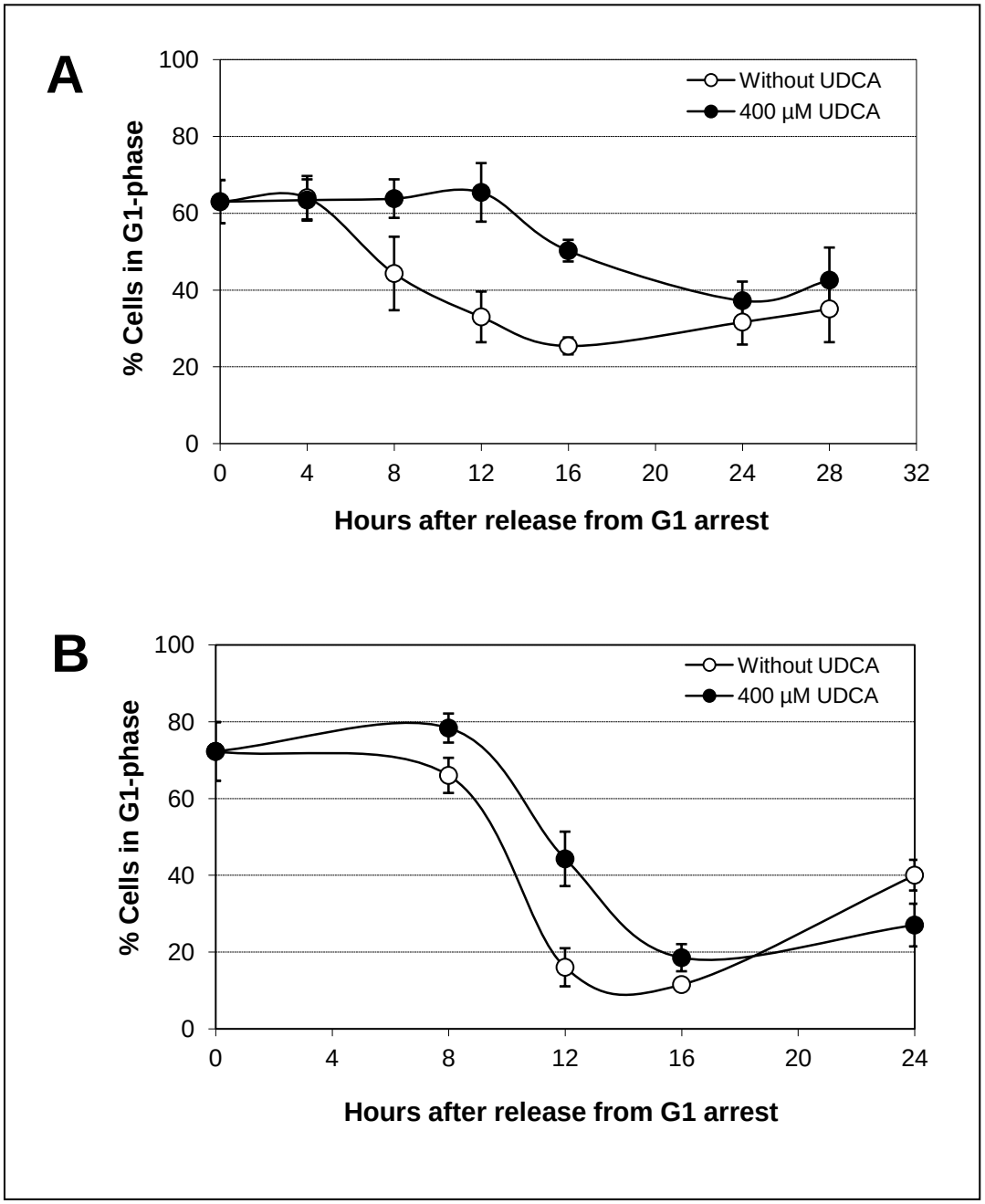

Figure 7.10. Effect of UDCA on G1→S transition. HCT8 cells (A) or HCT116 cells (B) were starved by serum depletion (Figure. 7.9.). After serum addition they were allowed to progress in the presence or absence of UDCA (400 µM). Percentage of cells in G1-phase are plotted. Means ± SD of three experiments.

7.2.4.3. UDCA does not affect the G2/M→G1 transition

To investigate the effect of UDCA on the G2/M→G1 transition cells were synchronized in G2/M cell cycle phase with nocodazole and then allowed to progress in the regular medium, in the presence or absence of UDCA (400 µM). Nocodazole induced an arrest in G2/M cell cycle phase of 90 % in HCT8 (Figures 7.11) and HCT116 cells (Figure 7.7).

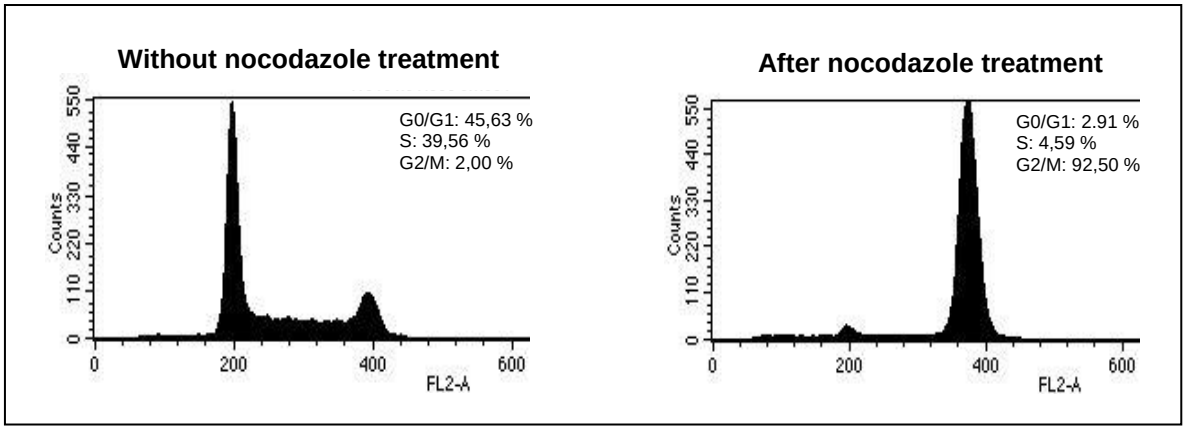

Figure 7.11. Synchronization of HCT8 cells in G2/M phase with nocodazole. FACS analysis of HCT8 cells treated or not for 12 h with nocodazole (50 ng/ml). FACS measurements were performed by Marie-Luise Hanski.

Three hours after of removal of nocodazole, practically all the cells were in G1-phase. Cells treated with UDCA behaved in the same way as the nontreated, i.e. UDCA did not affect the G2/M → G1 transition (Figure 7.12).

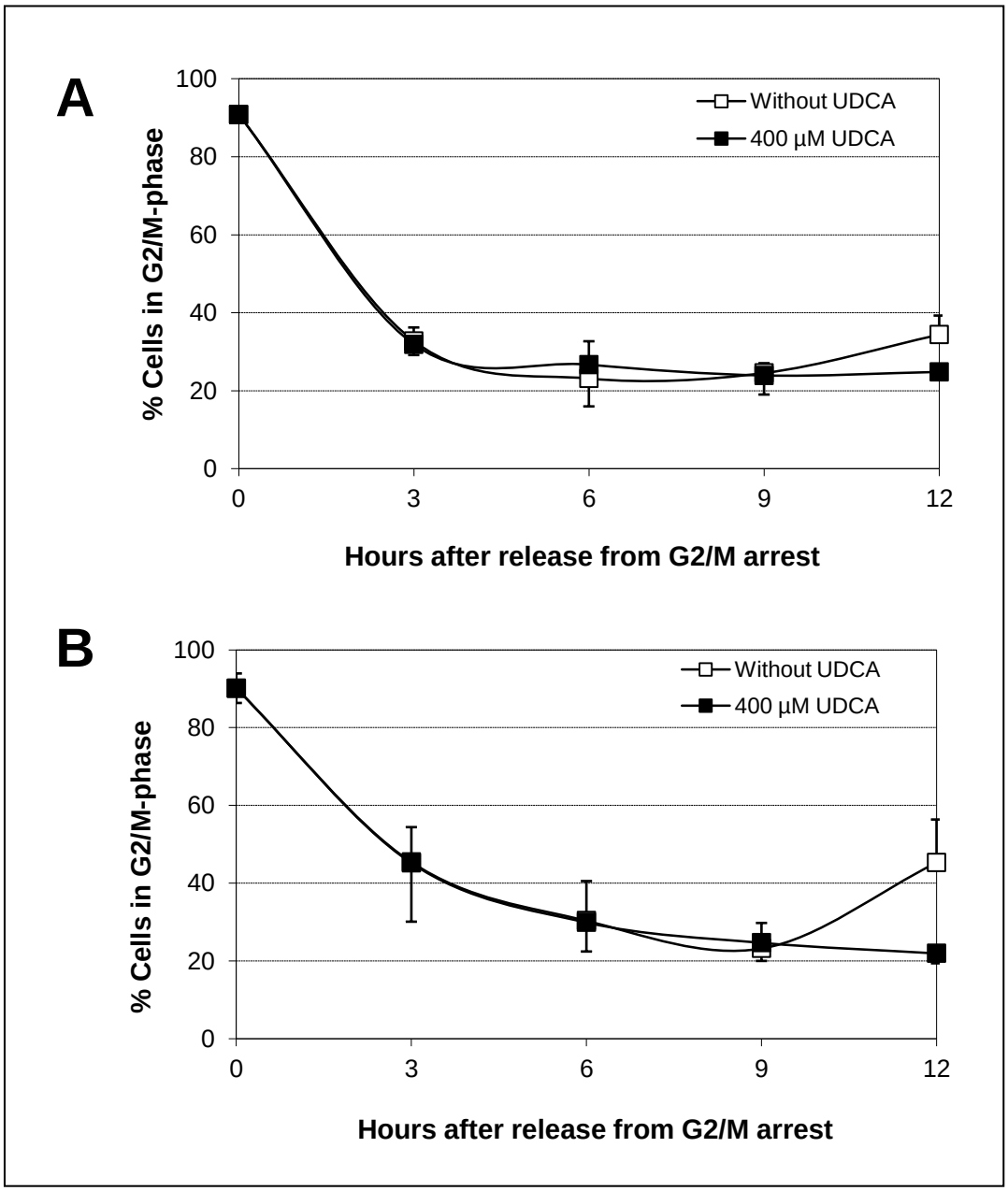

Figure 7.12. UDCA does not affect the G2/M→G1 transition. HCT8 cells (A) and HCT116 cells (B) were arrested in G2/M by nocodazole (Figure 7.11). After removal of nocodazole they were allowed to progress in the presence or absence of UDCA (400 µM).

These results show that UDCA-induced slow down of the cell cycle is associated with a delay of the G1→S transition.

7.3. Identification of the molecular targets of UDCA

7.3.1. The proteins regulating the G1→S transition: p21, cyclin A, CDK2, CDK6 and Rb are affected by UDCA

The main proteins responsible for the progression of the cells from the G1-phase to the S-phase of the cell cycle are cyclin dependent kinases (CDKs). They inactivate Rb protein by phosphorylation, which results in the release and activation of the transcription factor E2F-1. The function of E2F-1 is to transcribe proteins that are necessary for the progression into the S-phase. CDKs are activated by cyclins and are inhibited by p21 and p27 proteins. The analysis of expression of these proteins showed that 3 days of treatment with UDCA inhibited the expression of CDK2, CDK6, cyclin A and Rb proteins (Figure 7.13). It induced Rb hypophosphorylation and increased the expression of p21 protein. The expression of CDK4, p27, cyclin D1, cyclin E was either not affected or the effect was not consistent in all the cell lines, suggesting that these proteins are not the common targets of UDCA.

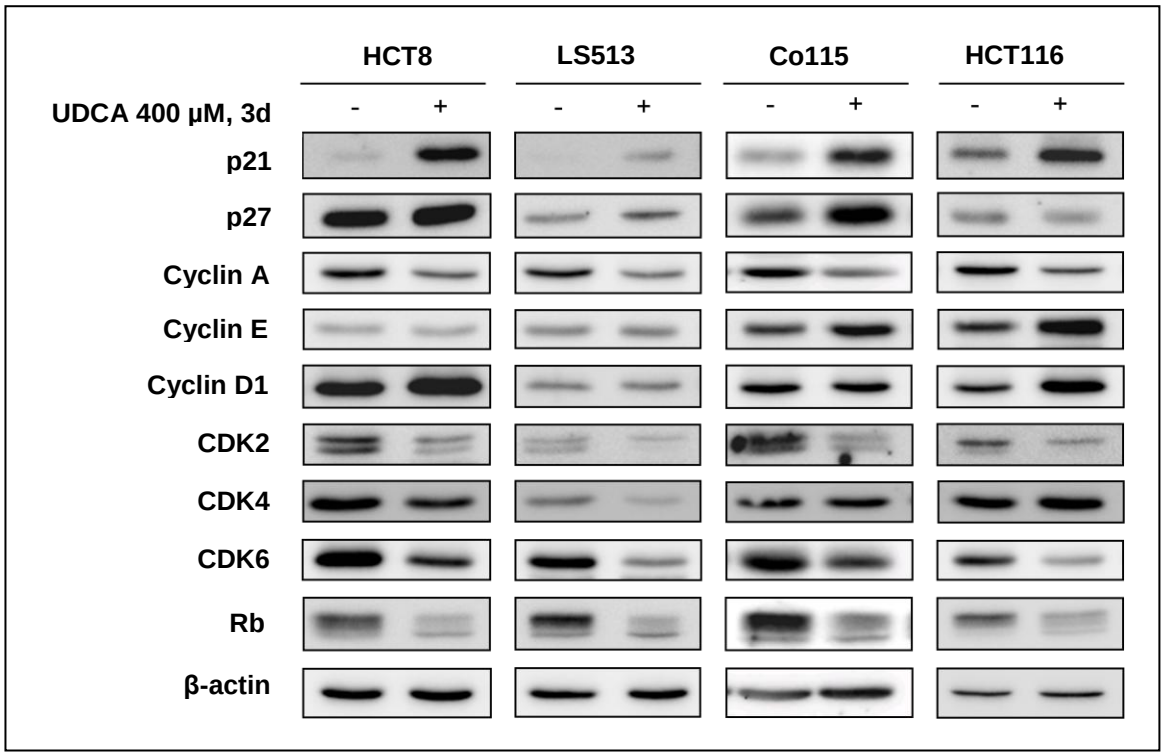

Figure 7.13. UDCA targets genes involved in the G1→S transition. Cells were treated for 3 days with 400 µM UDCA. Lysates in Sato buffer were analysed in western blot. β-actin was taken as a loading control. This figure shows a representative experiment for each cell line. At least 3 experiments were done in each case.

7.3.2. UDCA downregulates the transcription factor c-Myc

c-Myc is a transcription factor known to regulate many genes involved in the G1→S transition. It was found that 3 days of UDCA treatment decreased c-Myc expression in all cell lines (Figure 7.14).

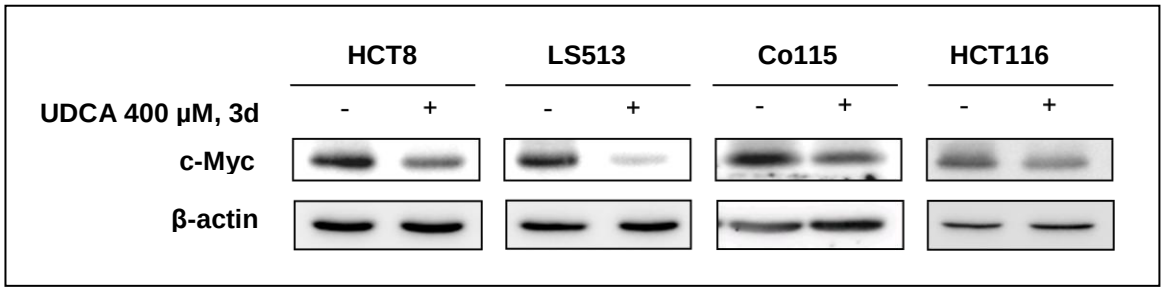

Figure 7.14. UDCA decreases c-Myc expression in all cell lines. Cells were treated for 3 days with 400 µM UDCA. c-Myc expression was determined in lysates by western blot. β-actin was taken as a loading control. This figure shows a representative result of at least 3 experiments. The samples are the same as in Figure 7.13.

7.3.3. c-Myc, p21, cyclin A and Rb are affected by UDCA during the G1→S-phase transition

Once the targets of UDCA were identified, their involvement in the G1→S-phase transition was investigated. It could indicate a role in the delay of the cell cycle after treatment.

Expression of p21, cyclin A, CDK2, CDK6 and Rb proteins was determined in HCT8 cells at different time points during G1→S transition in the presence or absence of UDCA. In parallel, cell cycle was determined by FACS. The exit from G1-phase occurred after 4 hours of serum stimulation in the nontreated cells. After 8 hours practically all the cells were in S-phase, whereas in the presence of UDCA the cells remain in the G1 cell cycle phase (Figure 7.15, see also figure 7.10 A).

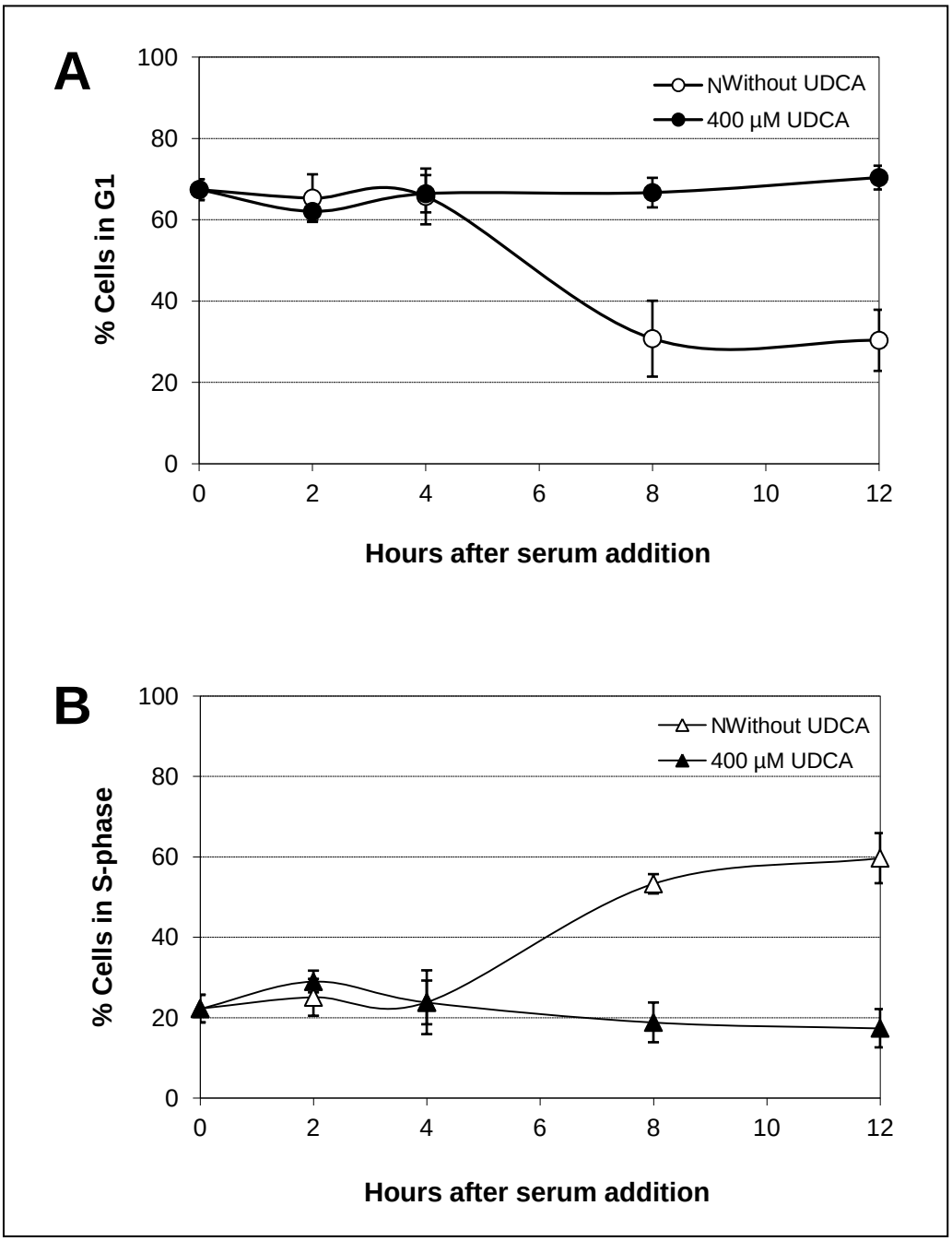

Figure 7.15. Effect of UDCA on G1→S-phase transition. HCT8 cells were starved for 24 h and then allowed to grow after serum addition in the presence or absence of UDCA (400 µM). Cell cycle was analysed by FACS. A.: Release from G1-phase; B.: Entrance in S-phase.

Serum depletion induced an increase in p21 expression, a decrease in c-Myc, cyclin A, CDK2 and Rb expression. In addition, Rb was hypophosphorylated. UDCA presence inhibited the upregulation of c-Myc after serum stimulation. p21 expression was increased after serum stimulation and then decreased to basal level when the cells entered S-phase. UDCA delayed the time course of p21 expression. Cyclin A expression gradually

increased as the cells progressed from G1-phase to S-phase. In UDCA-treated cells this increase was not observed. The expression of Rb practically did not change, whereas serum stimulation induced hyperphosphorylation after 4 hours. In the presence of UDCA hyperphosphorylation was observed after 8 hours of serum stimulation. UDCA did not affect CDK2 and CDK6 protein expression within 12 h (Figure 7.16).

To sum up, c-Myc, p21, cyclin A and Rb were affected by UDCA during the G1→S-phase transition, but not CDK2 and CDK6. In addition, c-Myc and p21 were the earliest molecules affected by UDCA.

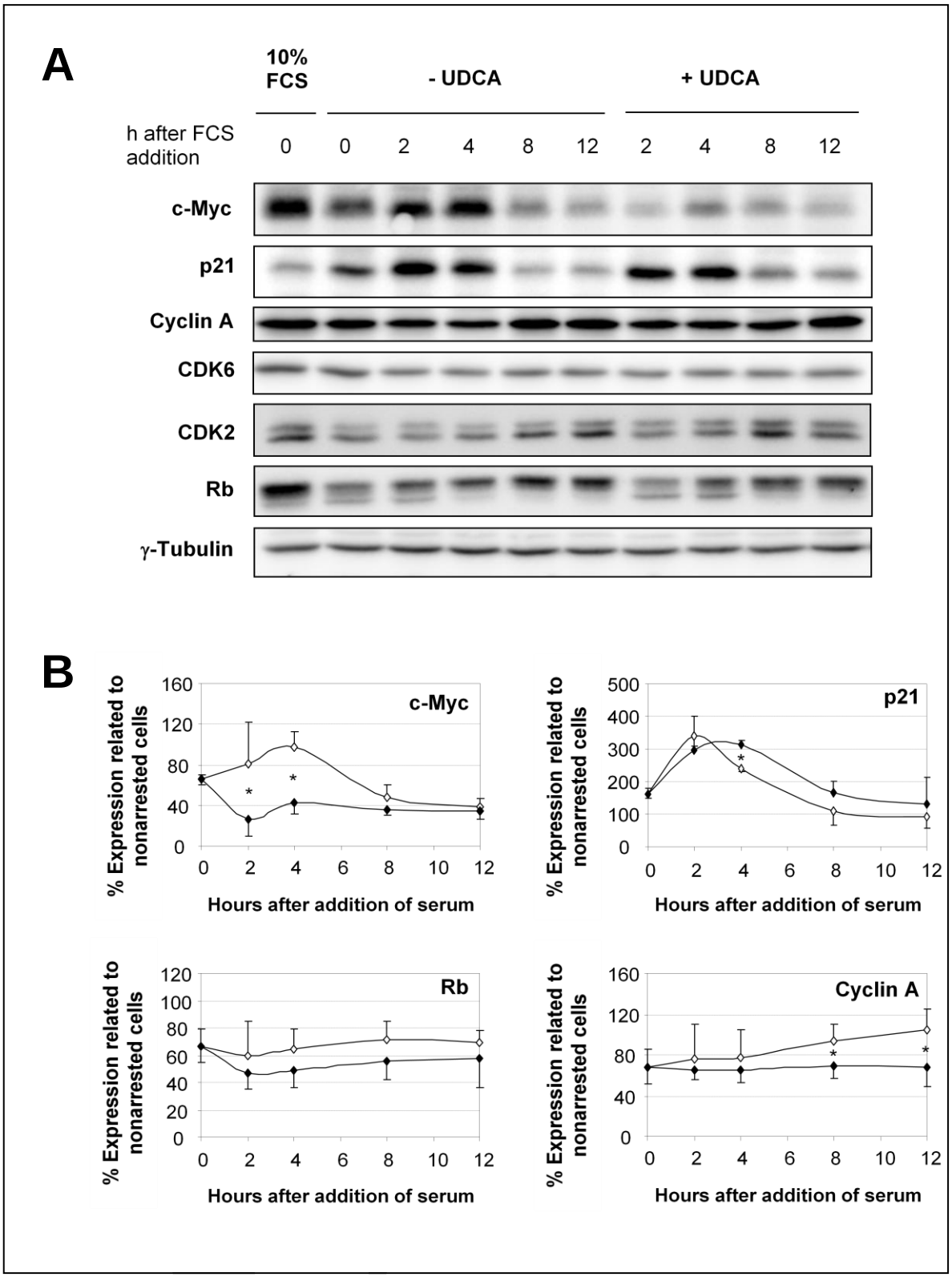

Figure 7.16. UDCA affects c-Myc, p21, Rb and Cyclin A during the G1→S transition. HCT8 cells were starved for 24 h and then serum was added in the presence (full symbols ♦) or absence (empty symbols ◊) of UDCA (400 µM). A.: Cells were lysed in Sato buffer and analysed by western blotting. As a control, a lysate of cells grown in medium with 10% FCS for 24 h was taken (non arrested cells); B.: The intensity of the bands related to that in the nonarrested cells, and normalized to γ-tubulin is shown. Means ± SD of at least 3 experiments. (* $p<0.05$).

7.3.4. Decrease of c-Myc expression contributes to the inhibition of proliferation

Since c-Myc was one of the first molecules whose expression is affected by UDCA during G1→S transition (Figure 7.16) the question was asked if it played a role in the inhibition of proliferation after treatment. After 3 days of treatment c-Myc was downregulated by UDCA in all cell lines (Figure 7.14). In order test the effect on proliferation, c-Myc expression was suppressed in HCT8 cells and cell number was determined by cell count. Cells with decreased levels of c-Myc grew slower than the ones with normal expression of the protein (Figure 7.17). This means that c-Myc suppression is sufficient to inhibit proliferation.

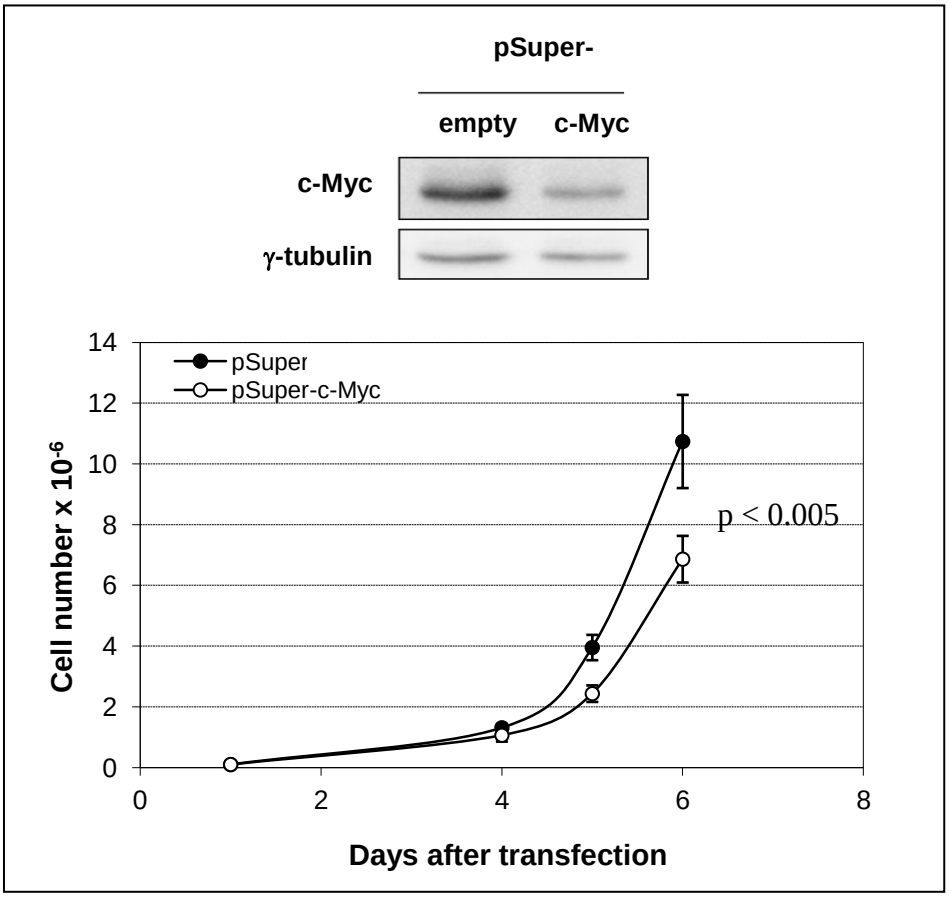

Figure 7.17. c-Myc suppression inhibits proliferation. HCT8 cells were transfected with pSuper vector or pSuper-c-Myc plasmid. 1 day after transfection they were replated. Cells were counted in duplicates 4, 5 and 6 days after transfection. Expression of c-Myc was determined by western blot, 2 days after transfection. Means ± SD of 3 experiments.

7.3.5. UDCA inhibits E2F-1 activity

One of the targets of UDCA which is affected during the G1→S transition is Rb protein. This molecule has the function of binding to the transcription factor E2F-1. When bound to Rb, E2F-1 is not active. Phosphorylation of Rb releases E2F-1, which can bind to DNA and induce the transcription of genes involved in proliferation. Since UDCA induces Rb hypophosphorylation (Figure 7.13), this could result in inhibition of E2F-1 activity and consequently inhibition of proliferation. The luciferase reporter assay showed that the activity of the endogenous E2F-1 was very low and UDCA decreased the activity minimally (data not shown). When E2F-1 was overexpressed, UDCA treatment also induced a decrease in the activity of the exogenous E2F-1 (Figure 7.18).

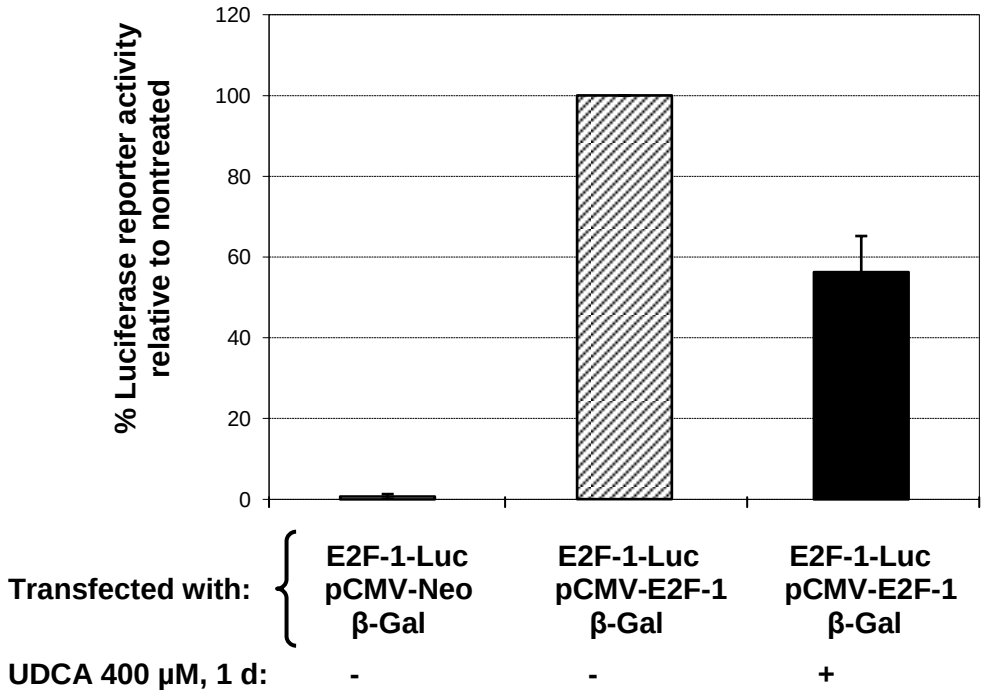

Figure 7.18. UDCA inhibits E2F-1 activity. HCT8 cells were transfected with E2F-1 (0,1 µg), E2F-1-Luc (1 µg) and β-Gal (50 ng) plasmids. After 1 day the cells were treated with UDCA (400 µM). Luciferase activity was measured 2 days after transfection and 1 day after start of treatment. β-Gal activity was used for normalization. Mean ± SD of 3 experiments.

This experiment indicates that UDCA partially inhibits the binding of E2F-1 to the promoter, and therefore it inhibits its activity.

7.3.6. UDCA inhibits PCNA binding to chromatin

One of the last steps of the G1→S transition is the binding of PCNA to chromatin. UDCA-treated cells progress slower in the cell cycle and the G1→S transition is delayed. The effect of UDCA on PCNA binding to chromatin was investigated, in order to check if this step of the G1→S transition was affected by UDCA. For this, HCT8 cells were treated with UDCA for 3 days and the PCNA protein not bound to chromatin was extracted, as described in Materials and Methods (Point 6.2.1.9.2).

Insoluble nuclear PCNA, which plays a role in DNA replication and is bound to chromatin, was detected by immunohistochemistry in the adherent cells. The expression of PCNA was not affected by UDCA, but less PCNA was bound to chromatin in the treated sample. UDCA treatment reduced the percentage of cells with chromatin-bound PCNA from 26 % to 2 % (Figure 7.19). Another experiment showed complete inhibition (data not shown). This result shows that after treatment almost no PCNA is bound to chromatin.

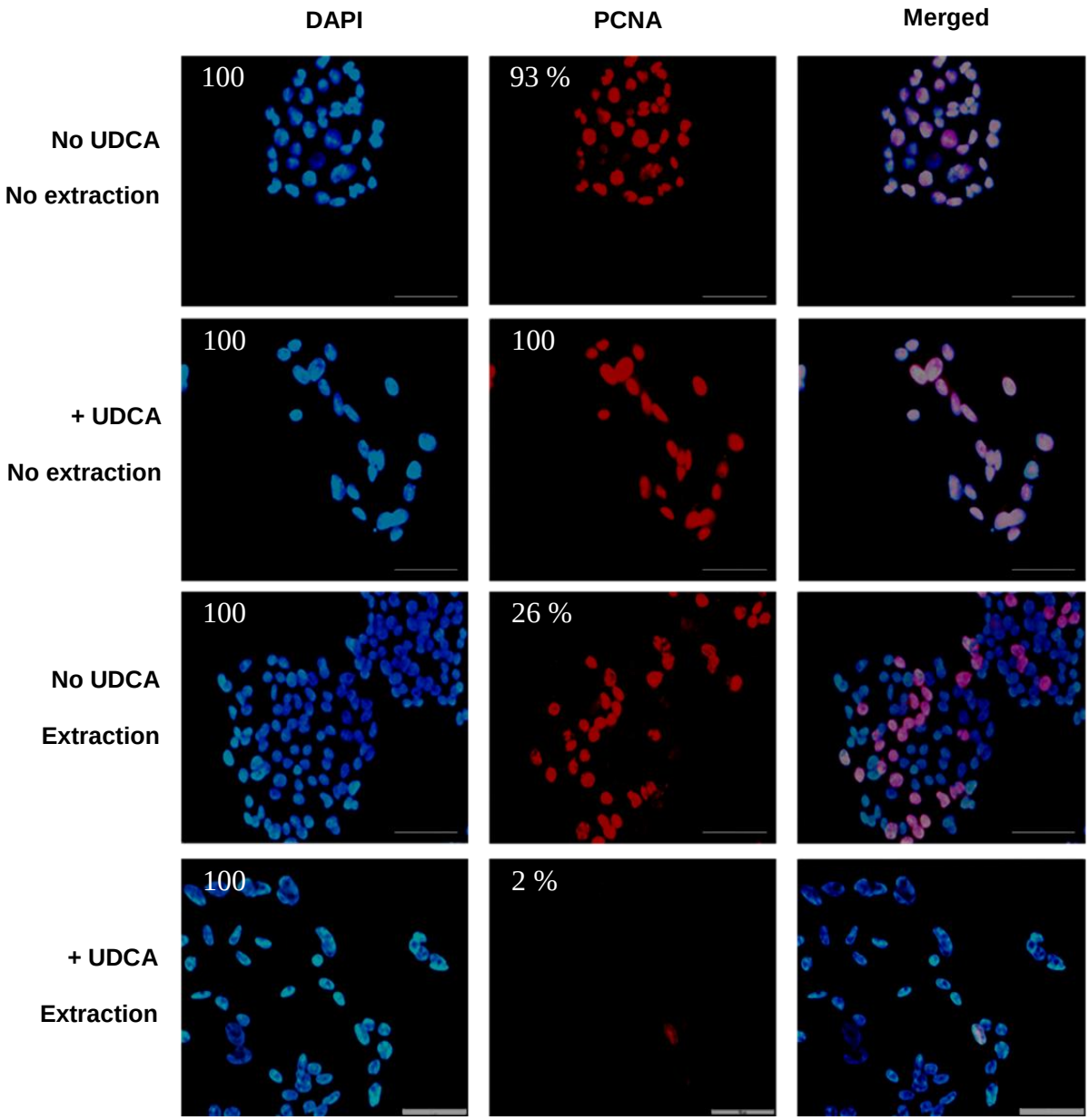

Figure 7.19. UDCA inhibits PCNA binding to chromatin. Cells were either not treated or treated with UDCA for 3 days (400 µM). The soluble PCNA was removed by extraction with Triton X-100. Total (No extraction) or chromatin-bound PCNA (Extraction) expression was detected by immunohistochemistry. This experiment was done twice. The percentage of stained cells is shown. Bars = 50 µm.

7.4. Regulation of UDCA targets

7.4.1. p21 upregulation by UDCA is p53 independent

One of the main regulators of p21 is p53. This protein is known to induce p21 transcription. Since p21 is upregulated by UDCA in all cell lines, this could be a result of the upregulation of p53 after UDCA treatment. Expression of p53 in cells treated with 400 µM UDCA for 3 days was determined by western blot and compared with the expression of p21. In contrast to p21, p53 was not upregulated by UDCA in LS513 and Co115 cell lines. In HCT8 and HCT116 there was upregulation after treatment. (Figure 7.20).

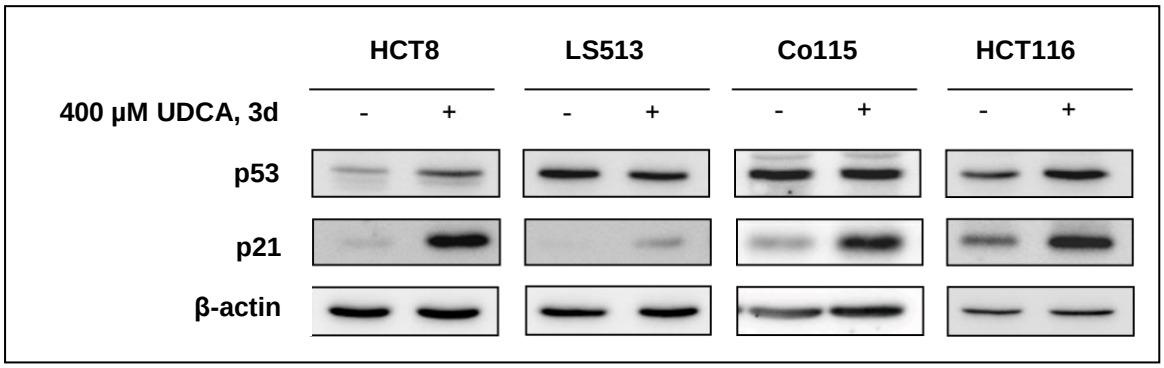

Figure 7.20. Upregulation of p21 by UDCA is independent from p53. Cells were treated with 400 µM UDCA for 3 days, lysed in Sato buffer and expression of p53 and p21 proteins was determined by western blot. β-actin was taken as a loading control. This figure shows a representative experiment for each cell line. At least 3 experiments were done for each cell line. These samples are the same as the ones in figure 7.13.

We can conclude that the increase of p21 expression induced by UDCA is not mediated by p53.

7.4.2. Regulation of UDCA targets by c-Myc

As c-Myc suppression is sufficient to inhibit proliferation, and it is one of the earliest proteins affected after treatment, the question was posed whether it mediated the effect of UDCA on p21, cyclin A, CDK2, CDK6 and Rb proteins.

Suppression of c-Myc induced upregulation of p21 and downregulation of CDK2 and CDK6 but no change in cyclin A expression. In the case of Rb, suppression of c-Myc

induced an increase of the expression of the lower band of the protein, which represents the hypophosphorylated form (Figure 7.21).

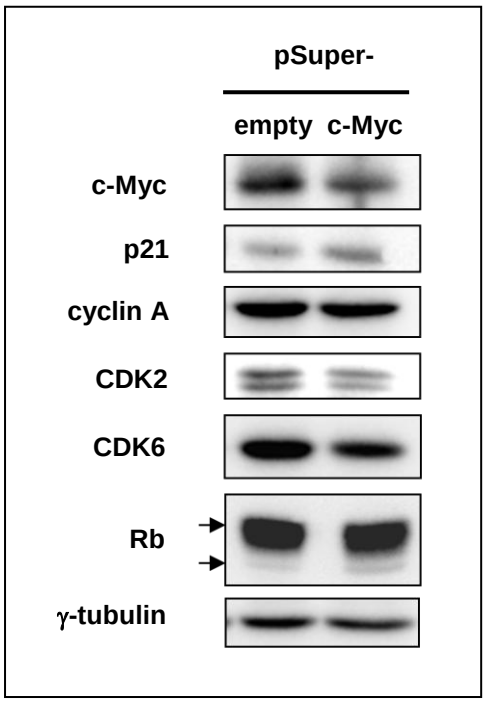

Figure 7.21. Effect of c-Myc suppression on the expression of UDCA targets. HCT8 cells were transfected with empty pSuper vector or pSuper-c-Myc. After 1 day the cells were replated and after 2 days of transfection lysed in Sato buffer. Western blot was made to determine the expression of c-Myc, p21, cyclin A, CDK2, CDK6 and Rb. γ-tubulin was taken as loading control. Blot representative for 3 experiments.

The conclusion from this experiment was that p21, CDK2 and CDK6 are downstream targets of c-Myc, suggesting that their downregulation after UDCA treatment could be a result of the suppression of c-Myc.

The effect of c-Myc overexpression on the expression of the same proteins was also investigated. After overexpression of c-Myc no change on the expression of any molecule was observed (Figure 7.22). Since this was done using transient transfection, the transfection efficiency was tested and it was found that it was too low to induce any effect on the downstream targets (less than 1 %) (data not shown).

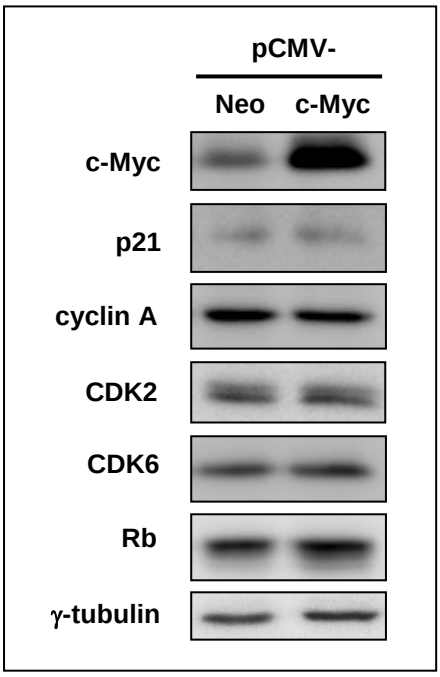

Figure 7.22. Effect of c-Myc overexpression on the expression of UDCA targets. HCT8 cells were transiently transfected with pCMV-Neo (control) or pCMV-c-Myc by using Lipofectamine 2000. After 1 day cells were replated and after 2 days of transfection lysed in Sato buffer and the expression of c-Myc, p21, cyclin A, CDK2, CDK6 and Rb was analysed by western blotting. γ-tubulin was taken as loading control. Blot representative for 3 experiments.

7.4.3. Regulation of c-Myc by UDCA

UDCA induced decrease in c-Myc expression, which was sufficient to inhibit proliferation in HCT8 cells (Figure 7.17). We asked if UDCA regulates c-Myc, whether at the protein level or at the transcriptional level.

7.4.3.1. Is c-Myc regulated by UDCA transcriptionally?

To know if the downregulation of c-Myc was at transcriptional level, the mRNA concentration was checked in HCT8 cells after UDCA treatment. Cells were treated with 400 µM UDCA for 1, 2 and 3 days and a one-step real time RT-PCR was carried out. In parallel, expression of c-Myc was checked in cell lysates by western blot. The decrease in c-Myc expression was observed at protein level but not at mRNA level (Figure 7.23).

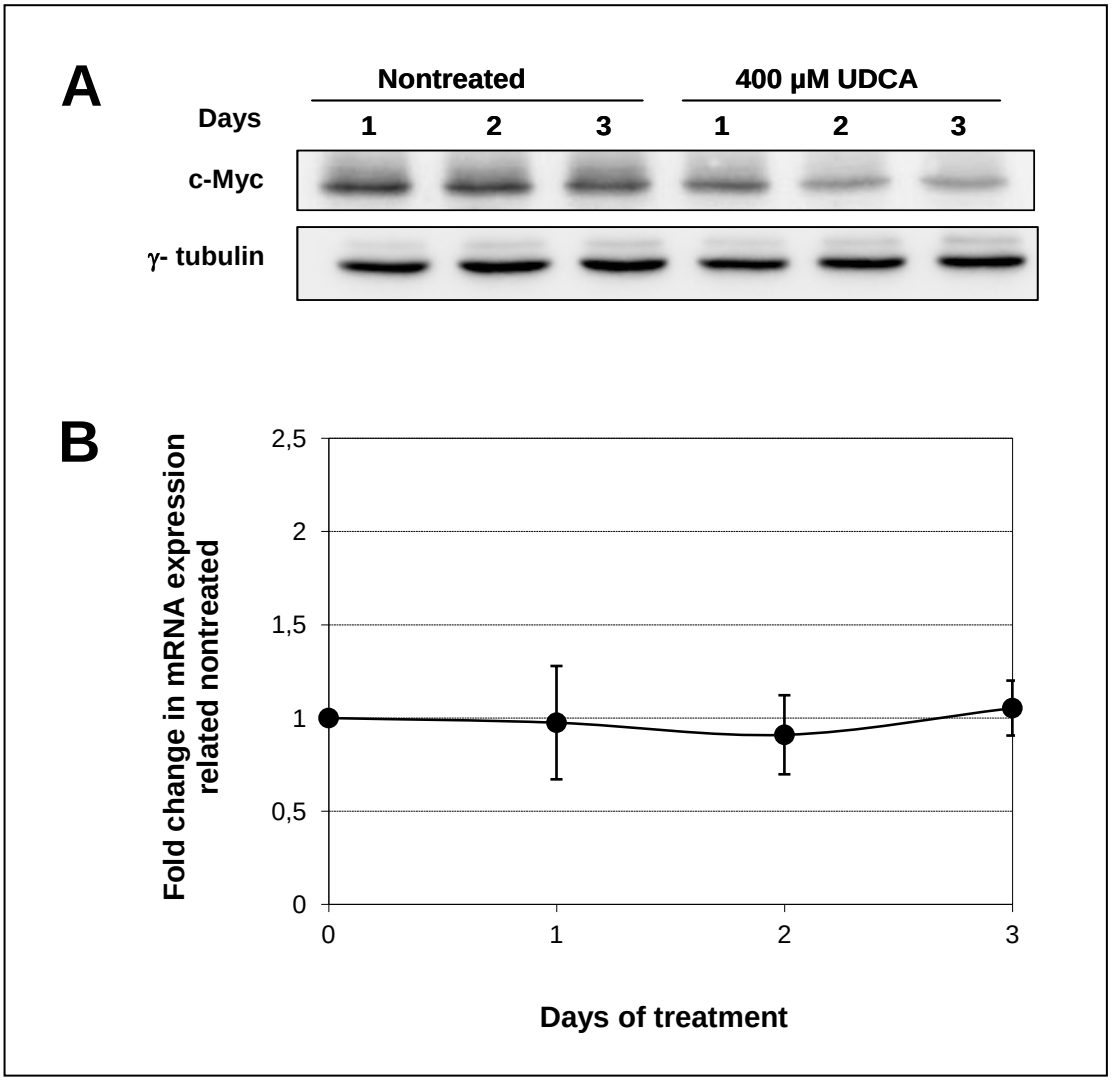

Figure 7.23. UDCA regulates c-Myc on the protein level. HCT8 cells were treated with 400 µM UDCA and harvested after 1, 2 or 3 days. A.: Protein expression was determined by western blot using γ-tubulin as loading control. Blot representative for 3 experiments. B.: mRNA was analysed by RT-PCR. The fold change in mRNA expression related to the nontreated sample for each time point is plotted. Mean ± SD of 3 experiments. Experiment done by Santosh Subramanian.

Moreover, transcriptional regulation of c-Myc by UDCA was also determined by luciferase reporter assay. HCT8 cells were transfected with a luciferase reporter containing c-Myc promoter, they were treated with UDCA and luciferase activity was measured. No decrease but a minor increase of the luciferase activity was observed after treatment with UDCA, (Figure 7.24). This increase was however not observed at mRNA level (Figure 7.23 B).

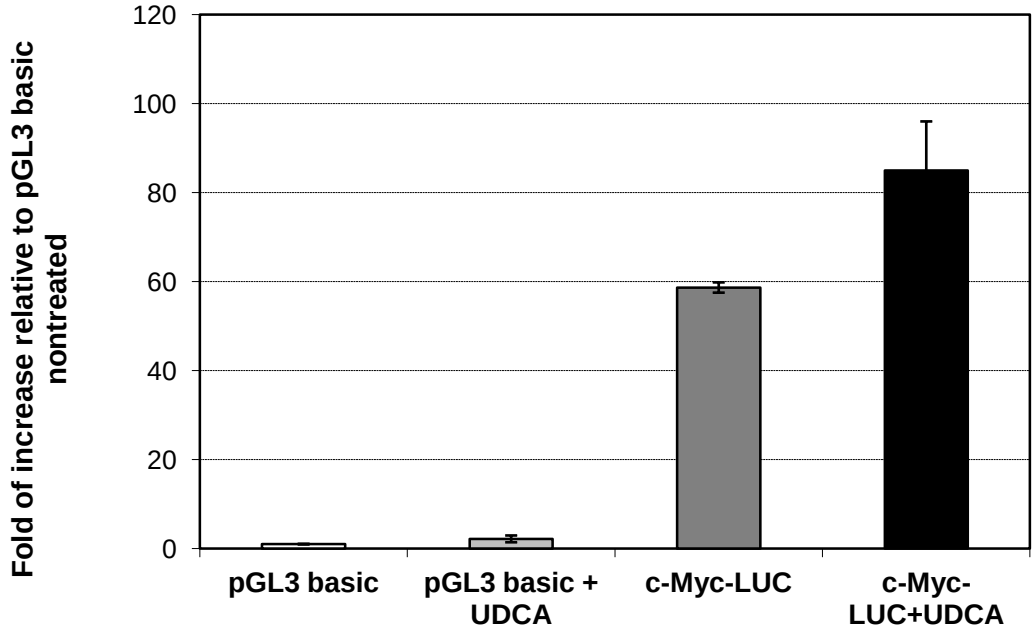

Figure 7.24. UDCA does not decrease c-Myc at transcriptional level. HCT8 cells were transfected with 500 ng of luciferase reporter plasmid (pGL3 (control) or c-Myc-Luc (CG362)) and 50 ng of β-Gal, treated with 400 µM UDCA for 2 days and luciferase activity was measured. β-Gal activity was used for normalization. Mean values ± SD of 3 experiments.

Together these results indicate that UDCA regulates c-Myc not at transcriptional but at protein level.

7.4.3.2. Is c-Myc downregulated by UDCA due to degradation?

To answer if c-Myc downregulation was potentially due to degradation of the protein, the effect of UDCA on c-Myc phosphorylation was checked, since the phosphorylation at Thr58 is related to degradation of the protein (53). HCT8 cells were treated with UDCA and phosphorylation of c-Myc was determined by western blot. It was observed that while c-Myc protein expression decreased after treatment, its specific phosphorylation increased (Figure 7.25).

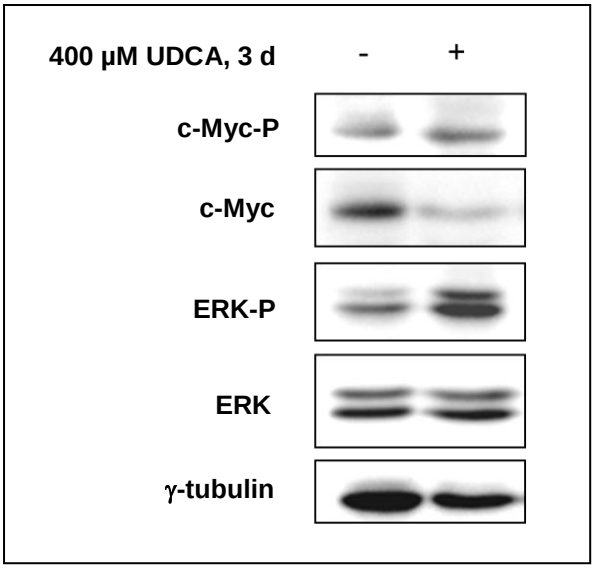

Figure 7.25. UDCA induces a higher specific phosphorylation of c-Myc and ERK proteins. HCT8 cells were treated with UDCA (400 µM, 3 days) and lysed in sample buffer. c-Myc, phospho-c-Myc (c-Myc-P), ERK and phospho-ERK (ERK-P) expression was detected by Western blot. γ-tubulin was used as loading control. This blot is representative of 3 experiments.

The effect of UDCA on ERK, a kinase that phosphorylates c-Myc at serine 62 (53), was also investigated. UDCA induced ERK phosphorylation in HCT8 cells but did not affect its expression (Figure 7.25). Increase in ERK phosphorylation was concomitant with decrease in c-Myc expression and the delay of the G1→S transition (Figure 7.26).

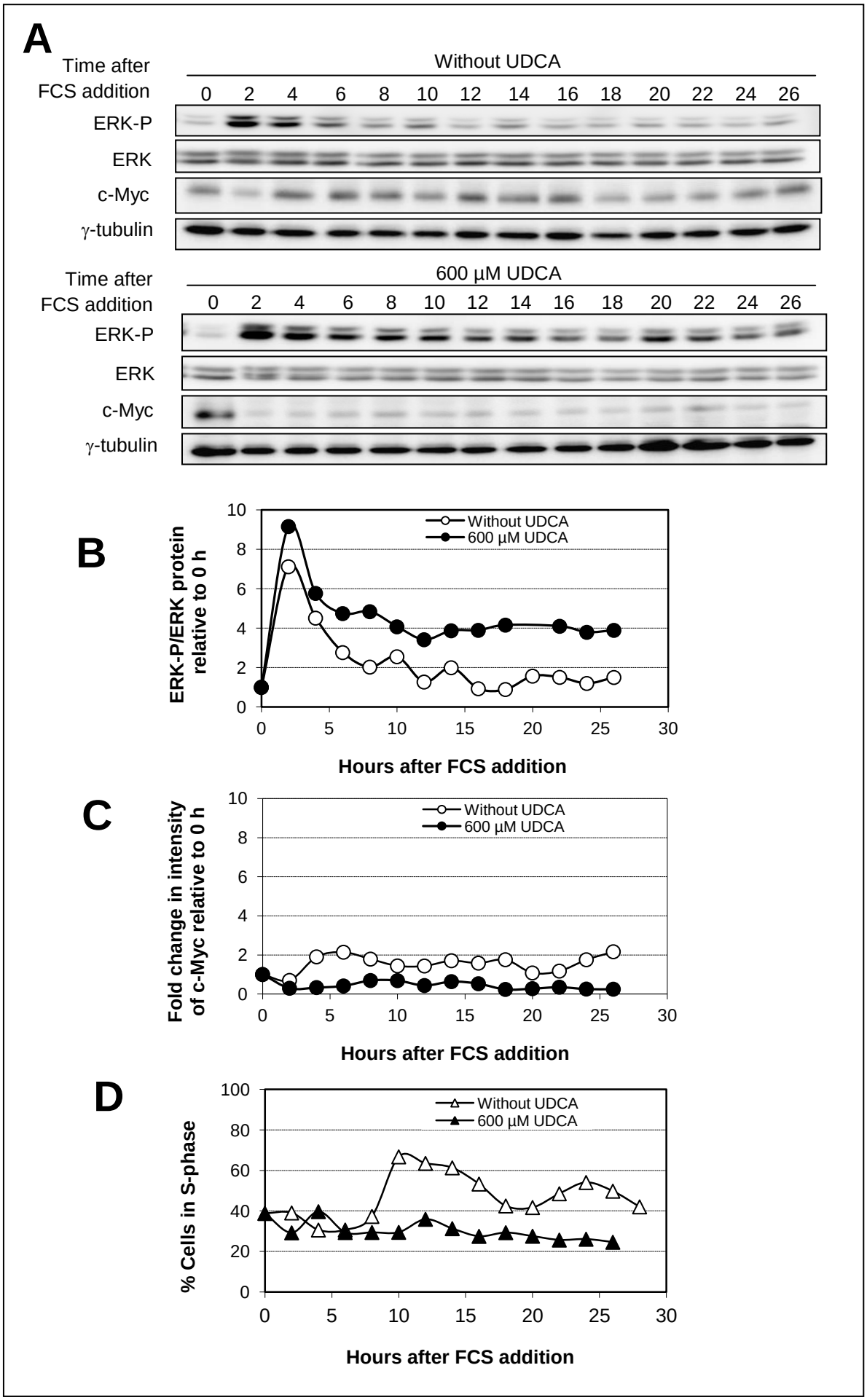
A
Time after FCS addition
Without UDCA
0 2 4 6 8 10 12 14 16 18 20 22 24 26
ERK-P
ERK
c-Myc
γ-tubulin
Time after FCS addition
600 µM UDCA
0 2 4 6 8 10 12 14 16 18 20 22 24 26
ERK-P
ERK
c-Myc
γ-tubulin
B
ERK-P/ERK protein relative to 0 h
Without UDCA
600 µM UDCA
Hours after FCS addition
C
Fold change in intensity of c-Myc relative to 0 h
Without UDCA
600 µM UDCA
Hours after FCS addition
D
% Cells in S-phase
Without UDCA
600 µM UDCA
Hours after FCS addition

Figure 7.26. Increase in ERK phosphorylation is concomitant with the decrease in c-Myc expression and the G1→S transition delay. HCT8 cells were starved for 24h and then serum was added in the presence or absence of UDCA (600 µM). Cell lysates were prepared in sample buffer every 2 hours. A.: Expression of c-Myc, ERK and phospho-ERK (ERK-P) was analysed by western blot. B.: Intensity of ERK-P expression relative to the total protein. C.: Intensity of c-Myc expression relative to 0 h. D.: Cell cycle was analysed by FACS and the percentage of cells in the S-phase was plotted. Experiment done by Santosh Krishna Subramanian and Marie-Luise Hanski.

The conclusion from these results is that c-Myc is downregulated at protein level by degradation. The decrease in c-Myc protein amount is concomitant with and increased phosphorylation of c-Myc and an increase in ERK phosphorylation.

This allows the hypothesis that activated ERK does not stabilize c-Myc in HCT8 cells.

7.5. Investigation of the abrogation of the inhibition of proliferation by UDCA after c-Myc overexpression

The question was posed if c-Myc overexpression would abrogate the effect of UDCA on proliferation. As transient transfection had low efficiency (data not shown), we tried to make stable transfectants for c-Myc overexpression in HCT8 cells, by selection with G418. Unfortunately no clone with increased levels of c-Myc expression was obtained.

In another experiment HA-tagged-c-Myc plasmid was transfected in HCT8 cells and the effect of UDCA on proliferation was determined by inmunohistochemistry. Incorporation of EDU was used as measure of proliferation (see Materials and Methods point 6.2.1.10). Two experiments were done and in both no cells overexpressing c-Myc and incorporating EDU at the same time were found (Figure 7.27).

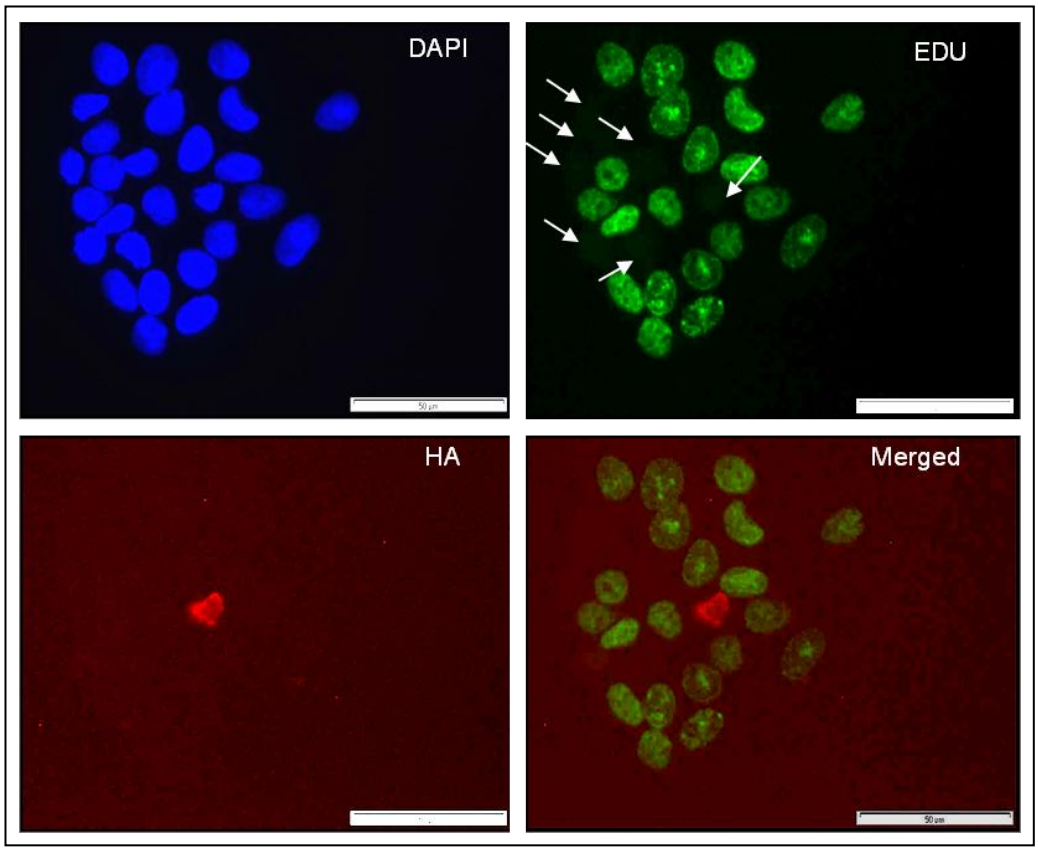

Figure 7.27. c-Myc overexpressing cells are not proliferating. HCT8 cells were transfected with a plasmid expressing c-Myc. Cells were replated after 1 day and treated with UDCA (400 µM) for 1 day. c-Myc expressing cells (red) and cells incorporating EDU (green) were detected by immunohistochemistry. Nuclei were stained with DAPI (blue) (Bars = 50 µm).

The hypothesis derived from this experiment is that c-Myc overexpression prevents cells from growing, which could explain why no stable clone for c-Myc overexpression was obtained.

7.6. Effect of w*nt* pathway defect on the antiproliferatory action of UDCA

Since the *wnt* pathway is often affected in colorectal cancer, and c-Myc is a downstream target of it, the question was posed whether UDCA treatment would affect the proliferation of cells with deficient *wnt* pathway differently as in cells with normal w*nt* status. An isogenic pair of cell lines was used in which one of them had normal *wnt* status (Rat2-wnt1) and the other was deficient for *wnt* (Rat2-mv7). It was found that UDCA inhibited

the proliferation of both cell lines to the same extent, which indicates that the inhibition is independent from the *wnt* status (Figure 7.28).

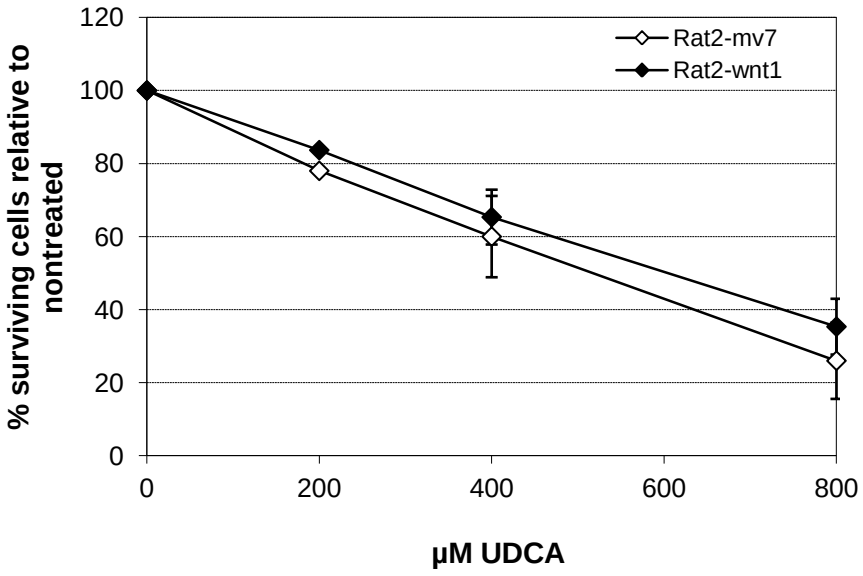

Figure 7.28. UDCA inhibits proliferation independently from the *wnt* pathway**.** An isogenic pair of *wnt* deficient and proficient cells, Rat2-mv7 and Rat2-wnt1 respectively, were treated for 3 days with 200 or 400 µM UDCA and proliferation was analysed by MTT test. Mean values ± SD of 3 experiments.

8. DISCUSSION

Until now the treatment existing for prevention of colon cancer has many side effects and is not always effective (100-102). UDCA has practically no side effects (103, 104) and it has been found that it reduces the risk of cancer development in patients who suffer from both primary sclerosing cholangitis and ulcerative colitis (78, 79). We investigated the mechanisms of antiproliferative action of UDCA in colon cancer cells.

8.1. UDCA inhibits proliferation of $p53^{wt}$ colon cancer cells

The cell lines used to investigate the antiproliferative mechanism of UDCA were $p53^{wt}$, to avoid conditioning the prevention to the mutation of this gene, which often occurs in a late stage of tumor development (3), and we found that UDCA treatment inhibited the proliferation of all of them (Figure 7.1), also detected by inhibition of BrdU incorporation (Figure 7.2),

8.2. UDCA-induced inhibition of proliferation does not correlate with speed of growth

The effectiveness of UDCA treatment was not dependent on the speed of growth of the different colon cancer cell lines investigated (Table 7.1). A correlation would have been interesting as UDCA would rather target cancer cells than normal cells, as it is known that transformed cells proliferate faster than normal ones (105).

8.3. UDCA-induced inhibition of proliferation is not due to apoptosis

UDCA treatment induced no apoptosis in our system (Figure 7.3) as reported previously (81, 106, 107). However, some studies show that UDCA can induce apoptosis in other cell systems such as HT-29 cells or hepatocytes (108, 109).

8.4. UDCA induces senescence in LS513 cells

Senescence was induced after 3 days in LS513 cells and slightly after 6 days in HCT116 cells (Figures 7.4 and 7.5). Induction of cellular senescence, which is another way of stopping cell proliferation (110), has previously been described to be induced by UDCA in HCT116 cells, in higher concentrations (500 µM UDCA) (111). Since in our experiment HCT116 cells showed no senescence after 3 days, there must have been another reason for the inhibition of proliferation at this time point. In HCT8 and Co115 cells senescence was not induced.

8.5. UDCA decreased S-phase population in all cell lines investigated, slowed down the cell cycle and induced G1 arrest in HCT116 and HCT8 cells

UDCA treatment decreased in all cell lines the S-phase population (Figure 7.6). In addition, HCT8 and HCT116 cells showed an increase in G1-phase population, and in LS513 a higher percentage of cells in G2/M phase was observed (Table 7.2).

In HCT116 cells UDCA delayed the G1→S transition and slowed down the cell cycle. This was also observed in HCT8 cells. The delay was longer in HCT8 cells and the inhibition of proliferation was also higher than in HCT116 cells, suggesting that cells had impaired proliferation due to the G1→S transition delay.

In LS513 cells there was a slight increase in G2/M phase population after treatment, and in the treated cells the percentage of BrdU incorporation was less than the percentage of cells in S-phase indicating that some cells in S-phase were not proliferating, i.e. they were in S-phase arrest. It has been reported that S-phase arrest is concomitant with p21 expression, ERK activation (112) and cyclin A downregulation (113) in cancer cells.

Co115 cells showed practically no change in the cell cycle except for the S-phase decrease.

8.6. Cell cycle regulating proteins c-Myc, p21, cyclin A, CDK2, CDK6 and Rb were affected by UDCA

UDCA induced c-Myc, cyclin A, CDK2, CDK6 and Rb expression decrease, p21 upregulation and Rb hypophosphorylation, suggesting that they could play a role in the inhibition of proliferation.

8.6.1. c-Myc was downregulated by UDCA which contributed to inhibition of proliferation

We found that c-Myc suppression was sufficient to inhibit proliferation in HCT8 cells (Figure 7.17), which demonstrates that the downregulation of c-Myc contributes to the antiproliferatory effect of UDCA. It is known that c-Myc induces proliferation (114-116) by regulation of several pathways, including the activation of cyclin E/CDK2 and E2F-1 and progression into S-phase (54, 117). It has also been reported that inactivation of c-Myc can induce cellular senescence in primary tumors (71). This suggests that downregulation of c-Myc by UDCA could mediate the decrease in S-phase population observed after treatment, and the induction of senescence in LS513 cells.

8.6.2. Expression of the CDK-activator cyclin A was decreased after UDCA treatment

Cyclin A is one of the central proteins regulating cell cycle progression. It activates CDK2 kinase to promote progression into S-phase (118) and in G2 phase it activates CDK1 to induce entry into mitosis (27, 28). Interestingly, cyclin A is necessary for c-Myc-induced cell cycle progression (119). Downregulation of cyclin A or inhibition of its synthesis could therefore result in an inhibition of DNA synthesis, less entry into mitosis, and inhibition of proliferation (120, 121).

8.6.3. CDK2 and CDK6 kinases were downregulated by UDCA

CDK2, which is activated by cyclin E and cyclin A, is involved in S-phase entry by regulating many processes including DNA replication, centrosome duplication, and

elimination of the CDK inhibitor p27 (21, 28, 122, 123). CDK6 is activated in early G1 phase by cyclin D1, and phosphorylates Rb to activate E2F-1 and induce transcription of pro-proliferative proteins, like cyclin E (124, 125). Downregulation of these kinases would therefore affect the cell cycle progression and inhibit proliferation.

8.6.4. UDCA upregulated the CDK inhibitor p21

p21 induces cell cycle arrest, in G1 phase and G2, by inhibiting cyclin/CDK complexes (30, 126-128). It also affects DNA replication by inhibiting PCNA binding to chromatine (126, 129). In addition, p21 is necessary for the assembly of cyclin D1/cdk4 and cyclin D1/CDK6 complexes, promoting cell G1-phase progression, although when it is overexpressed to high levels, it blocks proliferation by inhibition of cyclin/CDK complexes (35). Thus, p21 upregulation by UDCA could contribute to inhibition of proliferation.

8.6.5. Retinoblastoma protein was activated by UDCA

Rb is less phosphorylated after UDCA treatment. Hypophosphorylated form of Rb is active and inhibitory for E2F transcription factors, and therefore for cell cycle progression and proliferation (130).

8.7. The effect on CDK4, p53, p27, cyclin E and cyclin D1 expression is not the main cause for the antiproliferative effect of UDCA

CDK4 (as well as CDK6) is known to be activated by cyclin D1 and to phosphorylate Rb in early G1, promoting the release of E2F-transcription factors and progression through G1-phase (131). In our system, only in HCT8 and LS513 this kinase was downregulated, which can contribute to the inhibition of proliferation in these two cell lines, whereas in Co115 and HCT116 cells it was not changed. Since this downregulation is not common to all cell lines, the molecule cannot be considered as a general target of UDCA in colon cancer cells. Also p53, p27, cyclin E and cyclin D1 were affected only in some cell lines. p53 overexpression was observed in HCT8 and HCT116 cells, and p27 overexpression was induced in Co115 cells after treatment, which could contribute to the inhibition of

proliferation by UDCA, since these two proteins are known to inhibit cell cycle progression (132-134). This also indicates that UDCA can induce p21 expression in a p53-independent manner. In conclusion, UDCA can inhibit proliferation of colon cancer cells without affecting the expression of CDK4, p53, p27, cyclin E and cylin D1. This does not indicate that these targets cannot contribute to this effect, as the expression some of them were affected by UDCA in some cell lines, and the activity of the molecules after the treatment was not investigated.

8.8. UDCA inhibits E2F-1 transcriptional activity

Transcriptional activity of E2F-1 was inhibited by UDCA (Figure 7.18). Rb in its hypophosphorylated form inhibits E2F-1 by binding to it (95, 135). Rb is phosphorylated by cyclin D/CDK4 and cyclin D/CDK6 complexes in early G1 phase and by cyclin E/CDK2 in late G1, to promote G1→S transition, and by cyclin A/CDK2 in S-phase, to promote progression through this phase, by release of the transcription factor E2F-1 (42, 43, 45). E2F-1 induces the transcription of G1→S-phase genes, such as cyclin A (25). The downregulation of cyclin A observed after treatment could therefore be mediated by the inhibition of E2F-1 activity. This effect would lead to inhibition of the G1→S transition (Figure 8.1), and could contribute to the inhibition of proliferation.

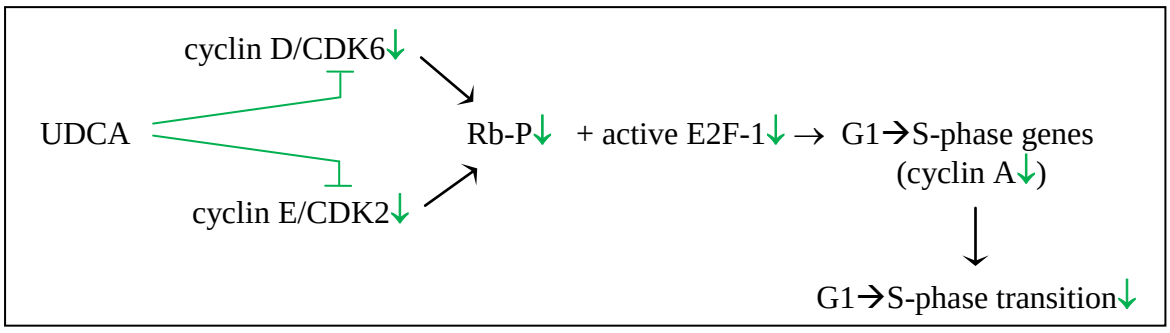

Figure 8.1. Inhibition of G1→S transition by UDCA could be due to inhibition of cyclin/CDK complexes by downregulation of CDK2 and CDK6 expression, which induce Rb-phosphorylation (Rb-P) and release of active E2F-1 (95, 135). E2F-1 decrease in activity would result in less transcription of G1→S-phase genes such as cyclin A (25). Green: Present observation. Black: Connections reported by others.

8.9. The G1→S transition delay is mediated by UDCA effect on c-Myc, p21, Rb and cyclin A

UDCA inhibited the upregulation of c-Myc after serum stimulation in quiescent HCT8 cells (Figure 7.16), which could be the reason for the delay of the G1→S transition as it is known that entrance into cell cycle from quiescence is triggered by c-Myc, whose expression is very low when the cells are in the G0 phase and is induced after growth factor stimulation to promote cell cycle entry (47, 59, 136).

It was observed that p21 was upregulated as cells entered cell cycle and in the G1→S transition it was repressed (Figure 7.16). p21 activates cyclin D/CDK complexes in early G1 to induce G1 cell cycle phase progression (137), and that repression of p21 in late G1 is mediated by c-Myc (138), which is known to induce transcriptionally the p21 repressor gene *AP-4* (59). In UDCA-treated HCT8 cells, the decrease in p21 expression occurred at a later time point, most probably due to the decrease in c-Myc level.

Rb hyperphosphorylation after serum stimulation, which is necessary for cells to progress through G1-phase and for the entry into S-phase, was delayed by UDCA (Figure 7.16). Phosphorylation of Rb is mediated by cyclin/CDK complexes, whose activity is inhibited by p21 (135), which leads to E2F release, induction of cyclin A expression and entry into S-phase. It has been shown that cyclin A plays a role in G1→S transition (118). In nontreated cells, cyclin A was induced at before S-phase entry. This was not observed within 12 hours in UDCA-treated cells, which did not enter S-phase (Figure 7.16).

Taken together these results suggest that UDCA delays G1→S transition by downregulation of c-Myc, which results in less p21 repression in late G1, leading to inhibition of Rb phosphorylation and less cyclin A expression (Figure 8.2).

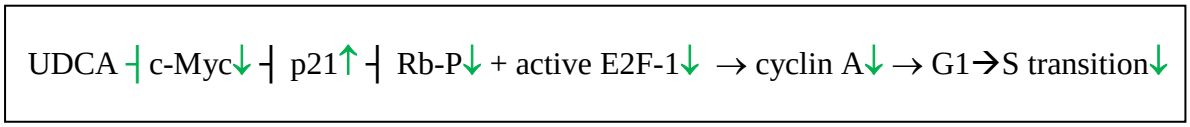

Figure 8.2. c-Myc, p21, Rb and cyclin A play a role in G1→S transition delay by UDCA. UDCA decreases c-Myc, which represses p21, which inhibits Rb phosphorylation and E2F-1 activation, resulting in less cyclin A expression and inhibition of G1→S transition. Green: Present observation. Black: Connections reported by others.

8.10. PCNA binding to chromatin is inhibited by UDCA

PCNA binding to chromatin was inhibited by UDCA (Figure 7.19), which can result in inhibition of DNA replication. p21 has been demonstrated to bind to PCNA, preventing its binding to DNA at the G1→S transition checkpoint (32, 126, 129). PCNA p21 binding site overlaps with the DNA polymerase δ- and replication factor C- interaction sites, so that the DNA replication complex cannot be formed (139). Since p21 was overexpressed after UDCA treatment, it is probable that it mediated the UDCA-induced inhibition of PCNA binding to chromatin and S-phase progression (Figure 8.3).

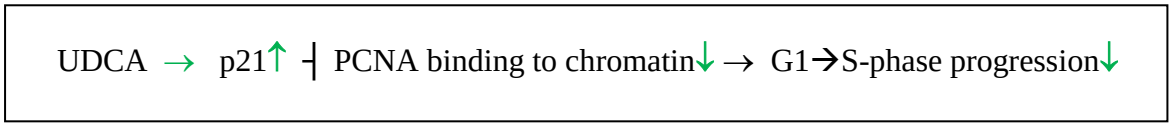

Figure 8.3. PCNA binding to chromatin inhibition by UDCA could be induced by the upregulation of p21 we observed after treatment, and this would lead to inhibition of G1→S-phase progression. Green: Present observation. Black: Connections reported by others.

8.11. c-Myc suppression is sufficient to induce p21 upregulation, CDK2 and CDK6 downregulation and Rb dephosphorylation

In the present work, the suppression of c-Myc by siRNA induced not only decrease of proliferation but also affected some of the UDCA targets (Figure 7.21). p21 was upregulated, as reported previously (59, 138) and CDK2 and CDK6 were downregulated after c-Myc suppression. It has been shown that c-Myc induces transcription of CDK2 (119), but until now nothing has been reported about c-Myc regulation of CDK6. We suggest that the effect on expression observed after UDCA treatment of these proteins is mediated by the suppression on c-Myc. Rb was slightly less phosphorylated after c-Myc suppression, which is expected as p21 inhibits and the kinases induce Rb phosphorylation (Figure 8.4).

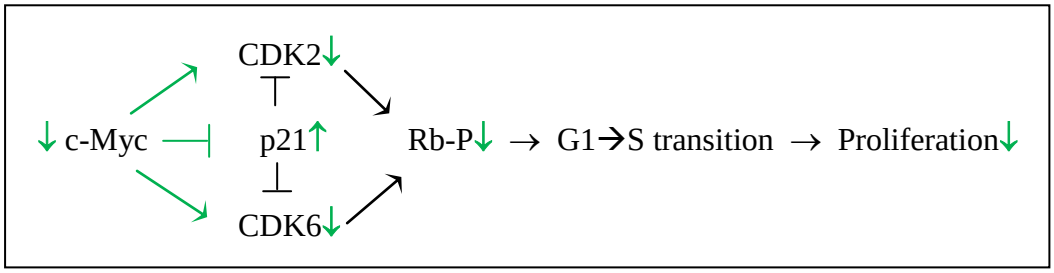

Figure 8.4. c-Myc suppression leads to inhibition of proliferation, CDK2 and CDK6 downregulation, Rb hypophosphorylation. Green: Present observation. Black: Connections reported by others.

8.12. c-Myc is suppressed at protein level. The suppression is concomitant with persistent ERK phosphorylation

Decrease of c-Myc expression was observed at protein level after UDCA treatment in HCT8 cells, but there were no indications of suppression at transcriptional level, since mRNA level was not affected and there was no decrease in the luciferase activity of the c-Myc promoter (Figures 7.23 and 7.24). In addition, UDCA induced c-Myc phosphorylation at Thr58. It has already been shown that c-Myc is phosphorylated in the first place by ERK kinase at serine 62 and subsequently by GSK3β at threonine 58, after which the protein is degraded on the proteasome pathway (53, 140). In addition, we found that UDCA induced persistent ERK phosphorylation which was concomitant with the decrease of c-Myc expression (Figure 7.26). Interestingly, persistent ERK phosphorylation has been shown to induce cell cycle arrest, which would suggest that UDCA-induced ERK phsophorylation could contribute to the inhibition of proliferation. Since c-Myc phosphorylation at Thr58 can only take place once it is phosphorylated at Ser62, its degradation might be induced by persistent ERK phosphorylation induced by UDCA (Figure 8.5).

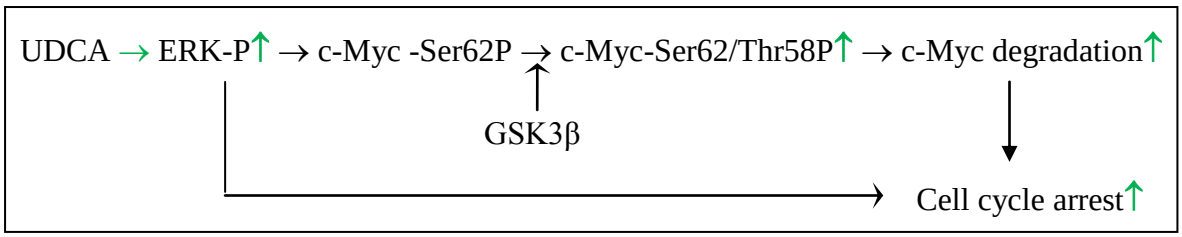

Figure 8.5. UDCA could induce degradation of c-Myc via and induction of its phosphorylation, and it could be mediated by persistent ERK phosphorylation, which leads to cell cycle arrest. Green: Present observation. Black: Connections reported by others.

8.13. c-Myc overexpression could induce cell death

Cells overexpressing c-Myc were not proliferating (Figure 7.27). It has already been reported that c-Myc induces apoptosis when overexpressed (47, 56, 141), therefore it is possible that in HCT8 cells, c-Myc overexpression also induced cell death. Because of this, it was not possible to determine whether there would be an abrogation of the effect of UDCA after c-Myc overexpression. This could have confirmed that the downregulation of the protein expression is essential for the inhibition of proliferation.

8.14. The *wnt* pathway defficiency does not affect the inhibition of proliferation by UDCA

The present results indicate that *wnt* status does not affect the inhibition of proliferation by UDCA. c-Myc is known to be transcriptionally regulated by the w*nt* pathway (49), and no transcriptional regulation of it was observed after treatment. Interestingly this suggests that treatment with UDCA would also be effective in those patients with mutation in the *wnt* pathway genes, such as APC, which occurs frequently in colon cancer (142). This result indicates that UDCA acts upon c-Myc downstream of the *wnt* pathway.

8.15. The proposed mechanisms for the inhibition of proliferation by UDCA

Taking together the present findings and the available background information, I propose the following mechanisms for the inhibition of proliferation of colon cancer cells by UDCA:

UDCA induces ERK phosphorylation. ERK phosphorylates c-Myc at serine 62. Then c-Myc is phosphorylated at threonine 58 and degraded. c-Myc suppression would induce accumulation of p21, decrease of CDK2 and CDK6, and this would result in less Rb phosphorylation and inhibition of E2F-1 activity. Cyclin A expression would be then decreased as it is transcriptionally regulated by E2F-1. Moreover, p21 accumulation would lead to inhibition of PCNA binding to chromatin. All these changes result in slowing down of the cell cycle, by delay of the G1/S cell cycle transition, S-phase progression and entry into mitosis, and inhibition of proliferation (Figure 8.6). This mechanism is proposed for HCT8 and HCT116 cells, since they are inducing G1 arrest and it has been demonstrated that the G1→S transition is delayed. In addition, none of the targets shown to be affected by UDCA play a role in the G2/M→G1 transition which was not affected by UDCA (Figure 7.12). It is possible that in LS513 and Co115 cells the mechanism was different, as they did not show G1 arrest, although the molecular targets were the same as in HCT8 and HCT116 cells. In the case of LS513, there was an increase in G2/M phase, which could be caused due to the suppression of cyclin A expression, which is necessary for the entrance into mitosis, or even by the expression of p21, as by blocking CDK2, it induces accumulation of inactive cyclin B/CDK1, which are essential for initiantion of mitosis (143). UDCA also induced an S-phase arrest in LS5513. p21 overexpression and decrease of cyclin A expression could play a role in this effect (112, 113). In this cell line there was also induction of senescence after treatment, possibly induced by the decrease in c-Myc expression (71). In Co115 cells, no effect on the cell cycle was observed, except for S-phase population decrease. This suggests that UDCA may have more than one antiproliferative mechanism in colon cancer cells, which could be also regulated by c-Myc.

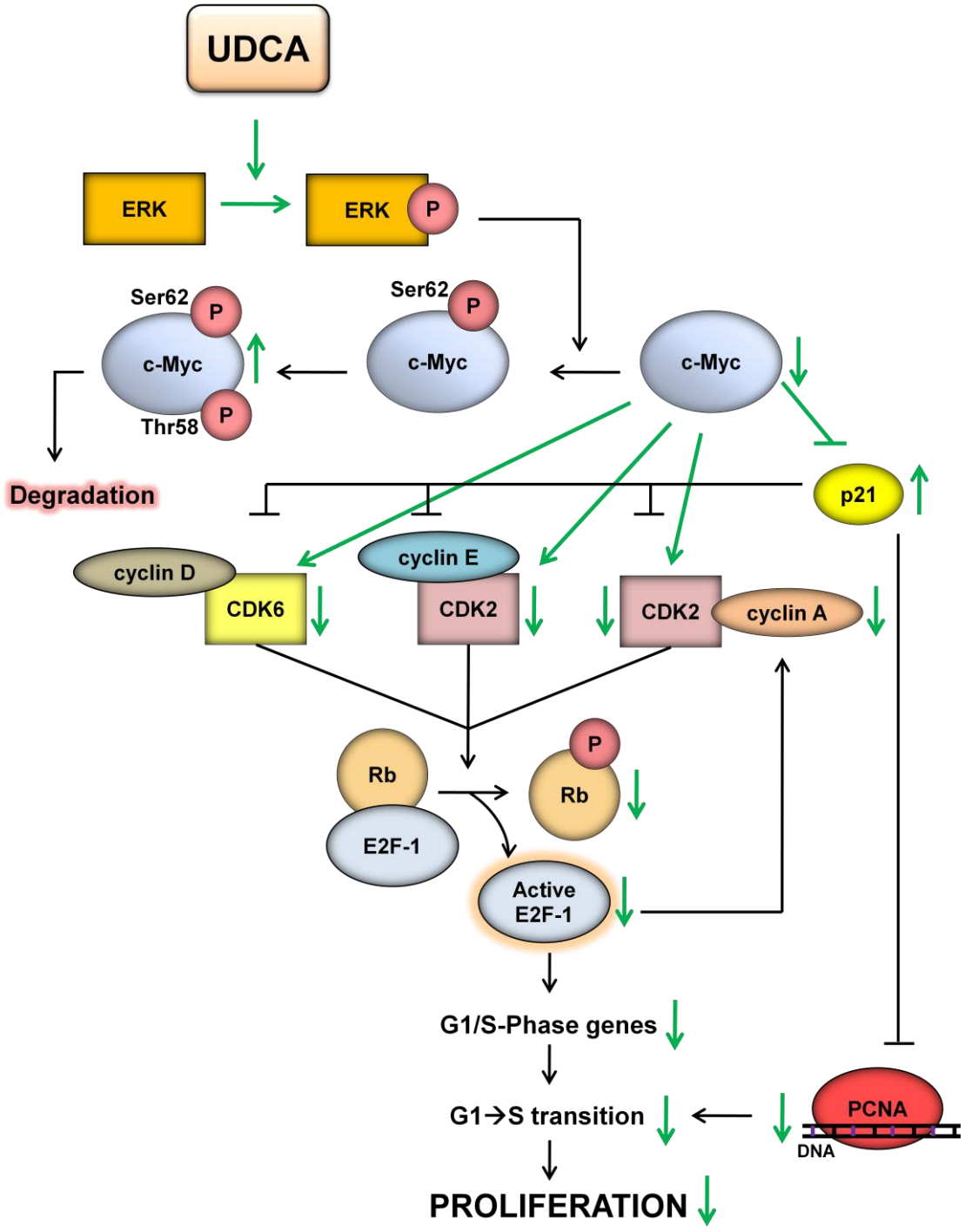

Figure 8.6. Proposed mechanism of inhibition of proliferation by UDCA in HCT8 and HCT116 cells. Green: Present observation. Black: Connections reported by others.

9. CONCLUSIONS

The following conclusions can be drawn from this work:

1. UDCA inhibits proliferation of $p53^{wt}$ colon cancer cells by different mechanisms, independently from the speed of growth.

2. The antiproliferatory effect of UDCA in HCT8, LS513, Co115 and HCT116 cells is not due to induction of apoptosis.

3. In HCT8 and HCT116 cells UDCA induces G1 arrest and a delay of the G1→S transition.

4. In LS513 cells UDCA induces senescence, S-phase arrest and a slight increase of G2/M-phase population.

5. In Co115 cells decrease in S-phase population is induced by UDCA.

6. UDCA treatment induces downregulation of c-Myc, cyclin A, CDK2 and CDK6 proteins, upregulation of p21 and decrease in Rb phosphorylation and expression.

7. E2F-1 transcriptional activity and PCNA binding to chromatin is inhibited in HCT8 cells after UDCA treatment.

8. Suppression of c-Myc, p21 upregulation, Rb hypophosphorylation, and cyclin A downregulation observed after treatment in all cell lines occurs during the G1→S transition in HCT8 cells and could play a role in the delay.

9. c-Myc suppression occurs not at transcriptional level, and it is concomitant with phosphorylation at threonine 58 and an increase in ERK phosphorylation after UDCA treatment.

10. p21 upregulation in HCT8 and HCT116 cells and is independent from p53.

11. c-Myc suppression in HCT8 cells contributes to the inhibition of proliferation and the p21 upregulation, CDK2 and CDK6 downregulation and Rb hypophosphorylation.

12. c-Myc overexpression can inhibit proliferation.

13. The wnt pathway status does not play a role in the inhibition of proliferation by UDCA.

10. FUTURE PERSPECTIVES

In this work an antiproliferatory mechanism of UDCA has been proposed. Since UDCA has practically no side effects and it is very well tolerated by patients even at high doses (73), by contrast to other chemopreventive agents (101), treatment with UDCA could be an effective therapy. The proposed antiproliferative mechanism of UDCA allows to evaluate the possibility of substituting other drugs with UDCA. The comparison of their mechanism of action to that of UDCA allows to determine possible similarities and to predict a possible similar action or even a higher effectiveness of the treatment.

Patients with chronic ulcerative colitis are regularly treated with 5-ASA. Therefore, it would be interesting to test the effect of treatment with both UDCA and 5-ASA against proliferation, initially *in vitro* and afterwards *in vivo*, to see if this would improve the antiproliferatory effect. In the case that it would be improved, the next question which could be asked, is whether the dose of 5-ASA could be reduced, in order to minimize its side effects, and which would be the optimal concentration of both chemopreventive agents. Interestingly, Chu et al. reported that Mesalazine (5-ASA) downregulates c-Myc in human colon cancer cells (88, 144), which allows to think of the hypothesis that the antiproliferative effect of both drugs could be though regulation of the same target (c-Myc).

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12. PUBLICATIONS AND PRESENTATIONS

1. **R. Peiró-Jordán**, S. Krishna-Subramanian, M.L. Hanski, M. Zeitz, C. Hanski. *The chemopreventive agent UDCA inhibits proliferation of colon carcinoma cells by suppressing c-Myc expression.* – Eur. Journal of Cancer Prevention. Manuscript accepted.

2. **R. Peiró-Jordán**, S. Krishna-Subramanian, M.L. Hanski, M. Zeitz, C. Hanski. *UDCA-mediated inhibition of proliferation in colon cancer cells is associated with c-Myc suppression.* 29th German Cancer Congress (Berlin, 2010) – Poster

3. Bhonde MR, Hanski ML, Stehr J, Jebautzke B, **Peiró-Jordán R**, Fechner H, Yokoyama KK, Lin WC, Zeitz M, Hanski C. *Mismatch repair system decreases cell survival by stabilizing the tetraploid G1 arrest in response to SN-38.* Int J Cancer. 2009 Sep 8.

4. **R. Peiró-Jordán**, S. Krishna-Subramanian, M.L. Hanski, J. Behrens, M. Zeitz, C. Hanski. *UDCA targets S-phase and suppresses CDK2 and cyclin A expression: effects on colon cancer cell proliferation.* DGVS-Visceral Medicine Congress (Berlin-2008) – Poster.

5. **R. Peiró-Jordán**, S. Krishna-Subramanian, M.L. Hanski, J. Behrens, M. Zeitz, C. Hanski. *Proliferation inhibition of colon cancer cells by UDCA: cellular response mechanisms.* 28th German Cancer Congress (Berlin, 2008) – Poster.

6. **R. Peiró-Jordán**, S. Krishna-Subramanian, M.L. Hanski, J. Behrens, M. Zeitz, C. Hanski. *Effects of ursodeoxycholic acid treatment on colon cancer cell proliferation.* Falk Workshop II Falk Gastro-Conference, Part I: Mechanisms of Intestinal Inflammation (Dresden, 2007) – Poster.

13. CURRICULUM VITAE

For reasons of data protection, the Curriculum Vitae is not included in the online version.