mTOR Inhibition Impairs Proliferation of Hepatocytes with DNA Damage during Chronic Liver Injury thereby Delaying Liver Tumor Development

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Declaration: Here with I declare that the study has been done by my own under the guidance of Priv.-Doz. Dr. med. Arndt Vogel and all the information provided is novel and true and has not been submitted to any other Institute or University to obtain any other degree.

Laura Elisa Buitrago Molina

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To my beloved parents Gloria and Luis,
my brother Joseto and my sister Ana I.

With Love

Summary

Hepatocellular carcinoma (HCC) is the one of the most prevalent and lethal cancer worldwide. HCC is a difficult-to-treat tumor as suggested by the mortality figures which strictly coincide with incidence rates. Mortality rates for HCC can reasonably be reduced by early detection or prevention. However, given the poor performance of existing therapies, strategies to prevent or delay tumor development in liver are urgently needed. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell proliferation and survival integrating mitogenic and nutrient signaling. HCC is a complex and heterogeneous tumor with several genomic alterations. There is evidence of aberrant activation of several signaling cascades such as mTOR signaling and p53/p21 pathway. Therefore, the aim of this study was to determine the interaction between mTOR and p53/p21 signaling pathways in HCC and to find a new treatment to prevent this devastating disease. For this purpose, an analogue of rapamycin, RAD001 was used. This mTOR inhibitor was given to Fah- and Fah/p53- and Fah/p21-deficient mice. The results from this study show that RAD001 treatment did not reduce liver injury produced by FAA accumulation and did not affect baseline hepatocellular proliferation in healthy mice. Interestingly however, RAD001 efficiently inhibited proliferation of damaged hepatocytes and sustained their apoptosis sensitivity during chronic liver injury. Additionally, RAD001 treatment significantly lowered p53 and/or p21 levels in Fah- and Fah/p21-deficient mice indicating that activation of mTOR contributes to p53 accumulation in hepatocytes during chronic liver injury. Furthermore, the ability of RAD001 to inhibit cell cycle was markedly attenuated in Fah/p53-deficient mice suggesting that p53 pathway is required to suppress proliferation of hepatocytes with DNA damage. Mechanistically, RAD001 influenced the expression of several cell cycle related proteins and the building of CDK-cyclin complexes involved in the transition from G0 to G1 phase of cell cycle. Finally, long-term treatment with RAD001 noticeably delayed tumor development in liver.

Keywords: Liver, Cancer, mTOR

Zusammenfassung

Hepatocellular Carcinoma (HCC) ist die weltweit am häufigsten auftretende und auch tödlichste Krebsform. HCC ist ein schwer zu behandelnder Tumor, was durch die Sterblichkeitsrate angedeutet wird, welche absolut mit der Rate der Neuerkrankungen korreliert. Die Sterblichkeitsrate durch HCC kann durch Früherkennung oder Prävention deutlich reduziert werden. Bedingt durch die schwache Wirkung bestehender Therapien, sind Strategien zur Vorbeugung oder Verzögerung der Tumorentwicklung dringend notwendig. Das Angriffsziel für Rapamycin (mTOR) ist bei Säugern eine Serin/Threonin Kinase, welche die Zell Proliferation und Überlebensintegrierende mitogene und nährende Signalisierung reguliert. HCC ist ein komplexer und heterogener Tumor mit verschiedenen genomischen Veränderungen. Hinweise deuten auf eine abweichende Aktivierung verschiedener Signalkaskaden hin, wie z.B. die mTOR Signalisierung und der p53/p21 Pfad. Das Ziel dieser Arbeit war es deshalb, die Interaktion zwischen mTOR und p53/p21 signalisierenden Pfaden in HCC zu bestimmen und eine neue Behandlung zur Vorbeugung dieser verheerenden Krankheit zu finden. Zu diesem Zweck wurde ein Analog von Rapamycin, RAD001, verwendet. Dieser mTOR Inhibitor wurde Fah-, Fah/p53- und Fah/p21- defizienten Mäusen verabreicht. Die Ergebnisse dieser Studie zeigen, dass die Behandlung mit RAD001, die durch FAA Anreicherung ausgelösten Leberschäden nicht reduziert. Jedoch hat RAD001 die basale hepato-zelluläre Proliferation in gesunden Mäusen nicht beeinflusst. Interessanterweise inhibierte RAD001 effizient die Proliferation geschädigter Hepatocyten und anhaltend deren Sensitivität gegenüber Apoptose während chronischer Leberschädigung. Zusätzlich verringert RAD001 signifikant die Levels von p53 und/oder p21 in Fah- defizienten Mäusen. Diese Tatsache weist darauf hin, dass die Aktivierung von mTOR zur p53 Akkumulation in Hepatocyten während chronischer Leberschädigung beiträgt. Weiterhin wurde die Fähigkeit von RAD001, den Zellzyklus zu inhibieren, in Fah/p53- defizienten Mäusen merklich abgeschwächt. Das deutet darauf hin, dass der p53 Pfad zur Unterdrückung der Proliferation von Hepatocyten mit DNA Schädigung notwendig ist. RAD001 beeinflusst die Expression verschiedener mit dem Zellzyklus verknüpften Proteine und den Aufbau von CDK-Cyclin Komplexen, welche in den Übergang von der G0

in die G1 Phase des Zellzyklus involviert sind. Die Langzeitbehandlung mit RAD001 hat schließlich die Tumorentwicklung in der Leber nachweislich verzögert.

Schlagwörter: Leber, Krebs, mTOR

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Abbreviations

4E-BP1	4E-Binding Protein 1
AFP	Alpha Fetoprotein
AMPK	AMP-Activated Protein Kinase
ATM	Ataxia Telangiectasia Mutated
BrdU	5-Bromo2'-Deoxy-Uridine
CDK	Cyclin-Dependent Kinases
eEF1A	Eukaryotic Elongation Factor 1A
eEF2	Eukaryotic Elongation Factor 2
EGFR	Epidermal-Growth Factor Receptor
eIF-2 alpha	Eukaryotic Initiation Factor 2 alpha
eIF-4A	Eukaryotic Initiation Factor 4A
eIF-4E	Eukaryotic Initiation Factor 4E
eIF-4F	Eukaryotic Initiation Factor 4F
eIF-4G	Eukaryotic initiation factor 4G
FAA	Fumarylacetoaccetate
FAH	Fumarylacetoaccetate Hydrolase
H&E	Hematoxylin and Eosin staining
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HGD	Homogentisic Acid Oxidase
HPD	4-Hydroxyphenylpyruvate Dioxygenase
HT1	Hereditary Tyrosinemia Type 1
IGF	Insulin-like Growht Factor
IRS1	Insulin Receptor Substrate 1
MAAI	Maleylacetoacetate Isomerise
MAPKK	Mitogen-activated Protein Kinase Kinase
mLST8	Mammalian LST8/G-protein ß-subunit like Protein
mSIN1	Mitogen-activated Protein-kinase-associated Protein 1
mTOR	mammalian Target of Rapamycin
mTORC1	mTOC Complex 1

mTORC2	mTOC Complex 2
NTBC	2-(2-Nitro-4-Fluoromethylbenzoyl)-1,3-Cyclohexanedione
PDCD4	Programmed Cell Death Protein 4
PH	Partial Hepatectomy
PI3K	Phosphoinositide-3 Kinase
PRAS40	Proline-rich Akt Substrate 40 kDa
pten	Phosphatase and Tensin Homolog
rb	Retinoblastoma Protein
RHEB	Ras-like GTPase
S6	Ribosomal Protein S6
S6K1	p70 Ribosomal Protien S6 Kinase 1
SA	Succinylacetone
SA-ß-Gal	Senescence-Associated ß-Galactosidase
TAT	Tyrosine Aminotransferase
TSC	Tuberous Sclerosis Complex
TUNEL	TdT-Mediated dUTP-Biotin Nick End Labeling

Introduction

Cancers arise by an evolutionary process as somatic cells mutate and escape the restraints that normally control their expansion. Suppressing the emergence of such autonomous cells is an evolutionary imperative where cells in regenerative tissues retain the potential for neoplastic disturbance throughout life. Consequently, multiple mechanisms have arisen to anticipate uncontrolled cell division. In combination, these tumor-suppressing mechanisms are remarkably effective.

1.1 Hepatocellular Carcinoma

Hepatocellular carcinoma is the most common type of liver cancer in adults, accounting for 80% of all liver malignancies (Befeler and Di Bisceglie, 2002; Parkin et al., 2001). While many cancers may originate in other parts of the body and spread to the liver, HCC originates from hepatocytes. There are two different growth patterns that occur in patients with HCC: as a single tumor that grows larger or as smaller cancer nodules throughout the liver, which grow into multiple tumors.

Liver cancer is the sixth most common cancer and the third leading cause of cancer-related death worldwide. It is the major cancer in Southeast Asia and sub-Sahara Africa, but the incidence of this tumor is much lower in Western Europe and the USA. However, the incidence and mortality rates of HCC have doubled in the USA over the past 25 years, and this upward trend is expected to continue over the coming decades (El-Serag et al., 2003; El-Serag and Mason, 1999). Each year, it is estimated that more than 700,000 people are diagnosed with liver cancer and over 600,000 people die from the disease (Thorgeirsson and Grisham, 2002).

The most common risk factor is a chronic hepatitis B (HBV) or C (HCV) infection. Cirrhosis, which is frequently caused by hepatitis, is associated with 50-80% of HCC patients (Liu et al., 2004). However, other risk factors for HCC include race, cirrhosis from non-viral causes such alcoholism, hemochromatosis and primary biliary cir-

rhosis. Furthermore, concomitant risk factors such as HCV infection in addition to alcoholism, tobacco use, diabetes or obesity increase the relative risk of HCC development, as numerous studies in humans and animal models have shown (Chokshi and Marrero, 2001; Davila et al., 2005; El-Serag et al., 2001; Fong et al., 1994; Hassan et al., 2002; Ming et al., 2002; Ohata et al., 2003).

HCV accounts for almost 90% of all cases of HCC in Japan; and in China, HBV infection is diagnosed in about 80% of patients with HCC (Beasley, 1988; Di Bisceglie, 1995; El-Serag, 2002). In Europe and North America, however, despite a significant lower incidence rate of 3 to 4 per 100,000 population, a distinct increase in cases of HCC has been reported as a result of intravenous drug use, unsafe sexual practices and other causes (Allen and Venook, 2004; El-Serag and Mason, 1999; Wingo et al., 1995). Because of a lack of effective HCV vaccination, underlying HCV infection is largely responsible for that increase. As a result of the interval between the onset of infection and the development of liver cirrhosis, the incidence of HCV-related HCC will continue to increase over the next few years (Colombo and Donato, 2005). In contrast to Asian populations, the percentage of Western patients with HCC but without underlying cirrhosis is considerable, and development of HCC in cirrhotic individuals in the West is associated with a wider spectrum of underlying diseases. In the West, the percentage of virally engendered cirrhosis is lower than that in Asian regions, but alcohol-toxic or cryptogenic hepatic damage is observed more frequently in Western countries (Sherman, 2005).

Patients with HCC are more likely to live longer when the cancer is detected before it has spread or if it is in the early stages (Bolondi et al., 2001; Llovet et al., 2003). The five-year survival rate for patients with resectable early stage cancer ranges from 30% to 60%. However, patients with advanced HCC have a five-year survival rate of less than 5%. When HCC is detected in the early stages, surgery, local ablation or liver transplantation are likely curative treatment options (Makuuchi and Sano, 2004; Shimozawa and Hanazaki, 2004). On the other hand, current therapies for tumors that have spread throughout the liver have not achieved the success rate that has been seen in several other tumors (Bruix and Sherman, 2005; Tang, 2001). Given the poor performance of existing therapies, strategies to prevent or reduce liver tumor development are urgently needed (Kensler et al., 2003).

HCC is a complex and heterogeneous tumor with several genomic alterations. There is evidence of aberrant activation of several signaling cascades such as the Wnt-ßcatenin pathway, EGFR-RAS-MAPKK pathway and c-Met pathway (Llovet and Bruix, 2008). Among others, an increasing number of human diseases has been linked to the dysregulation of mTOR including diabetes, obesity and cancer (Kenerson et al., 2005; Um et al., 2004; Vellai et al., 2003). Intriguingly, most of these are due to aberrant hyperactivity of the mTOR pathway, which makes inhibitors of mTOR potentially effective therapeutics for the treatment of these diseases (Tsang et al., 2007). Additionally, it has been shown that the p53/p21 pathway is altered in more that 50% of the human cancers. Therefore, this study was focused to determine the interaction of mTOR and p53/p21 signaling pathways in the development of tumors in the liver.

1.2 Mammalian Target of Rapamycin (mTOR) signaling

mTOR is a 250 kDa serine/threonine kinase that integrates mitogenic and nutrient signaling to regulate cell growth by controlling mRNA translation, ribosome biogenesis, autophagy and metabolism (Guertin and Sabatini, 2007). mTOR is part of two large complexes: a rapamycin and nutrient-sensitive multiprotein complex called mTOR Complex 1 (mTORC1) (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002) and a growth-factor-sensitive but nutrient-insensitive mTOR-containing complex called mTOR Complex 2 (mTORC2). Unlike mTORC1, rapamycin does not bind to mTORC2 (Jacinto et al., 2004; Loewith et al., 2002). The discovery of mTORC2 provided conclusive evidence to the suspicions that rapamycin did not inhibit all of mTOR's functions (Guertin and Sabatini, 2007).

1.2.1 mTOR Complex 1 (mTORC1)

This complex is characterized by functioning as a nutrient/energy/redox sensor and controlling protein synthesis. It is activated by insulin, growth factors, amino acids and oxidative stress. mTORC1 is composed of mTOR, regulatory associated protein of mTOR (RAPTOR), mammalian LST8/G-protein ß-subunit like protein (mLST8) and proline-rich Akt substrate 40 kDa (PRAS40) (figure 1) (Hara et al.,

2002; Kim et al., 2002; Loewith et al., 2002; Sancak et al., 2007). RAPTOR positively regulates mTOR activity and recruits mTORC1 substrates. On the other hand, PRAS40 negatively regulates mTOR activity in a manner that depends upon its phosphorylation state. The molecular function of mLST8 is still ambiguous (Hara et al., 2002; Sancak et al., 2007).

1.2.1.1 Upstream regulation of mTOCR1 signaling

A major understanding of mTOC1 regulation was reached by the discovery that the TSC1 and TSC2 bipartite protein complex negatively controls its activity (figure 1) (Crino et al., 2006). Mutations in either the *tsc1* or *tsc2* gene cause the hamartomatous syndrome tuberous sclerosis complex (TSC). The discovery of the connection between TSC and the mTORC1 pathway provided the first molecular link between mTOR and cancer (Guertin and Sabatini, 2007). TSC2 possesses a GTPase activating protein (GAP) activity and inactivation of the TSC1/2 complex, either by mutation or by cellular growth signals, leading to the activation of the ras-like GTPase RHEB (Crino et al., 2006). Although it is difficult to detect a direct biochemical interaction between endogenous RHEB-GTP and mTORC1, *in vitro* experiments indicate that soluble GTP-loaded RHEB directly activates the kinase activity of mTORC1. In contrast, PRAS40 inhibits RHEB-GTP-dependent mTORC1 activation in a dose-dependent manner in an *in vitro* kinase assay, and it stably associates with mTORC1 in cells (Sancak et al., 2007). Although RHEB-GTP and PRAS40 are adversaries in a cell-free system, in intact normal cells, Akt forces them to cooperate. For instance, Akt, and possible other kinases, phosphorylates PRAS40 on T246, diminishing its ability to inhibit mTORC1 (Huang and Porter, 2005; Kovacina et al., 2003; Sancak et al., 2007). Therefore, Akt both promotes RHEB-GTP-loading and relieves mTORC1 from PRAS40 repression.

Growing evidence argues that the connection between Akt and TSC2-RHEB-mTORC1 is a critical step in PI3K-meditated tumorigenesis. For instance, rapamycin slows the proliferation of transformed cells null for *pten,* a tumor suppressor gene, or expressing constitutively active Akt (Guertin and Sabatini, 2005). Studies in mouse models support this idea showing that the neoplastic phenotypes induced by *pten* deletion of transgenic activation of Akt are sensitive to rapamycin (Majumder et al., 2004; Neshat et al., 2001; Podsypanina et al., 2001). In mice, depletion of Akt activity can also thwart *pten*-deletion-driven as well as RAS-driven and

chemically induced tumorigenesis (Chen et al., 2006; Skeen et al., 2006). Based on *in vitro* studies in mouse embryo fibroblasts, it is argued that the oncogenic activity of Akt in these models depends on mTORC1 (Skeen et al., 2006).

In contrast to growth-factor-driven activation of mTORC1, hypoxia, AMPK activation resulting from depletion of cellular energy, Wnt-GSK3 signaling, and glucocorticoids all inhibit mTORC1 by promoting TSC1/2 activation (Inoki et al., 2006; Reiling and Sabatini, 2006; Wang and Proud, 2006). Amino acid deprivation may also activate TSC1/2, although other evidence argues that mTORC1 and S6K1 respond to amino acid availability independently of TSC1/2 (Nobukuni et al., 2005; Sarbassov et al., 2005a).

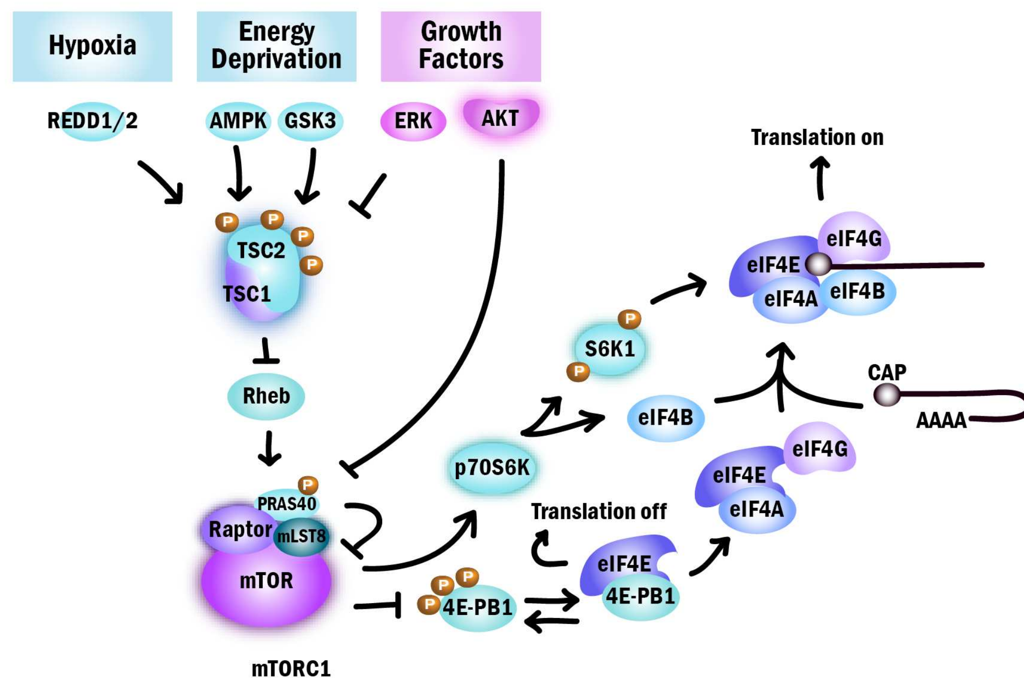

Figure 1 The mTOR signaling
The mTOR kinase is the catalytic component of two distinct multiprotein complexes called mTORC1 and mTORC2. In addition to mTOR, mTORC1 contains RAPTOR, mLST8, and PRAS40. mTORC1 drives cellular growth by controlling numerous processes that regulate protein synthesis and degradation. Diverse positive and negative growth signals influence the activity of mTORC1, many of which converge upon the TSC1/2 complex. Activation and inhibition induced by direct phosphorylation is indicated by a phosphate (P) (Guertin and Sabatini, 2007).

1.2.1.2 Downstream effectors of mTORC1

Ribosomal p70 S6 kinase (S6K1) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), both regulators of mRNA translation, are the best characterized downstream effectors of mTOR as part of the mTORC1 complex (Bjornsti and Houghton, 2004). mTOR-dependent phosphorylation of S6K1 allows translation of the translational apparatus (ribosomal proteins, elongation factors eEF1A, eEF2 and the poly-A-binding protein), whereas cap-dependent translation is regulated by phosphorylation of 4E-BP1 (Guertin and Sabatini, 2007). When activated by mTORC1, S6K1 promotes protein synthesis by phosphorylating PDCD4 (programmed cell death protein 4) and targeting it for degradation (Dorrello et al., 2006). PDCD4 blocks protein translation by binding and preventing the eIF-4A helicase from the unwinding secondary structure in the 5′ untranslated region (5’UTRs) of mRNA. The ribosomal protein S6 is perhaps the best-known S6K1 substrate. S6 is one of the 30 distinct ribosomal proteins, which together with 18S rRNA, constitutes the smaller 40S ribosomal subunit. The importance of S6 in ribosome function and protein translation is underlined by the deletion of S6 in mice, which results in a block of ribosome biogenesis and cell cycle progression (Tang et al., 2001). Despite being an indirect measure of mTORC1 activity and lacking a clear function, S6 phosphorylation is widely used in research and in the clinic as a biomarker of mTORC1 activity.

The second established signaling pathway down-stream of mTOR is 4E-BP1. It is a small unstructured polypeptide that when hypophosphorylated binds to eIF-4E, it thereby prevents the formation of the eIF-4F complex and its binding to the cap of mRNA. Structural studies indicate that 4E-BP proteins behave as molecular mimics of eIF-4G by competing for a binding to eIF-4E. Upon phosphorylation by mTOR, 4E-BP1 releases eIF-4E, which binds the cap at the 5’ ends of mRNA transcripts and assembles with other translation initiation factors to initiate cap-dependent translation (figure 1) (Guertin and Sabatini, 2007). The cap consists of the structure m^7GpppX (where X is any nucleotide). eIF-4F is a trimeric complex comprised of eIF-4G, eIF-4A and eIF-4E, that mediate the binding of the mRNA to the 43S pre-initiation complex to form the 48S initiation complex. eIF-4E binds directly to the cap structure. eIF-4G is a scaffolding protein, which binds eIF-4A and eIF-4E. eIF-4A serves as an ATP-dependent helicase that functionally interacts with a cofactor, eIF-4B. eIF-4B is an initiation factor that promotes the binding of mRNA to 40S ri-

bosomes *in vitro* and promotes ATP-dependent helicase activity of eIF-4A to unwind secondary structures present within the 5'UTRs of certain mRNAs, until the initiator AUG. eIF-4E appears to be the limiting factor in eIF-4F complex formation and to be highly regulated upon growth factor stimulation (Holland et al., 2004).

The eIF-4F complex is thought to particularly enhance translation of transcripts possessing complex 5'-untranslated regions, which encode proteins associated with G1 cell-cycle progression and S-phase initiation. Then inhibition of mTOR signaling may result in a prolonged G1 phase or arrest in G1. mTOR might therefore also be viewed as a gatekeeper, which only allows G1 progression under nutrient-replete conditions (Bjornsti and Houghton, 2004). Interest in the regulation of mTOR has increased substantially in recent years, largely because of an apparent link between deregulation of translation and cancer cell survival (Shaw and Cantley, 2006).

1.2.2 mTOR Complex 2 (mTORC2)

Like mTORC1, mTORC2 also contains the mLST8 protein, but rather than RAPTOR, mTORC2 includes the rapamycin-insensitive companion of mTOR (RICTOR). This makes mTORC2 distinctive from mTORC1 due to the fact that mTORC2 does not likely bind to the rapamycin-FK506-binding protein 12 complex. In addition to mSIN1 (also known as mitogen-activated protein-kinase-associated protein 1) proteins, mTORC2 also contains the protein observed with RICTOR (PROTOR), a protein that is only found in higher eukaryotes (Pearce et al., 2007; Woo et al., 2007). PROTOR specifically interacts with RICTOR, but not RAPTOR, because the interaction is tighter than the RICTOR-mTOR interaction and independent of mTOR.

The functions and regulatory mechanism of mTORC2 are still largely unknown. It has been shown in several studies that mTORC2 should be considered as part of the PI3K-Akt pathway as it directly phosphorylates Akt on one of the two sites that are necessary for Akt activation in response to growth-factor signaling (Hresko and Mueckler, 2005; Sarbassov et al., 2005b). Additional studies indicate that mTORC2 regulates protein kinase C α phosphorylation, actin cytoskeleton organization, and Akt phosphorylation at Ser473 (Jacinto et al., 2004; Sarbassov et al., 2005b).

1.2.3 Negative feedback regulation of PI3K-Akt signaling

Surprisingly, patients suffering from TSC do not develop more aggressive tumors like those patients suffering from diseases linked to *pten* mutations. The reason for this difference may be the existence of a strong negative feedback loop. In many cell types, PI3K-Akt signaling is strongly repressed upstream in the PI3K pathway by the activation of mTORC1 signaling (Manning, 2004). It has been shown that loss of TSC1/2 function results in a decrease of Akt phosphorylation (Jaeschke et al., 2002; Kwiatkowski et al., 2002). Additionally, it appears that when mTORC1 is active, S6K1 directly phosphorylates and inhibits IRS1 (Zick, 2005). When S6K1-deficient mice are challenged with a high fat diet, they become resistant to obesity because loss of the feedback loop enhances their insulin sensitivity (Um et al., 2004). These data suggest that tumors in TSC patients are less aggressive because the feedback loop suppresses PI3K-Akt signaling.

Mechanistically, when Akt is inactive, TSC1/2 inhibits RHEB while PRAS40 inhibits mTORC1 (figure 2, left). Upon activation, Akt promotes mTORC1 activity by phosphorylating both TSC1/2 and PRAS40, resulting in GTP-loading of RHEB, which directly activates mTORC1 and release of mTORC1 from PRAS40 repression (figure 2, middle). When TSC is knocked down, RHEB strongly activates mTORC1 and then Akt is inhibited by the negative feedback loop. Even though PRAS40 is dephosphorylated in this state, its ability to repress mTORC1 is overrun by the greatly elevated RHEB activity (figure 2, right) (Guertin and Sabatini, 2007).

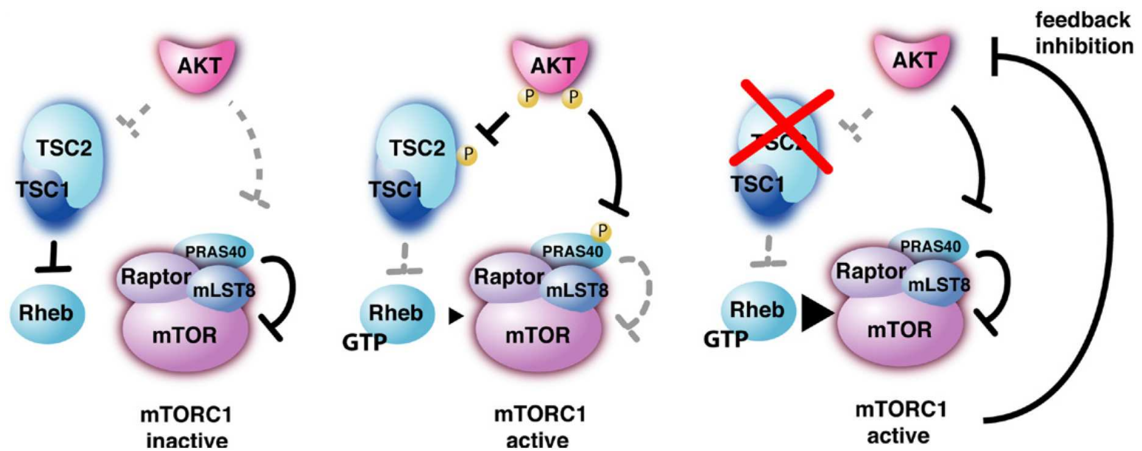

Figure 2 Model of mTORC1 co-regulation by RHEB and PRAS40
(Guertin and Sabatini, 2007)

1.2.4 Role of mTOR in HCC

Previous studies have shown that activation of the mTOR pathway in HCC ranged from 15% to 41% in experimental models of HCC (Sahin et al., 2004; Semela et al., 2007). A recent report showed that the mTOR pathway is activated in a subset of patients with early HCC. That activation of mTOR signaling is due to ligand-dependent signals from EGF and IGF signaling rather than from a mutation-dependent mechanism. Additionally, patients displaying activation of the mTOR pathway also presented higher levels of AFP, less differentiated tumors and a higher incidence of recurrence (Villanueva et al., 2008). These data suggest the relevance of mTOR signaling in HCC and show it as an attractive target for clinical trials.

1.3 RAD001 (Everolimus), a mTOR inhibitor

As a sensor of the availability of growth factors, nutrients and energy sources, the mTORC1 kinase plays a central role in tumor biology. Consequently, mTORC1 inhibitors have a broad antitumor activity as shown pre-clinically in experimental tumor models as well as clinically in cancer patients. Strikingly, certain tumor types appear to be predisposed to respond to mTORC1 inhibition, a phenomenon related to de-

regulation of critical elements of the PI3K/mTORC1 pathway (Lane and Breuleux, 2009).

The macrolide antibiotic rapamycin is a product of the bacteria *Streptomyces hygroscopicus*, which was isolated from a soil sample taken from Easter Island (known locally as Rapa Nui). In the mid-1970s, it was identified as a potent antifungal agent and was found to be a potent suppressor of the immune system (Bjornsti and Houghton, 2004).

Rapamycin and its analogues, like sirolimus (rapamycin), temsirolimus (CCI-779) and everolimus (RAD001), are highly specific inhibitors of mTOR, and differ only slightly in chemical structure that results in improved chemical stability and pharmaceutical properties. Rapamycin first binds the 12-kDa immunophilin FK506-binding protein (FKBP12). The FKBP-rapamycin complex then binds to the FK-rapamycin binding (FRB) domain of mTOR, resulting in inhibition of the function of mTOR (figure 3). Whether rapamycin or the FKBP-rapamycin complex directly inhibit the kinase activity of mTOR is controversial (Bjornsti and Houghton, 2004). This inhibition suppresses cytokine-driven T-cell proliferation, inhibiting the progression from G_1 to the S phase of the cell cycle.

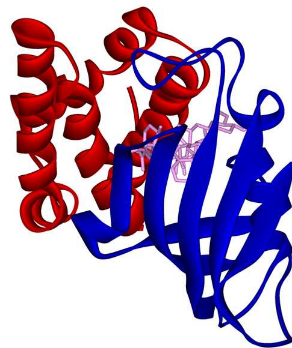

Figure 3 Ribbon diagram of FKBP12 in complex with RAD001 (Everolimus) interacting with the rapamycin-binding domain of mTOR. Red: Rapamycin-binding domain of mTOR; blue: FKBP12; pink RAD001

RAD001 [40-O-2-hydroxyethyl)-rapamycin], or Everolimus, is an analogue of rapamycin that can be administrated orally. The immunosuppressive activity of RAD001 *in vitro* is about 3-fold lower than rapamycin, but its activity *in vivo* seems compara-

ble to rapamycin due to some more favorable pharmacokinetic properties. RAD001 inhibits the growth factor-stimulated *in vitro* proliferation of vascular sooth muscle cells and prevents allograft rejection in rats (Schuler et al., 1997). S6K1 inhibition and antineoplastic effect by RAD001 have been shown in human cancer cell lines *in vitro* and in xenograft models *in vivo*, an optimal effect being achieved with 2.5 mg/kg/day in melanoma, lung, pancreas and colon carcinoma. Similarly, RAD001 demonstrates a concentration-dependent anti-tumor activity in a rat pancreas carcinoma model with an intermittent dosing schedule. RAD001 has also shown antiangiogenic activity (Lane et al., 2009) and inhibits the human vascular endothelial cells proliferation. RAD001 leads to an increase in the number of cells in the G0/G1 phase of the cell cycle. At a concentration of 10nM, there was a 10-70% increase, indicating a delay in cell cycle progression at the early G0/G1 phase. An *in vivo* xenograft model confirmed this observation (Vignot et al., 2005). When combined with other chemotherapeutic agents, RAD001 generally showed at least additive antitumor activity (Baselga et al., 2009; Sosman and Puzanov, 2009).

RAD001 is currently undergoing evaluation in phase I studies as an anti-neoplastic agent and is already in phase III trials as an immunosuppressor acting in synergy with cyclosporine in kidney transplantation (Okamoto et al., 2009).

1.4 p53

The tumor suppressor p53 functions primarily as a transcription factor that can mediate its different downstream functions by activating or processing a large number of target genes (Mirza et al., 2003; Polyak et al., 1997; Vogelstein et al., 2000; Zhao et al., 2000). p53 transcriptionally regulates a variety of biological activities (Vousden and Prives, 2009) that include DNA metabolism (Helton and Chen, 2007), apoptosis (Vousden, 2006), cell-cycle regulation (Kastan et al., 1992), senescence (Garbe et al., 2007), energy metablism (Green and Chipuk, 2006; Matoba et al., 2006), angiogenesis (Menendez et al., 2006; Pal et al., 2001; Teodoro et al., 2006; Zhang et al., 2000), immune response (Taura et al., 2008), cell differentiation, motility and migration (Molchadsky et al., 2008; Qin et al., 2009; Roger et al., 2006; Singh et al., 2007; Tedeschi and Di Giovanni, 2009), and cell-cell communication (Yu et al., 2006). Additionally, recent studies have demonstrated how p53-

dependent activation of micro RNA genes can participate in the modulation of various biological activities (Braun et al., 2008; He et al., 2007; Sinha et al., 2008; Yamakuchi et al., 2008).

1.4.1 p53 in the cell cycle regulation

p53 also induces cell-cycle arrest by stimulating the expression of the cyclin-dependent kinase inhibitor p21 (p21). This CDK inhibitor regulates the cell cycle via perturbation of cyclins which ensure the successful transitions from S phase to G1. CDKs inhibition by p21 results in suppression of both G1-to-S and G2-to-mitosis transitions by causing hypophosphorylation of retinoblastoma (Rb) and also preventing the release of E2F. Moreover, p53 can stimulate 14-3-3 protein which sequesters the CDK1-cyclin B1 complex out of the nucleus, resulting in a G2 block (Zheng et al., 2006).

1.4.2 Role of p53 in apoptosis

Activation of p53 may not just induce cell-cycle arrest but also apoptosis. Interestingly, p53 regulates the expression of a key subset of the Bcl-2 family genes, including Bax, Noxa, p53-upregulated modulator of apoptosis (PUMA) and BH3-interacting domain death agonist (Bid). PUMA plays an important role in p53-mediated apoptosis. In vitro, over-expression of PUMA is followed by increased Bax expression, Bax conformational change, translocation to the mitochondria, cytochrome c release and reduction in the mitochondrial membrane potential (Liu et al., 2003). Noxa is also considered to be a mediator of p53-induced apoptosis. It has been shown that Noxa can localize to the mitochondria and interact with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9 (Oda et al., 2000). Therefore, PUMA and Noxa might mediate the apoptosis that is elicited by genotoxic damage or oncogenes activation due to their expression is induced by p53. Additionally, it has been reported that Myc oncoprotein potentiates apoptosis through both p53-dependent and p53-independent mechanisms (Meyer et al., 2006).

1.4.3 p53 and its relation with the mTOR signaling

Many studies have shown that there is a significant crosstalk at different levels between mTOR and p53 signaling. The p53 pathway is activated by cellular stress signals of DNA damage or hypoxia and results in cell-cycle arrest, senescence or apoptosis. Dependent on p53 activation, the TSC1/TSC2 complex is activated directly or via positive regulation of the AMP kinase. As a result, mTOR activity is suppressed, an effect reported also via activation of the LKB1 tumor suppressor identified upstream of AMP (Feng et al., 2007; Feng et al., 2005; Levine et al., 2006; Shaw et al., 2004). Higher upstream in the PI3K-Akt-mTOR pathway, p53 positively regulates PTEN activity (Levine et al., 2006).

1.4.4 p53 is mutated in many cancers

The p53 pathway is crucial for effective tumor suppression. p53 is one of the most commonly mutated genes in human cancers. Mutational frequency of the p53 gene varies across tumor types but on average, 50% of all tumors will have sustained a lesion at the p53 locus. During tumorigenesis, the developing tumor cell is exposed to several cellular stresses that culminate in DNA damage. It may be likely that those tumors without p53 mutations have some lesions at points elsewhere in the p53 pathway, which would act to functionally inactivate the p53 response to DNA damage. ATM and MDM2 are examples of alternative targets for mutation that affect p53 upstream of gene activation and can effectively prevent the transcription of downstream p53 target genes. This is also valid for mutation of genes downstream in the p53 pathway that would reduce the effectiveness of p53-mediated cell cycle arrest and apoptosis.

Studies in animal models in which p53 has been constitutively or conditionally altered demonstrate that p53 transcriptional activity is key to tumor suppression (Christophorou et al., 2006; Lozano, 2007; Martins et al., 2006). Mouse knock-in approaches were used to show that the tissue-specific predisposition to cancer and tumor onset correlated with the DNA binding and transactivation potential of p53 mutants in cell systems or *in vitro* (Iwakuma and Lozano, 2007; Liu et al., 2000; Ryan and Vousden, 1998).

1.4.5 p53 in Hepatocellular Carcinoma

Mutations in p53 tumor suppressor gene are the most common alterations detected in HCC (Hussain et al., 2007). It has been reported that p53 increases the frequency of HCC prediction from 79% to 86% (El Far et al., 2006). The existence of specific mutations in p53 in different human cancers involves environmental carcinogens and endogenous processes. For example, somatic mutations at the third base in codon 249 of p53 in HCC have been related to exposure to aflatoxin B1 (AFB1), in association with HBV infection (Hussain et al., 2007). Additionally, chronic infection with HBV and HCV viruses and exposure to oxidative stress induce DNA damage and mutation in cancer-related genes, including p53. Thus, it can be considered that p53 mutations might operate in either HCC initiation or progression, depending of the context (Fabregat, 2009).

A previous study showed that attenuated p53 function and telomere-induced chromosomal instability play a critical and cooperative role in the progression of chronic liver damage to HCC (Farazi et al., 2006). *In vitro* studies using human HCC-derived cell lines showed that six of them show p53 abnormalities, suggesting that alterations in p53 may be important events in the transformation of hepatocytes into HCC (Nagao et al., 1999). Additionally, p53 is often mutated in high-grade HCC (Jeng et al., 2004). It has been shown that loss of p53 occurs in HCC and diversity of the p53 aberrations increases with the progression of chronic liver damage to HCC (Guan et al., 2007).

1.5 p21

During evolution, higher eukaryotes have evolved different checkpoint mechanisms in order to monitor and respond to cellular stresses, arresting cell proliferation until the errors are fixed or the environmental conditions become proper to transmit the genetic material. When these checkpoints are disturbed, the integrity of the genome is compromised and cancer development could be promoted. The tumor suppressor protein p53 mediates the DNA-damage-induced checkpoint through the activation of numerous growth inhibitory or apoptotic genes. Among these, the small 165 amino acid protein p21 mediates p53-depenent G1 cell-cycle arrest

(Brugarolas et al., 1995; Deng et al., 1995). p21 belongs to the Cip/Kip family of CDK inhibitors that includes p21, p27 and p57. p21 primarily binds and inhibits the kinase activity of the CDK2 and CDK1, inducing growth arrest at specific stages in the cell cycle (figure 4).

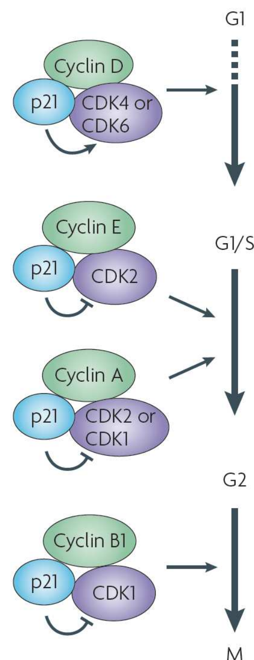

Figure 4 p21 promotes and inhibits the kinase activity of several CDK/cyclin complexes
Under determined conditions, the cell-cycle progression through G1 is promoted by p21, which advocates the kinase activity of CDK4 or CDK6 in a complex with cyclin D. p21 also suppresses E2F1-dependent gene transcription and progression into and through the S phase by inhibiting CDK2-cyclin E and the subsequent inhibition of CDK2-dependent phosphorylation of Rb and the sequestration of E2F1. In addition, p21 suppresses the kinase activity of CDK2-cyclin A and CDK1-cyclin A, which are needed for progression through the S phase and into G2 respectively. Furthermore, p21 inhibits the progression through G2 and G2/M by suppressing the kinase activity of CDK1-cyclin B1 (Abbas and Dutta, 2009).

Furthermore, p21 binds to the proliferation cell nuclear antigen (PCNA), interfering with its PCNA-dependent DNA polymerase activity and thereby inhibiting DNA replication and modulating numerous PCNA-dependent DNA repair processes (figure 5).

p21 was considered initially as a potential tumor suppressor since it inhibits proliferation and is the major downstream target of p53. However, evidence from recent studies suggests that, under certain conditions, p21 could also act as an oncogene due to its role in apoptosis (Gartel, 2009).

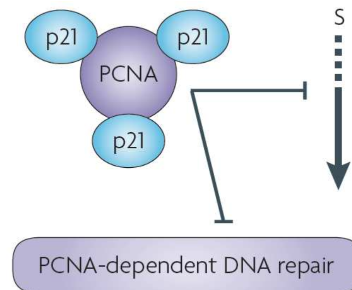

Figure 5 p21 inhibits PCNA
p21 binds to and inhibits PCNA and then suppresses the DNA synthesis and modulation of PCNA-dependent DNA repair pathways (Abbas and Dutta, 2009).

1.5.1 p21 as a tumor suppressor

Tumor suppressor functions for p21 were found in p21-null mice that develop a variety of neoplasias that include tumors of hematological, endothelial and epithelial origin. Additionally, p21 could protect mice against chemical carcinogens because its loss greatly accelerated the development of malignant skin carcinoma induced by chemicals (Topley et al., 1999). Furthermore, the absence of p21 mainly resulted in higher grade, poorly differentiated tumors in a skin carcinogenesis mouse model (Philipp et al., 1999).

It has been shown that p21 alone is sufficient to suppress the regenerative capacity of hepatocytes and kidney proximal tubular cells. Loss of p21 is not compensated by other cell-cycle inhibitors. In its absence, proliferation of cells with DNA damage resulted in rapid cancer formation (Willenbring et al., 2008a). By mediating p53-dependent cell-cycle arrest, p21 plays a significant role in tumor suppression since the absence of p21 makes mice more susceptible to tumorigenesis (Martin-Caballero et al., 2001).

1.5.2 Oncogenic activities of p21

In contrast to the tumor suppression functions, p21 may also act as an oncogene. In p53-deficient mice, loss of p21 results in a significantly extended survival of the animals (De la Cueva et al., 2006). Additionally, loss of p21 in Atm-deficient mice delays thymic hymphomagenesis. In mouse models for oligodendrogliomas (ODGs), loss of p21 reduced the development of tumors (Miettinen et al., 2001).

Specifically in liver, studies have shown that p21 checkpoint activation was associated with an increased HCC risk at the cirrhosis stage (Wagayama et al., 2002). Additionally, other studies present evidence that telomere shortening and an accumulation of DNA damage, molecular characteristics of HCC, coincide with loss of p21 (Plentz et al., 2007). Moreover, p21 is overexpressed in other cancers such as prostate, cervical, breast and squamous cell carcinomas; and p21 upregulation correlates positively with tumor grade, invasiveness and aggressiveness and is a poor prognostic indicator, suggesting that p21 may also act as an oncognene (Abbas and Dutta, 2009).

1.5.3 p21, a modulator of apoptosis

Although best known for its growth-inhibitory functions, p21 also inhibits apoptosis. Through its ability to promote cell-cycle inhibition, especially in the face of genotoxic insults or microtubule-destabilizing agents, p21 protects cells from apoptosis because an active cell cycle is required to sense these agents and trigger apoptosis. The cytostatic effect of p21 with the consequent inhibition of apoptosis is counteracted by several mechanisms. The cellular response can be switched from cell-cycle arrest to apoptosis by the selective transcriptional repression of CDKN1A, the selective activation of pro-apoptotic genes or defects in p21 expression downstream of p53 (Kaneuchi et al., 1999; Okaichi et al., 1999; Samuels-Lev et al., 2001). Additionally, post-transcriptional modifications of p21 such as its phosphorylation (Li et al., 2002; Meng et al., 2007; Oh et al., 2007), which affects protein stability or cytoplasmatic localization of p21 (Li et al., 2002; Zhou et al., 2001), and its cleavage by caspase-3 also account for the differential effects on cell-cycle arrest versus apoptosis (Zhang et al., 1999).

p21 also can protect against apoptosis in different stimuli such as those induced by growth factor deprivation or p53 overexpression (Roninson, 2002). Under these conditions, the anti-apoptotic effect of p21 cannot be attributed to its cytostatic effects because apoptosis does not depend on cell-cycle progression. Rather, it may depend on the ability of p21 to regulate gene transcription through its multiple protein-protein interactions or its function in DNA repair. For example, when p21 is localized in cytoplasm, it binds to and inhibits the activity of protein involved in the induction of apoptosis (procaspase-3 and caspase-8), stress-activated kinases (SAPKs) and apoptosis signal-regulating kinase 1 (ASK1) (figure 6) (Dotto, 2000; Roninson, 2002). Additionally, p21 induces the upregulation of genes encoding anti-apoptotic proteins, and suppresses the induction of pro-apoptotic genes (Dotto, 2000).

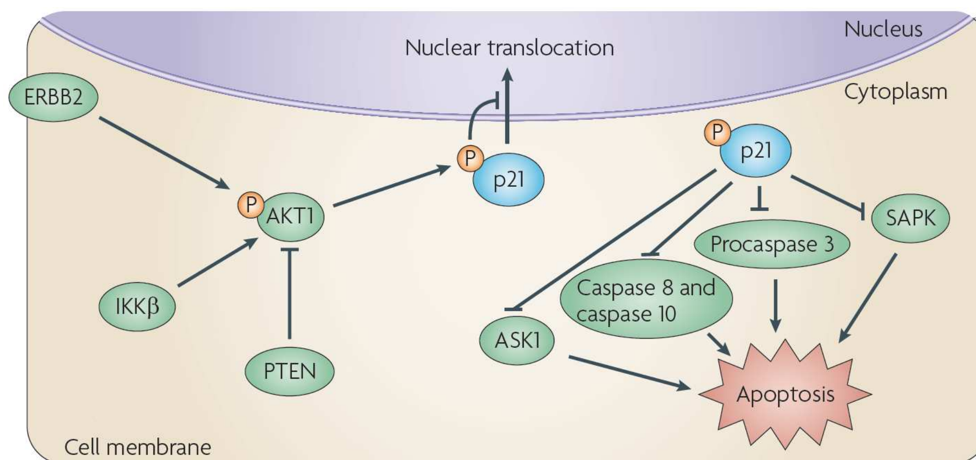

Figure 6 Cytoplasmic activity of p21
Nuclear translocation of p21 is inhibited when phosphorylated at Thr145 by activated Akt1 downstream of ERBB2 or IKKß signaling. At the cytoplasm, p21 exhibits anti-apoptotic activity, inhibiting several proteins involved in apoptosis. It is not clear whether the phosphorylation of p21 is only required for retaining p21 in the cytoplasm or also for its cytoplasmic activities. ASK1, apoptosis signal-regulating kinase 1, also known asSAPK, stress-activated protein kinase (Abbas and Dutta, 2009).

1.6 Hereditary Tyrosinemia type 1

The liver plays a central role in the pathophysiology of many inborn errors of metabolism, because it is the major site of catabolic, synthetic and detoxification reactions. In humans, the most severe disease of the tyrosine catabolic pathway is Hereditary Tyrosinemia type 1 (HT1), an autosomal recessive inborn error of metabolism. The first report with typical clinical and biochemical findings was presented by Sakai in 1957 (Pitkanen et al., 2000). This disease is characterized by progressive liver and kidney damage and neurological crisis. In the acute form, symptoms appear in early childhood and are characterized by a rapid deterioration of hepatic and renal functions, leading to death during the first year because of liver failure. The chronic form includes symptoms such as progressive hepatic dysfunction during infancy and death from liver cirrhosis or HCC. Cardiomyopathy is also a common feature of the disorder (Langlois et al., 2006).

HT1 is caused by deficiency of the enzyme fumarylacetoaccetate hydrolase (FAH), which carries out the last step in the tyrosine catabolic pathway (figure 7). This enzyme is a homodimer of two 43 kDa subunits, consisting of 419 amino acids residues per monomer (Pitkanen et al., 2000). The absence of FAH leads to an accumulation of the primary metabolite in HT1, fumarylacetoaccetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA) and tyrosine. It has been shown that FAA, but not MAA nor SA, is highly mutagenic and a potent alkykator, causing oxidative damage by reacting with glutathione and sulfhydryl groups of proteins. FAH is mainly expressed in the liver and in the kidneys (Grompe, 2001).

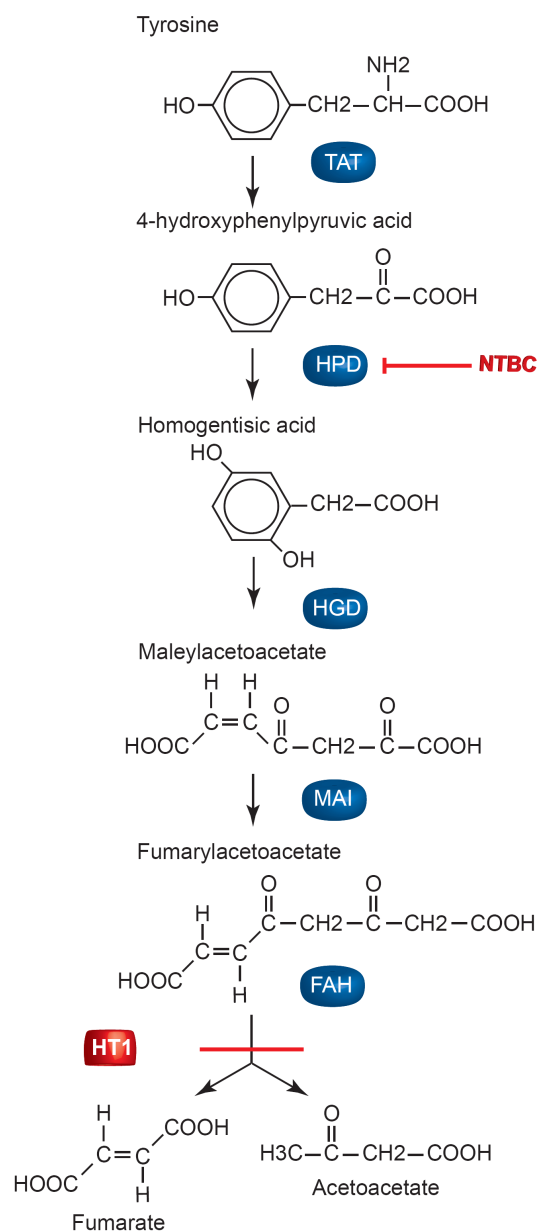

Figure 7 Schematic tyrosine catabolic pathway
Three types of hereditary Tyrosinemia have been described (HT). In HT1 fumarylacetoacetase (FAA) is defective. This defect in FAA activity leads to the accumulation of toxic metabolites. The enzymes that are involved in the pathway are shown in blue. They are: Tyrosine aminotransferase, 4-hydroxyphenylpyruvate dioxygenase, homogentisic acid oxidase, and maleylacetoacetate hydrolase, the enzyme deficient in hereditary tyrosinemia type 1. NTBC was used in this study as an inhibitor of 4-hydroxyphenylpyruvate dioxygenase.

Prevention of FAA accumulation has beneficial therapeutic effects in HT1. A dietary restriction in tyrosine and phenylalanine is given to HT1 patients to limit the accumu-

lation of the toxic metabolite FAA. However, treatment with NTBC (2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione) has become the mainstay for the management of HT1. The principle of this treatment is to reduce metabolic flow through the tyrosine catabolic pathway by blocking the activity of 4-hydroxyphenylpyruvate dioxygenase (HPD), enzyme acting upstream of FAH in the tyrosine catabolic pathway, preventing the formation of the toxic compound FAA (figure 7). NTBC rapidly improves liver and kidney functions but does not completely prevent the formation of HCC in long-term therapy (Al-Dhalimy et al., 2002).

Alternative causes of elevated concentration of plasma tyrosine exist and are attributable to genetic defects different than those responsible for tyrosinemia type I. These have been categorized as tyrosinemia type II and tyrosinemia type III. Each of these two disorders are more benign than type I, with different clinical presentations. Tyrosinemia type II is caused by a deficiency of tyrosine aminotransferase (TAT) and clinically presents with hyperkeratotic plaques on the hands and soles of the feet and affected individuals may have photophobia. Tyrosinemia type III is an extremely rare disorder, caused by a deficiency of 4-hydroxyphenylpyruvic dioxygenase (HPD), and is associated with metabolic acidosis and growth failure in infancy (Russo et al., 2001).

Importantly, tyrosine per se is not toxic to the liver or kidney but rather causes only dermatological, ophthalmologic, and possible neurodevelopmental problems. Patients with Tyrosinemia type II and III have highly elevated blood tyrosine levels but do not manifest liver disease or renal tubular dysfunction (Grompe, 2001).

1.7 Murine Hepatic Cancer Model

A murine model of Fah deficiency has been developed by targeted disruption of exon 5 of the Fah gene (Grompe et al., 1993). Homozygous mutant mice die in the neonatal period due to hepatic dysfunction. Fah-deficient mice have a grossly altered pattern of hepatic mRNA expression, particularly in genes responsive to cAMP, they suffer from severe hypoglycemia, and the endoplasmic reticulum of hepatocytes is disorganized. The neonatal lethality and misexpression of liver mRNA in Fah-deficient mice can be rescued by the drug NTBC. When NTBC

treatment is stopped in adult Fah-deficient mice, they develop a phenotype similar to humans with HT1, including rapidly progressive hepatic dysfunction and death within 2 months after drug withdrawal (Overturf et al., 1996). Low-dose treatment with NTBC causes less liver injury, allowing the survival of the mice. However, all of these mice develop liver cancer. The hepatocytes undergo progressive malignant changes from dysplasia to hepatomas and then to HCC, thus providing the opportunity to study the molecular changes associated with tumor progression in the liver (Grompe et al., 1995).

2 Aim of the Study

The aim of this study was to determine the interaction between mTOR and p53/p21 signaling pathways that are implicated in hepatocellular carcinogenesis and to find a new treatment to prevent this devastating disease. For this purpose, an analogue of rapamycin, RAD001, was used. This mTOR inhibitor was given to Fah-, Fah/p53- and Fah/p21-deficient mice.

3 Materials and Methods

3.1 Mice

In this study, the C57BL/6-Fah^{tm1Mgo} (*Fah*$^{-/-}$) mouse strain was used (Grompe et al., 1993). *Fah*$^{-/-}$ mice were crossed with C57BL/6-p21^{tm1} (*p21*$^{-/-}$) and C57BL/6J-Tg(Alfp-cre)1Gsc - FVB.129-Trp53^{tm1Brn} (*p53*^{fl}/^{fl}/*Alfp-Cre+*) mice through successive generations to produce C57BL/6-Fah^{tm1Mgo} - C57BL/6-p21^{tm1} (*Fah/p21*$^{-/-}$) and C57BL/6-Fah^{tm1Mgo} - C57BL/6J-Tg(Alfp-cre)1Gsc - FVB.129-Trp53^{tm1Brn} (*Fah*$^{-/-}$/*p53*$^{fl/fl}$/*Alfp-Cre,* i.e., *Fah/p53*$^{-/-}$) mice double knockout mice. *Alfp-cre* mediated deletion in *p53* mice is hepatocyte-specific. Those mice express the cre-recombinase open frame (ORF) under the control of both the mouse albumin regulatory elements and the alpha-fetoprotein enhancers (Alfp-cre) (Kellendonk et al., 2000).

Animals were monitored by two inspections per week. All Fah-deficient animals were treated with NTBC in the drinking water at a concentration of 7.5mg/L unless otherwise indicated (a gift from S. Lindstedt, Gøtheborg, Sweden). C57BL/6 wild-type mice were purchased from Jackson Laboratories.

Bromodeoxyuridine (BrdU) was given to the mice in drinking water at a concentration of 0.8mg/ml for 4 days before liver collection, unless otherwise indicated.

Mice were housed in rooms with constant temperature, humidity and 12h light/dark cycles. Food and water were available *ad libitum.* Animal care and experiments were all in accordance with the guidelines of the department of animal care at Hannover Medical School, Hannover, Germany.

3.2 Genotyping

3.2.1 DNA isolation from mouse tails

Three millimeter mouse tails were lysated in a buffer (10mM Tris-HCl pH 8.2, 400mM NaCl, 2mM EDTA), containing 2% SDS and 50U/ml proteinase K, overnight at 56℃. The next day, proteinase K was inactivated by heating for 10min at 96℃. Saturated NaCl was added to lysates and centrifuged for 10min at maximum speed. Finally, supernatant was taken and diluted by a ratio of 1:10 with distilled/deionized water.

3.2.2 Genotyping of *Fah* mice

Three primers were used for *Fah* genotyping:
066 – Forward primer 5'- CTA GGT CAA TGG CTG TTT GG -3'
067 – Common primer 5'- GGA CAT ACC AAT TTG GCA AC -3'
068 – Reverse primer 5'- TAA AAT GAG GAA ATT GCA TCG -3'

The PCR conditions were as follows: 2.5µl 10x reaction buffer (Genecraft), 0.5µl 2mM dNTPs, 1µl 10pmol primers, 0.5µl 5 units/µl Biotherm Taq DNA polymerase (Genecraft), 2µl 1:10 diluted DNA and 11.5µl distilled water. The PCR profile was as follows: initial denaturation at 94℃ for 2min., th en for cycling, denaturation at 94℃ for 40sec, annealing at 62℃ for 1min and extension at 72℃ for 1min. Thirty-four cycles of PCR amplification and a final extension step at 72℃ for 10min were performed. PCR products were run in a 1% agarose gel containing 1.5µl Gel Star Nucleic Acid Gel Stain (Lonza) per 100ml agarose gel. The primers amplified a fragment of 180bp for *Fah*$^{+/+}$ and 240bp for *Fah*$^{-/-}$ and both fragments for *Fah*$^{+/-}$.

3.2.3 Genotyping of *p21* mice

For *p21* genotyping, three primers were used:

PGK neo – 5'- GAA GAA CGA GAT CAG CAG -3'
p21 exon 144 – 5'- GAA CTT TGA CTT CGT CAG GG -3'
p21 geno U – 5'- ACA ACA CCT CCT GGT CAG AGG -3'

The PCR conditions were as follows: 5µl 5x Go Taq reaction buffer (Promega), 2µl 2mM dNTPs, 1µl 10pmol primers, 0.2µl 5 units/µl Go Taq DNA polymerase (Promega), 2µl 1:10 diluted DNA and 12.8µl distilled water. The PCR profile was as follows: initial denaturation at 95℃ for 2min., then for cycling, denaturation at 95℃ for 30sec, annealing at 62.5℃ for 30sec and extension at 72℃ for 1min. Thirty-five cycles of PCR amplification and a final extension step at 72℃ for 5min were performed. PCR products were run in a 1% agarose gel containing 1.5µl Gel Star Nucleic Acid Gel Stain (Lonza) per 100ml agarose gel. The primers amplified a fragment of ~700bp for $p21^{+/+}$ and ~100bp for $p21^{-/-}$ and both fragments for $p21^{+/-}$.

3.2.4 Genotyping of *p53 Alfp-cre* mice

The following pair primers were used for *p53* genotyping:
p53 A – 5'- CAC AAA AAC AGG TTA AAC CCA G -3'
p53 B – 5'- AGC ACA TAG GAG GCA GAG AC -3'

The PCR conditions were as follows: 2µl 10x reaction buffer (Genecraft), 2.5µl 2mM dNTPs, 1µl 10pmol primers, 2µl 25mM $MgCl_2$, 0.2µl 5units/µl Biotherm Taq DNA polymerase (Genecraft), 2µl 1:10 diluted DNA and 9.3µl distilled water. The PCR profile was as follows: initial denaturation at 94°C for 2min., then for cycling, denaturation at 94℃ for 30sec, annealing at 61℃ for 30sec and extension at 72℃ for 50sec. Thirty-five cycles of PCR amplification and a final extension step at 72℃ for 5min were performed. PCR products were run in a 1% agarose gel containing 1.5µl Gel Star Nucleic Acid Gel Stain (Lonza) per 100ml agarose gel. The primers amplified a fragment of 288bp for $p53^{+/+}$ and 370bp for $p53^{Lox/Lox}$ and both fragments for $p53^{+/Lox}$.

For *Alfp-cre* genotyping, the following pair primers were used:
Alfp-cre1 – 5'- GGA AAT GGT TTC CCG CAG ACC -3'
Alfp-cre2 – 5'- ACG GAA ATC CAT CGC TCG ACC -3'

The PCR conditions were as follows: 2.5µl 10x reaction buffer (Genecraft), 0.5µl 2mM dNTPs, 1µl 10pmol primers, 2µl 25mM $MgCl_2$, 0.2µl 5units/µl Biotherm Taq DNA polymerase (Genecraft), 2µl 1:10 diluted DNA, 10.8µl distilled water. The PCR profile was as follows: initial denaturation at 95°C for 2min., then for cycling, denaturation at 95℃ for 40sec, annealing at 62℃ for 1mi n and extension at 72℃ for 1min. Thirty-four cycles of PCR amplification and a final extension step at 72℃ for 10min were performed. PCR products were run in a 1% agarose gel containing 1.5µl Gel Star Nucleic Acid Gel Stain (Lonza) per 100ml agarose gel. The primers amplified a fragment of ~500bp for *Alfp-cre+.*

3.3 RAD001 (Everolimus)

RAD001, a rapamycin analogue, was provided by Novartis Pharma AG. RAD001 was formulated at 2% (w/v) in a microemulsion vehicle, which was diluted to the appropriate concentration in water just before administration by gavage. Different doses of RAD001 were tested; 10 mg/kg/day were subsequently used for the experiments.

3.4 Collection of samples

Shortly, mice were narcotized with a 10% ketamine/0.04% xylazine solution (12.5µl/g mouse). The abdomen cavity was longitudinally incised and the outer and inner skin was turned on one side and then to the other, so as to obtain an examination field as wide as possible. The complete liver was removed from the mouse.

3.4.1 Mouse liver collection

After taking out the liver from the mouse, it was split into parts and stored as follows: Left lateral, caudate lobes and half of the median lobe were frozen in liquid nitrogen and kept at -80℃ for protein analysis. Th e other half of the median lobe

was fixed in formalin and embedded in paraffin. The right lobe was embedded in OCT and kept at -20℃ (figure 8).

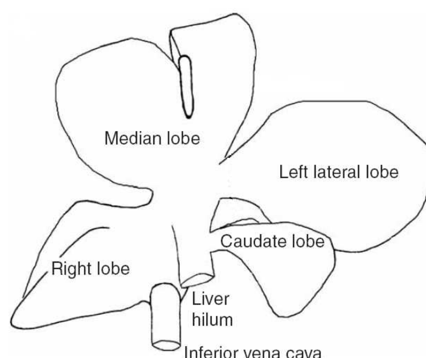

Figure 8 Mouse liver lobes

3.4.2 Mouse blood collection and serum measurements

Mice blood was taken from the orbital sinus using a glass capillary tube, collected in tubes containing heparin (Li-heparin LH/1.3 ml, Sarstedt) and processed as per manufacturer instructions. Bilirubin and glucose levels and transaminase activity were measured using an Olympus AU 400 System.

3.5 Western blot analysis

Liver tissue was homogenized in a cell lysis buffer (Cell Signaling) containing 1X Complete Protease Inhibitor mixture (Roche, Mannheim, Germany), centrifuged for 10min at 16000xg. Protein quantification was done from the supernatant by adding Bio-Rad Protein Assay Dye Reagent (80µl of 1:3000 protein extract/distilled water to 20µl reagent) and measuring OD at 595nm in a photometer. Then, 100µg protein extracts containing an electrophoresis sample buffer (4x ESB: 0,5M Tris Base, 40% glycerol, 12% SDS, 0,08% w/v bromophenol blue, 20% ß-mercaptoethanol) were resolved by SDS-PAGE (40mA for ~1.5h) and transferred onto a polyvinylidene di-

fluoride membrane (Millipore, Bedford, MA) using a western transfer apparatus (Bio-Rad Mini-Protean) with a transfer buffer (25mM Tris base, pH 8.3, 192mM glycine) for 1h at 100V. After blotting, membranes were blocked with 5% dry milk in TBS-Tween (50mM Tris base, pH 7.5, 150mM NaCl, 0.1% Tween) for 30min at RT. After that, membranes were incubated with the first antibody solution (5% dry milk in TBS-Tween for unphosphorylated antibodies and 5% BSA in TBS-Tween for phosphorylated antibodies) overnight at 4°C. The next day, membranes were washed with TBS-Tween and incubated with the respective secondary antibody (5% dry milk in TBS-Tween) for 1h at RT. After washings with TBS-Tween, detection of proteins was done using the chemiluminescence kit (Western Lightning®–ECL, Enhanced Chemiluminescence Substrate, PerkinElmer) and enhanced chemiluminescence films (Hyperfilm™ ECL, Amersham/GE Healthcare). Ponceau red staining was performed with each membrane to demonstrate equal protein loading of the samples.

3.5.1 Primary Antibodies

Name	**Company**
4E-BP1	Cell Signaling
Bax	Santa Cruz Biotechnologies
Bcl-x	BD Biosciences
Bid	R&D Systems
Bim	Sigma
c-jun	Cell Signaling
Caspase-9	Cell Signaling
CDK2	Santa Cruz Biotechnologies
CDK4	NeoMarkers
cIap1	Abcam
cyclin A	Santa Cruz Biotechnologies
cyclin B1	Santa Cruz Biotechnologies
cyclin D1	BD Biosciences
cyclin E	Santa Cruz Biotechnologies
eIF-4A	Cell Signaling
eIF-4E	Cell Signaling
Fas	Santa Cruz Biotechnologies

Flip-s	Stressgen
Mcl-1	Rockland
phospho-Akt (Ser473)	Cell Signaling
phospho-eIF-2α (Ser51)	Cell Signaling
phospho-eIF-4G (Ser1108)	Cell Signaling
phospho-S6 ribosomal protein (Ser240/244)	Cell Signaling
phospho-Rb (Ser780)	Cell Signaling
p21	BD Biosciences
p27	Santa Cruz Biotechnologies
p53	R&D Systems

3.5.2 Secondary Antibodies

Name	**Company**
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnologies
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnologies
Donkey anti-goat IgG-HRP	Santa Cruz Biotechnologies

3.6 Immunoprecipitation (IP)

Frozen liver tissue was grinded in liquid nitrogen, resuspended in RIPA buffer (1% NP40, 0,5% DOC, 0,1% SDS, 50mN Tris pH 8.0, 80mM NaCl, 50mM NaF, 1mM EDTA, 1mM EGTA, 20mM $Na_4P_2O_7$; Inhibitors: 0,1µM Vanadate, 2,5ng/ml Leupeptin, 0,15nM Benzamidine, 12,5ng/ml Pepstatin, 2,5mM PMSF) followed by sonication (20sec at amplification 4, cycle 1). Five-hundred micrograms of protein were pre-cleaned and incubated with 1µg of antibody for 2h at 4℃. Protein complexes were precipitated using protein A-sepharose beads for 1h at 4℃ and subjected to Western Blotting using the indicated antibodies.

3.7 Partial hepatectomy

C57BL/6 mice were operated on under sterile conditions between 8:00 and 11:00 A.M. (Mitchell and Willenbring, 2008). Shortly thereafter, the animals were narcotized with a 10% ketamine/0.04% xylazine solution (12.5µl/g mouse). The abdomen cavity was longitudinally incised just below to the rib cage. The left lateral and median lobes were individually ligated and removed. Then the abdomen was closed by suturing the inner and outer skin separately. The animals recovered on a 37℃ heating pad. The mortality of the animals following PH was <1%. Mice were treated either with RAD001 (10mg/kg/d) or a placebo 24 hours before and after partial hepatectomy. Two hours prior to harvest, the animals were injected with bromodeoxyuridine (BrdU, 100mg/kg) by intraperitoneal injection. For each time point studied, 3 animals were harvested.

3.8 Hepatocyte isolation and transplantation

Mouse liver was perfused using three different solutions (solution 1: EBSS without Ca and Mg with 0.5mM EGTA; solution 2: EBSS with Ca and Mg containing 10mM HEPES pH 7.4; solution 3: EBSS with Ca and Mg containing 10mM HEPES pH 7.4, 0.04mg/ml trypsin inhibitor, and 0.05mg/ml liberase blendzyme 3). After that, the liver was disrupted in FCS to stop collagenase activity by tearing and pipetting. Cells were filtered through a 100µM nylon mesh into a sterile falcon tube containing 10ml of media. Hepatocytes were pelled by centrifugation for 2min at 300rpm. Then wild-type hepatocytes were transplanted into *Fah/p21*$^{-/-}$ mice. One and one-half million cells were injected into the spleen. The animals were treated with 10mg/kg/d RAD001 or a placebo for 3 months after NTBC withdrawal and treated with BrdU containing 0.8mg/ml in drinking water for 3 days before harvesting. Five mice per group were harvested.

3.9 mAb Fas injection and caspase-3 activity

Fah-deficient mice (n=4) were treated with RAD001 for 14 days after NTBC withdrawal. At the end of the treatment, mice were injected intraperitoneally with a monoclonal anti-Fas antibody (Jo-2, purified anti-mouse CD95 (Fas); Becton Dickinson GmbH) at a dosage of 0.5µg/g mouse. The animals were harvested after 4h. Liver lysates were prepared by homogenization in hypotonic buffer (40mmol HEPES, 20% Glycerol, 4mmol DTT, pH 7.4). Lysates were centrifuged at 13,200rpm for 10min, and extracted proteins (50µg) were tested in triplicate experiments by measuring the proteolytic cleavage of a specific fluorogenic substrate for caspase-3 (Ac-DEVC-AMD, Caspase-3 (CPP32) Fluorogenic Substrate, Becton Dickinson GmbH). Samples treated with caspase-3 activity inhibitor (Ac-DEVD-CHO, Caspase-3 (CCP32) Inhibitor; Becton Dickinson GmbH) were used as reference.

3.10 Histology

3.10.1 Hematoxylin and Eosin (H&E) staining

Five micrometer sections were deparaffinazed in xylene and rehydrated in 3 different concentrations of ethanol solutions. After washing with distilled water, nuclei were stained with hematoxylin solution (Merck) for 15-30sec. Sections were washed with warm tap water to allow the stain to develop. After rinsing with distilled water, cytoplasms were stained with acidified eosin solution (Sigma-Aldrich) for 2min. Sections were rehydrated, cleared and mounted with a xylene-based mounting medium.

3.10.2 TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay

Detection of DNA strand breaks in apoptotic cells was performed with an *in situ* cell death detection kit (Roche). Five micrometer sections were deparaffinazed and rehydrated. After washing with distilled water, endogenous peroxidases were blocked

with 3% H_2O_2 for 10min. Antigens were unmasked by cooking sections in a microwave for 15min using citric acid retrieval buffer (10mM Tris Base, 0.05% Tween). Tissue permeabilization was done by incubating sections in a 20µg/ml proteinase K solution. Then, sections were fixed with 4% paraformaldehyde for 1h. After a second permeabilization with 0.1% Triton/0.1% sodium citrate, TUNEL analysis was performed by a fluorescein detection system, using reagents from a Roche kit. Sections were mounted with a DAPI-containing medium. Positive hepatocytes from 10 non-overlapping x200 fields per slide were counted.

3.10.3 5-bromo2'-deoxy-uridine (BrdU) staining

DNA synthesis was measured by BrdU incorporation into DNA strands. Shortly thereafter, 5µm sections were deparaffinized and rehydrated. After washing with distilled water, antigen unmasking was performed by incubating sections in citric acid retrieval buffer (10mM Tris Base, 0.05% Tween) in a water bath at 95℃ for 35min. After cooling down and washing with distiller water, DNA denaturation was performed in 2N HCl solution for 1h. Endogenous peroxidases were blocked with 3% H_2O_2 for 15min. To avoid non-specific binding of the antibody, sections were blocked with 10% normal goat serum for 30min. Then, sections were incubated with anti-BrdU antibody (1:200, 5% normal goat serum, GE Healthcare) overnight at 4℃. The next day, biotynilated goat anti-mouse IgG antibody (1:200, 5% normal goat serum, Invitrogen) was dropped into sections and incubated for 30min at RT. After washing, sections were incubated with HRP-Streptavidin Plus (Vector laboratories) for 30min at RT. Then, detection was done by adding AEC solution (Vector laboratories) until desired stain intensity developed. Sections were counterstained with hematoxylin and mounted with a water-based mounting media (Vector laboratories). Positive hepatocytes from 10 non-overlapping x200 fields per slide were counted.

3.10.4 Ki67 staining

Hepatocyte proliferation was checked by the presence of Ki67 cell cycle-related nuclear protein expressed in all phases of the active cell cycle. Shortly thereafter,

5µm sections were deparaffinized and rehydrated. After washing with distilled water, antigen unmasking was performed by incubating sections in EDTA retrieval buffer (1mM EDTA, 10mM Tris Base, 0.05% Tween) in a water bath at 95℃ for 35min. After cooling down and washing with distiller water, endogenous peroxidases were blocked with 3% H_2O_2 for 10min. Endogenous avidin/biotin were blocked with Avidin/Biotin solutions (Avidin/Biotin Blocking Kit, Vector laboratories) for 15min each at RT. To reduce unspecific binding of antibodies, sections were blocked with 5% normal goat serum for 1h at RT. Then, sections were incubated with anti-Ki67 antibody (1:500, 2% normal goat serum, Vector laboratories) overnight at 4℃. The next day, sections were incubated with broad-spectrum plus antibody (Vector laboratories) for 30min at RT. After washing, sections were incubated with HRP-Streptavidin Plus (Vector laboratories) for 30min at RT. Then, detection was done by adding AEC solution (Vector laboratories) until the desired stain intensity developed. Sections were counterstained with hematoxylin and mounted with a water-based mounting media (Vector laboratories).

3.10.5 Oval cell (A6) staining

Five micrometer sections were deparaffinazed and rehydrated. After washing with distiller water, endogenous peroxidases were blocked with 0.001M periodic acid and 0.1M sodium borohydride, both for 10 min at RT. Sections were incubated with anti-A6 antibody (1:100, provided by V. Factor (NIH, Washington DC)) overnight at 4℃. The next day, Alexa Fluor anti-rat antibody (1:100, Invitrogen) was applied to sections for 30min at RT. Finally, sections were mounted with a DAPI-containing medium.

3.10.6 p21 staining

Five micrometer sections were deparaffinazed and rehydrated. After washings with distilled water, the antigen unmasking was done by cooking the slides in a water bath with citrate retrieval buffer (10mM citrate acid, 0.05% Tween) for 35min at 96℃. After cooling down and washings, avidin/biotins were blocked using Avidin/Biotin solutions (Avidin/Biotin Blocking Kit, Vector laboratories) for 5min each

at RT. To prevent unspecific binding of antibody, sections were blocked with 5% normal horse serum for 30min at RT. Then, anti-p21 antibody (1:50, 2% normal horse serum, Santa Cruz Biotechnologies) was added to the sections and incubated overnight at 4℃. The next day, endogenous peroxida ses were blocked with 3% H_2O_2 for 10min at RT. Slides were incubated with biotynilated anti-goat antibody (ready-to-use antibody, Vector laboratories) for 30min at RT. Then, detection was done by adding AEC solution (Vector laboratories) until the desired stain intensity developed. Sections were counterstained with hematoxylin and mounted with a water-based mounting media (Vector laboratories).

3.10.7 Co-staining of Fah with BrdU

Five micrometer sections were deparaffinazed and rehydrated. After washings with distilled water, an antigen unmasking was done by cooking the slides in a water bath with citrate retrieval buffer (10mM citrate acid, 0.05% Tween) for 35min at 96℃. After cooling down and washings, DNA was dena turated by incubating slides in 2N HCl for 1h at RT. To reduce unspecific binding of antibodies, blocking was made by incubating slides with 5% normal goat serum for 15min at RT. Then, anti-BrdU antibody (1:200, 1% BSA, GE Healthcare) was applied to the slides and incubated overnight at 4℃. The next day, the slides we re incubated with Cy3 anti-mouse antibody (1:400, 1% BSA, Invitrogen) for 30min at RT. Anti-Fah antibody (1:1000, 1%BSA, provided by R. Tanguay) was added to the slides and incubated for 1h at RT. After that, the slides were incubated with Alexa Fluor 488 goat anti-rabbit antibody (1:1000, 1% BSA, Invitrogen) for 30min at RT. Finally, the slides were mounted with a DAPI-containing mounting medium.

3.10.8 Senescence-associated ß-Gal staining

Seven micrometer frozen sections were fixed in 0.2% glutaraldehyde for 3min. After washing with PBS, sections were incubated in freshly prepared senescence-associated ß-gal staining solution (1mg/ml of 5-bromo-4-chloro-3-indolyl ß-D galactoside (X-gal) in DMF, 40mM citric acid, sodium phosphate (pH 6.0), 5mM potas-

sium ferrocyanide, 5mM potassium ferricyanide, 150mM NaCl, 2mM $MgCl_2$) for 16-17h at 37℃. Sections were washed in PBS and counte rstained with eosin.

3.11 Microarray

Total RNAs from livers were extracted from 3 mice in each group using the Qiagen RNAeasy kit. The isolated RNAs were applied to murine Affymetrix GeneChip MOE 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, LA, USA) in the Microarray Core Facility of the Helmholtz Centre for Infection Research. Data analysis was performed using the Affymetrix Expression Analysis software GCOS 1.4.

3.12 Statistical analysis

Data are expressed as mean ± SD determined by one-way analysis of variance followed by Student T test to determine significance.

4 Results

4.1 RAD001 prevents proliferation of hepatocytes with DNA damage

Fah-deficient mice, that were taken off NTBC, mimic the liver failure and pathology of the human disease HT1 (Grompe et al., 1993). In order to analyze the role of mTOR in liver injury induced by FAA accumulation, Fah-deficient mice were taken off NTBC for 15 days and treated with either RAD001 or placebo. This time point was chosen because when mice are off NTBC for 15 days, they still have the same weight and overall health as mice on NTBC despite hepatic dysfunction.

First, the inhibition of mTOR activity by RAD001 treatment was verified. Phosphorylation of two major mTOR downstream targets, S6 and 4E-BP1, was analyzed by Western Blotting. Phosphorylation of S6 was evident in placebo-treated *Fah*$^{-/-}$ mice that were on and off NTBC. After RAD001 treatment, phosphorylation of S6 was markedly suppressed in those livers (figure 9). 4E-BP1 has at least five phosphorylation states, and the various phosphorylated forms of the protein are resolved into three bands by SDS-PAGE; those forms have been identified as α (least phosphorylated and fastest migrating), ß (intermediate) and γ (most phosphorylated and slowest migrating) (Yang et al., 2009). Placebo-treated Fah-deficient mice that were taken off NTBC, showed hyperphosphorylation of 4E-BP1; however, after RAD001 treatment, a slight reduction of 4E-BP1 phosphorylation was detected (figure 9).

Activation of Akt was analysed by Western Blotting. In placebo-treated *Fah*$^{-/-}$ mice, no activation of this kinase was observed. Suppression of mTORC1 activates Akt kinase in some cells due to a negative feedback loop between S6 kinase and insulin receptor substrate-1 (IRS1) (Guertin and Sabatini, 2007). However, no activation of Akt was seen in Fah-deficient mice on and off NTBC after RAD001 treatment (figure 9).

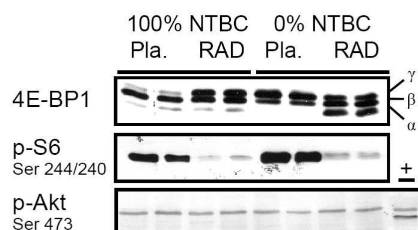

Figure 9 RAD001 significantly reduced the levels of S6 phosphorylation in Fah-deficient mice
RAD001 or placebo was administered to *Fah*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Phosphorylation of mTOR downstream targets S6 and 4E-BP1 were analyzed by Western Blotting. Positive control for phospho-Akt: Total liver lysate from insulin injected mice.

The mTOR pathway is involved in the initiation of cap-dependent translation. Therefore, the expression of eIF-4F components was also analyzed in Fah-deficient mice. In *Fah*$^{-/-}$ mice that were on NTBC, the expression of eIF-4E and eIF-4A was not altered by RAD001 treatment; contrary, no phosphorylation of eIF-4G and eIF-2α was detected in those mice. After NTBC withdrawal, induction of eIF-4E and phosphorylation of eIF-4G and eIF-2α was seen in *Fah*$^{-/-}$ mice; however, RAD001 treatment did not alter the expression of eIF-4E and eIF-4A, whereas eIF-4G and eIF-2α phosphorylation was moderately reduced (figure 10), indicating that the expression of translation initiation factors was not significantly affected by RAD001 treatment in *Fah*$^{-/-}$ mice.

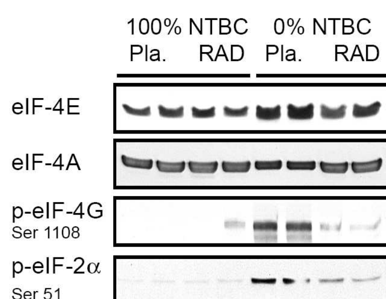

Figure 10 RAD001 treatment did not significantly affect the expression of translation initiation factors
RAD001 or placebo was administered to *Fah*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Immunoblots showing the expression of several translation initiation factors.

In addition, the effect of RAD001 in tyrosine aminotransferase (TAT), 4-hydroxyphenylpyruvate dioxygenase (HPD), homogentisic acid oxidase (HGD) and

maleylacetoacetate isomerise (MAAI), enzymes involved in the tyrosine catabolism pathway, was analyzed by microarrays. Previously, it was shown that accumulation of FAA leads to a negative feedback regulation of enzymes upstream in the tyrosine catabolism pathway (Langlois et al., 2006). However, a similar down regulation of all genes was evident in RAD001 and placebo-treated *Fah*$^{-/-}$ mice that were taken off NTBC, indicating that the RNA expression of those genes was not affected by the inhibition of mTOR; and thereby RAD001 treatment did not induce accumulation of the toxic intermediates in those mice (figure 11).

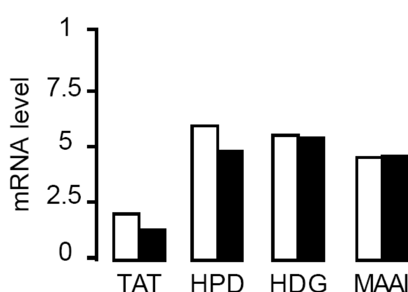

Figure 11 Expression levels of enzymes in the tyrosine catabolism pathway
mRNA levels of several enzymes involved in the tyrosine metabolism were measured by microarray analysis in mice that were on and off NTBC. Open bars: *Fah*$^{-/-}$ 100% NTBC placebo/*Fah*$^{-/-}$ 0% NTBC placebo, close bars: *Fah*$^{-/-}$ 100% NTBC RAD001/*Fah*$^{-/-}$ 0% NTBC RAD001.

The p53/p21 pathway is strongly activated in *Fah*$^{-/-}$ mice after NTBC withdrawal due to severe DNA damage induced by FAA (Willenbring et al., 2008a). Therefore, in this study the expression of several proteins involved in the pathway was analyzed by Western Blotting. Contrary to p27, no expression of p53 and p21 was detected in *Fah*$^{-/-}$ mice that were on NTBC (figure 12). As expected, up-regulation of p53 and p21 was observed in *Fah*$^{-/-}$ mice after NTBC withdrawal. Interestingly, the levels of those proteins were markedly reduced after RAD001 treatment and NTBC withdrawal (figure 12). Levels of p27 were slightly induced in *Fah*$^{-/-}$ mice that were taken off NTBC; however, RAD001 treatment did not affect them. These data suggest that RAD001 treatment blocks p53/p21 pathway in *Fah*$^{-/-}$ mice that were taken off NTBC.

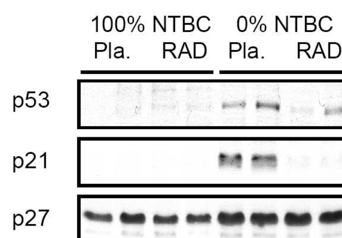

Figure 12 RAD001 significantly suppressed the p53/p21 pathway
RAD001 or placebo was administered to *Fah*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Immunoblots revealed reduced p53 and p21 protein levels in RAD001-treated *Fah*$^{-/-}$ mice that were off NTBC.

Histologies from *Fah*$^{-/-}$ mice that were on NTBC revealed no apparent pathology, whereas small foci of necroinflammation and some hepatocytes with multinucleation were evident in mice that were taken off NTBC (figure 13A). Treatment with RAD001 did not affect overall liver injury in livers from mice that were on and off NTBC (figure 13A).

It has been shown that proliferation is markedly reduced in Fah-deficient hepatocytes due to induction of p21 (Willenbring et al., 2008a). Here, hepatocyte proliferation was determined by BrdU incorporation. Several BrdU-positive hepatocytes were seen in both RAD001 and placebo-treated *Fah*$^{-/-}$ mice that were on NTBC, indicating that RAD001 treatment did not affect baseline proliferation of healthy hepatocytes (figure 13A,B). As expected, no BrdU-positive cells were found in *Fah*$^{-/-}$ mice that were off NTBC due to up-regulation of p21 (figure 13A). Interestingly, proliferation of hepatocytes was almost suppressed in RAD001-treated *Fah*$^{-/-}$ mice that were taken off NTBC despite the loss of p21 (figure 13A,B). Moreover, senescence was analyzed by SA-ß-Gal staining. No significant differences were found between RAD001 and placebo-treated *Fah*$^{-/-}$ mice that were on and off NTBC (figure 13A). Taking these together, the data suggest that treatment with RAD001 prevents the proliferation of hepatocytes with DNA damage despite the loss of p21.

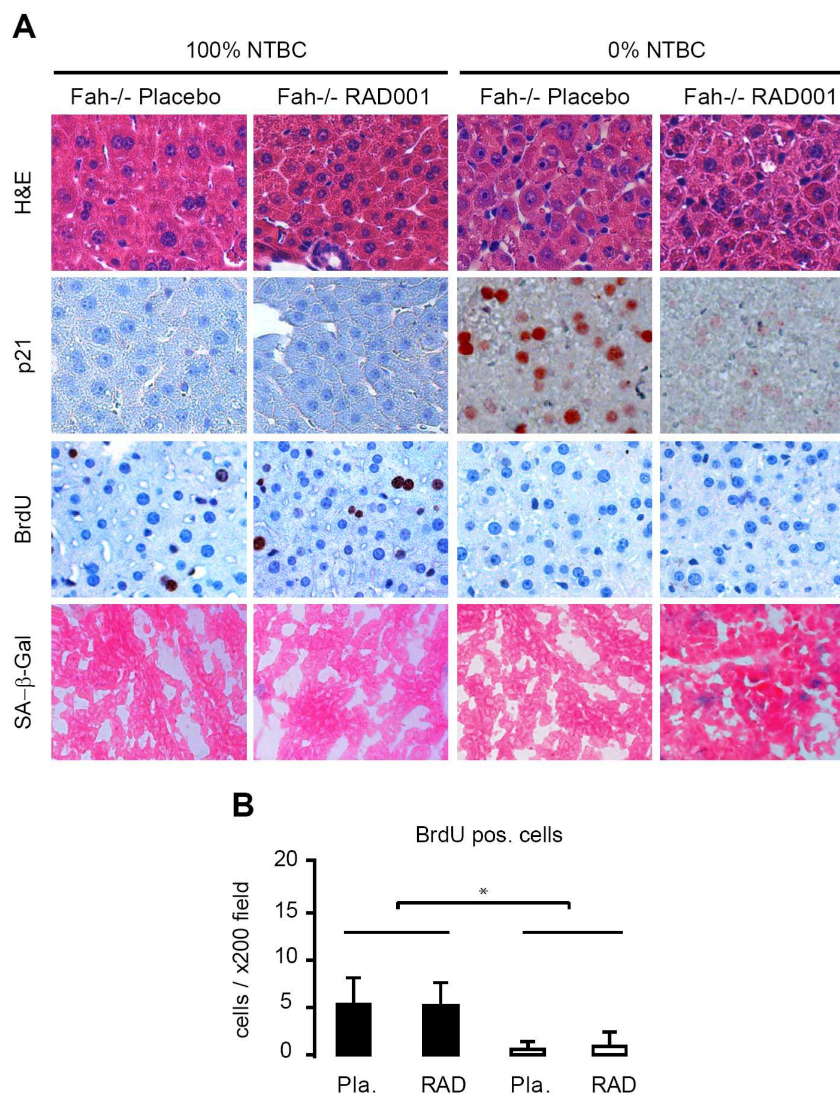

Figure 13 RAD001 significantly reduced the accumulation of p21 and suppressed proliferation of hepatocytes with DNA damage in *Fah*$^{-/-}$ mice taken off NTBC

RAD001 or placebo was administered to *Fah*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). **A** Representative H&E, p21, BrdU and SA-ß-Gal stainings of liver sections are shown (original magnification x200). **B** BrdU positive cells were counted at x200 magnification. Average ± SD is shown (*p≤0.0001, closed bars: on NTBC; open bars: off NTBC; Pla.:Placebo, RAD: RAD001).

4.2 RAD001-induced cell cycle arrest does not depend on p21

The data shown above suggest that the cell-cycle arrest induced by RAD001 treatment does not require p21. In order to confirm this observation, the drug was additionally tested in *Fah/p21*$^{-/-}$ mice that were on and off NTBC for 15 days.

Similar to the observation in *Fah*$^{-/-}$ mice, phosphorylation of S6 and 4E-BP1 was seen in placebo-treated *Fah/p21*$^{-/-}$ mice that were on and off NTBC (figure 14); however, RAD001 treatment blocked the phosphorylation of S6 and caused a slight shift of 4E-BP1 to the hypophosphorylated form in mice after NTBC withdrawal. Akt levels were not detectable in those mice (figure 14).

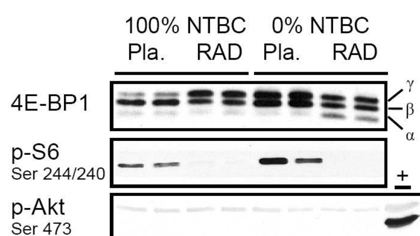

Figure 14 RAD001 reduces the phosphorylation levels of S6 in *Fah/p21*$^{-/-}$ mice taken off NTBC
RAD001 or placebo was given to *Fah/p21*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Immunoblots from total liver extract are shown. Phosphorylation of S6 and 4E-BP1 was reduced in RAD001-treated mice. Positive control for phospho-Akt: Total liver lysate from insulin-injected mice.

Expression of several translation initiation factors was analyzed by Western Blotting. In *Fah/p21*$^{-/-}$ mice that were on NTBC, eIF-4E and eIF-4A levels were not affected by RAD001 treatment. Contrary, no phosphorylation of eIF-4G and eIF-2α was seen in those mice (figure 15). After NTBC withdrawal, levels of eIF-4E and phosphorylation of eIF-4G and eIF-2α were increased (figure 15). However, RAD001 treatment significantly reduced the expression of those proteins. Levels of eIF-4A were not altered (figure 15). Similar to *Fah*$^{-/-}$ mice, RAD001 did not significantly affect the expression of translation initiation factors in *Fah/p21*$^{-/-}$ mice.

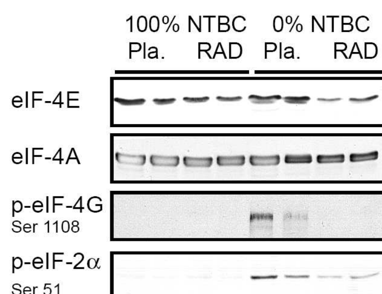

Figure 15 Expression of eIF-4F components in *Fah/p21*$^{-/-}$ mice
RAD001 or placebo was given to *Fah/p21*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Expression of several translation initiation factors was analyzed by Western Blotting.

Additionally, activation of p53/p21 pathway was also analysed. In *Fah/p21*$^{-/-}$ mice that were on NTBC, p27 expression levels were not changed after RAD001 treatment (figure 16). After NTBC withdrawal, the levels of p53 expression were increased in placebo-treated *Fah/p21*$^{-/-}$ mice; however, they were significantly reduced in RAD001-treated mice (figure 16). Moreover, p27 levels were slightly reduced in mice that were taken off NTBC after RAD001 treatment when compared with placebo-treated mice (figure 16). Immunoblot of p21 confirmed the absence of the protein in those mice. These data suggest that RAD001 treatment reduces the p53 expression in *Fah/p21*$^{-/-}$ mice that were taken off NTBC.

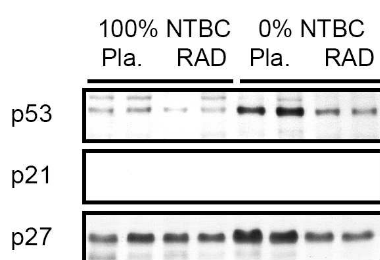

Figure 16 Protein levels of p53 were reduced in RAD001 *Fah/p21*$^{-/-}$-treated mice
RAD001 or placebo was given to *Fah/p21*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Immunoblots showing the expression of p53, p21 and p27.

Liver histology did not reveal significant differences between RAD001 and placebo *Fah/p21*$^{-/-}$-treated mice (figure 17A). Moreover, proliferation of hepatocytes was determined by BrdU incorporation. Similar to the observation in *Fah*$^{-/-}$ mice, treatment with RAD001 did not affect proliferation of healthy hepatocytes; a similar amount of

BrdU positive hepatocytes was seen in both RAD001 and placebo-treated *Fah/p21*$^{-/-}$ mice that were on NTBC (figure 17A,B). In *Fah/p21*$^{-/-}$ livers from mice that were taken off NTBC, BrdU staining showed a high number of positive hepatocytes. In contrast, however, few BrdU positive cells were found in RAD001-treated *Fah/p21*$^{-/-}$ mice that were off NTBC (figure 17A,B). SA-ß-Gal staining showed no significant differences between RAD001 and placebo-treated *Fah/p21*$^{-/-}$ mice that were on and off NTBC (figure 17A). Altogether, the data presented above suggest that the cell-cycle arrest that was induced in liver by RAD001 treatment is clearly independent of p21.

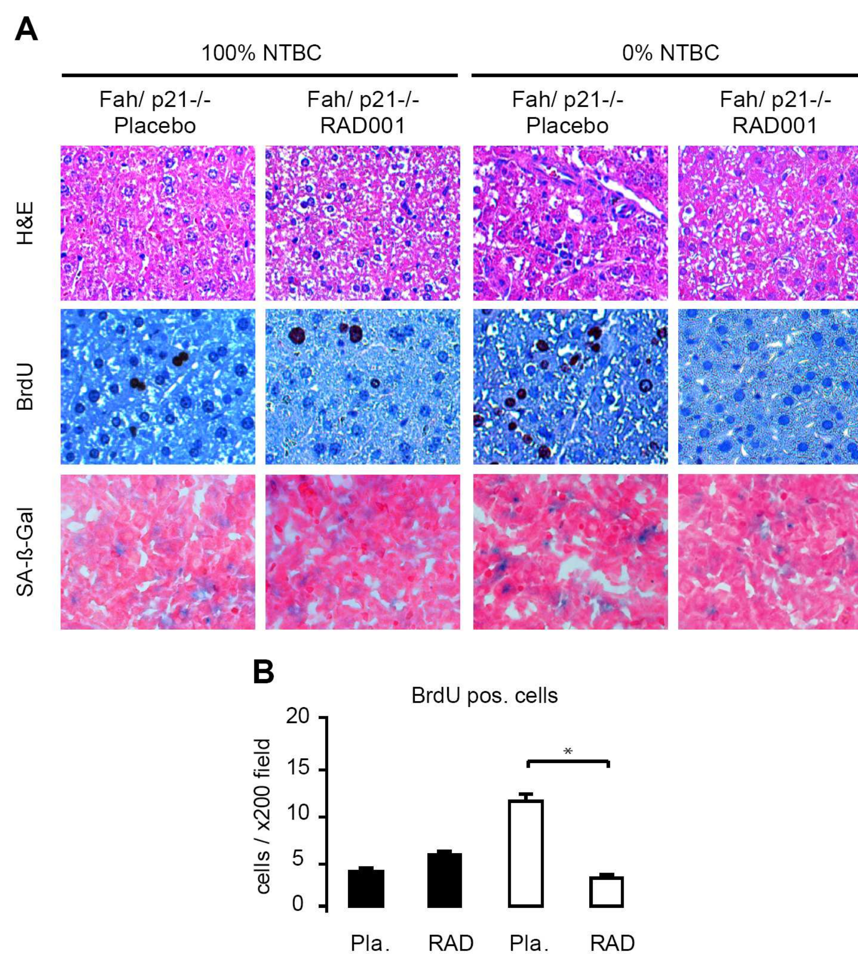

Figure 17 The ability of RAD001 to impair proliferation of hepatocytes is clearly independent on p21
RAD001 or placebo was given to *Fah/p21*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). **A** Representative H&E, BrdU and SA-ß-Gal immunohistostainings. **B** BrdU positive hepatocytes were counted in x200 magnification and results are represented as mean ± SD (*p≤0.0001, closed bars: on NTBC; open bars: off NTBC, Pla.:Placebo, RAD: RAD001).

4.3 RAD001-induced cell-cycle arrest is attenuated in absence of p53

In order to determine whether the cell-cycle arrest induced by RAD001 treatment requires p53, the effect of the drug was examined in *Fah/p53*$^{-/-}$ mice, in which p53 was specifically deleted in hepatocytes. Mice that were on and off NTBC were treated with either RAD001 or placebo for 15 days. In *Fah/p53*$^{-/-}$ mice that were on NTBC, RAD001 treatment did not affect the expression of 4E-BP1; phosphorylation of S6 and Akt was not detected in those mice (figure 18). Similar to the observations seen in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice, *Fah/p53*$^{-/-}$ mice that were taken off NTBC showed phosphorylation of S6 and 4E-BP1 and no expression of Akt. RAD001 treatment blocked the phosphorylation of S6 and did not affect phosphorylation of Akt. In contrast to *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice, treatment with RAD001 did not modify the phosphorylation of 4E-BP1 in *Fah/p53*$^{-/-}$ mice (figure 18).

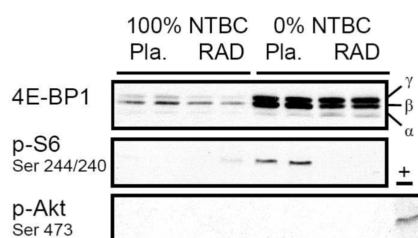

Figure 18 4E-BP1 and phosphorylation of S6 and Akt in *Fah/p53*$^{-/-}$ mice
RAD001 or placebo was administered to *Fah/p53*$^{-/-}$ mice for 14 days after NTBC withdrawal (n=4). Immunoblots showing the levels of designed proteins in livers. Positive control for phospho-Akt: Total liver lysate from insulin-injected mice.

Treatment with RAD001 did not affect the protein expression of eIF-4E and eIF-4A in *Fah/p53*$^{-/-}$ mice that were on NTBC; phosphorylation of eIF-4G and eIF-2α was not detected in those mice (figure 19). However, phosphorylation of eIF-4G and eIF-2α was increased in placebo-treated *Fah/p53*$^{-/-}$ mice that were taken off NTBC. Moreover, phosphorylation levels of eIF-4G were reduced after RAD001 treatment; interestingly, no changes in phosphorylation of eIF-2α were detected. No significant differences in eIF-4E and eIF-4A levels were seen in *Fah/p53*$^{-/-}$ mice that were taken off NTBC (figure 19).

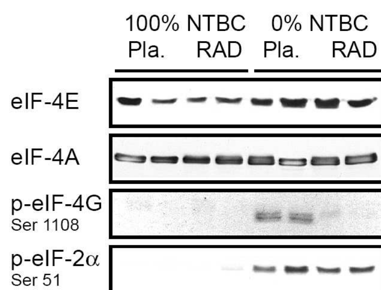

Figure 19 Expression of eIF-4F components in *Fah/p53$^{-/-}$* mice
RAD001 or placebo was administered to *Fah/p53$^{-/-}$* mice for 14 days after NTBC withdrawal (n=4). Immunoblots showing the levels of designed proteins in livers.

Furthermore, activation of p53/p21 pathway was analyzed. Low levels of p27 were detected in *Fah/p53$^{-/-}$* mice that were on NTBC. After NTBC withdrawal, significantly low levels of p21 were detectable in placebo-treated *Fah/p53$^{-/-}$* mice, which were reduced by treatment with RAD001 (figure 20). No significant changes in p27 levels in RAD001 and placebo-treated *Fah/p53$^{-/-}$* mice that were taken off NTBC were seen (figure 20). Immunoblot of p53 confirmed the absence of the protein in those mice.

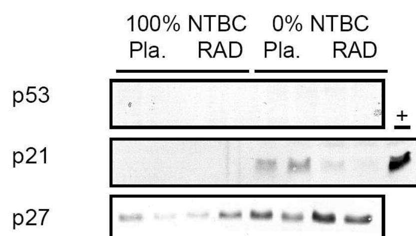

Figure 20 p21 expression is reduced after RAD001 treatment in *Fah/p53$^{-/-}$* mice that were taken off NTBC
Immunoblots showing the protein levels of p53, p21 and p27. Control for p21: total liver lysate from *Fah$^{-/-}$* that were off NTBC mice.

Histology did not reveal significant differences between RAD001 and placebo-treated livers of *Fah/p53$^{-/-}$* mice. Hepatocyte proliferation was determined by BrdU incorporation. Similar to the observations in *Fah$^{-/-}$* and *Fah/p21$^{-/-}$*, treatment with RAD001 did not affect proliferation of healthy hepatocytes (figure 21A,B). Additionally, placebo-treated *Fah/p53$^{-/-}$* mice that were taken off NTBC displayed BrdU-positive hepatocytes despite activation of p21 (figure 21A). Interestingly, however, BrdU-positive hepatocytes were still detectable after RAD001 treatment, although there was complete blocking of p21 expression (figure 21A). Treatment with RAD001 did not completely suppress the proliferation of hepatocytes in *Fah/p53$^{-/-}$*

mice that were taken off NTBC (figure 21A,B). Additionally, no significant differences were seen in SA-ß-Gal staining after RAD001 treatment (figure 21A). Altogether, these data suggest that the ability of RAD001 to impair the cell cycle is independent of p21 but requires an intact p53 pathway.

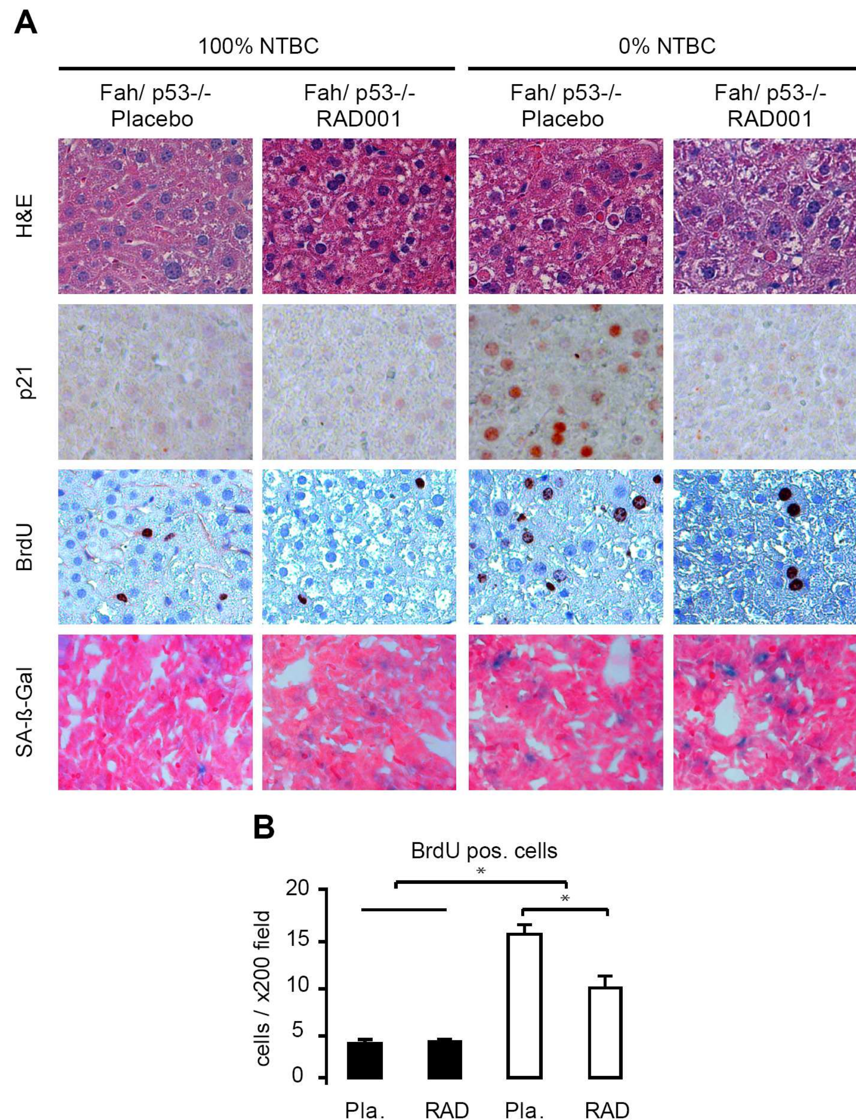

Figure 21 Loss of p53 significantly attenuated the anti-proliferative effects of RAD001
RAD001 or placebo was administered to *Fah/p53*$^{-/-}$ mice for 14 days after NTBC withdrawal. **A** Representative H&E, p21, BrdU and SA-ß-Gal immunohistostainings. **B** BrdU-positive hepatocytes were counted in x200 magnification and results are represented as mean ± SD (*p≤0.0001, closed bars: on NTBC; open bars: off NTBC, Pla.:Placebo, RAD: RAD001).

4.4 RAD001 delays proliferation of healthy hepatocytes following partial hepatectomy

The data above revealed that RAD001 treatment specifically inhibits proliferation of damaged hepatocytes. Surprisingly, however, treatment with RAD001 did not affect baseline proliferation of healthy hepatocytes: a similar number of BrdU-positive cells was detectable in RAD001 and placebo-treated *Fah*$^{-/-}$, *Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ mice that were on NTBC, suggesting that RAD001 is not, or at least less, effective in healthy cells. Therefore, to analyze the effect of RAD001 on healthy hepatocytes, C57BL/6 mice were treated either with RAD001 or placebo before and after 2/3 partial hepatectomy. Livers were collected at different time points.

Liver regeneration following 2/3 partial hepatectomy offers a unique in vivo system to study gene regulation and cell-cycle processes in adults, differentiated, non-transformed cells. After resection of the left lateral and median lobes of the liver, the remaining hepatocytes and non-parenchymal cells synchronously exit their resting G0 state. Entrance into G1 and the cell cycle is marked by a similar pattern of cyclin and CDK genes induction and expression. Peaks in DNA synthesis for mice occur around 36h after partial hepatectomy. Mitosis proceeds 6-8h after DNA synthesis. The re-growth of the liver following partial hepatectomy represents compensatory hyperplasia of the remaining cells and does not involve de-differentiation of the remaining cells. Within 4 days, the liver cell number is almost completely returned to normal, and within 7-10 days the extracellular matrix, micro-architecture and liver mass are restored. Many of the cyclins demonstrate dramatic patterns of cell cycle-related expression at the transcript level following partial hepatectomy in mice. Cyclins D exhibits induced transcript abundances that fluctuate and peak coincident with the G1 phase of the cell cycle.

In this study, phosphorylation levels of S6 and 4E-BP1 were determined. After 6 hours of partial hepatectomy, phosphorylation levels of S6 were increased, which were clearly reduced by RAD001 treatment (figure 22). In contrast, levels of 4E-BP1 were similar for RAD001 and placebo-treated C57BL/6 mice following partial hepatectomy. No phosphorylation of Akt was seen in those mice (figure 22).

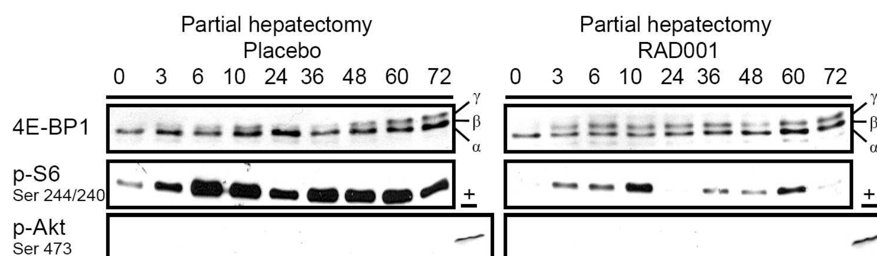

Figure 22 Levels of 4E-BP1 and phosphorylation of S6 and Akt in C57BL/6 mice RAD001 and placebo-treated after partial hepatectomy
Partial hepatectomy was performed as described in Materials and Methods. RAD001 or placebo was administrated to mice two days before surgery until harvesting. Immunoblots showing the levels of designed proteins in livers. Positive control for phospho-Akt: Total liver lysate from insulin-injected mice.

Moreover, the expression of eIF-4F components was determined. Protein levels of eIF-4E and eIF-4A were not altered after partial hepatectomy, and treatment with RAD001 did not affect them. Induction of eIF-4G protein was detected in placebo-treated mice after 6h of partial hepatectomy, which was markedly delayed up to 60h by RAD001 treatment. Similarly, increased phosphorylation of eIF-2α was detected in placebo-treated mice after 6h and 10h of partial hepatectomy, which was clearly delayed in RAD001-treated mice (figure 23). Additionally, no activation of Akt was detected in RAD001 and placebo-treated C57BL/6 mice after partial hepatectomy (figure 23).

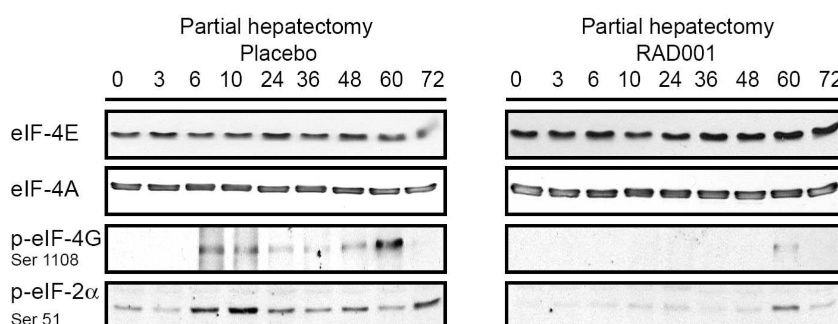

Figure 23 Expression of eIF-4F components in RAD001 and placebo-treated C57BL/6 mice after partial hepatectomy
Partial hepatectomy was performed as described in Materials and Methods. RAD001 or placebo was administrated to mice two days before surgery until harvesting. Immunoblots showing levels of designed proteins in livers.

Histology did not reveal significant differences between RAD001 and placebo-treated mice after partial hepatectomy (figure 24). Furthermore, hepatocyte proliferation was analysed by BrdU incorporation. Placebo-treated C57BL/6 mice dis-

played multiple BrdU-positive cells 36h after partial hepatectomy, which occurred at the peak of DNA synthesis. In contrast, no BrdU-positive cells were detectable in RAD001-treated mice 36h after partial hepatectomy. Interestingly, however, multiple BrdU-positive cells appeared 72h after partial hepatectomy in RAD001-treated mice, suggesting that treatment with RAD001 did not prevent but delayed entry for about the 36h into S-phase on cell cycle (figure 24).

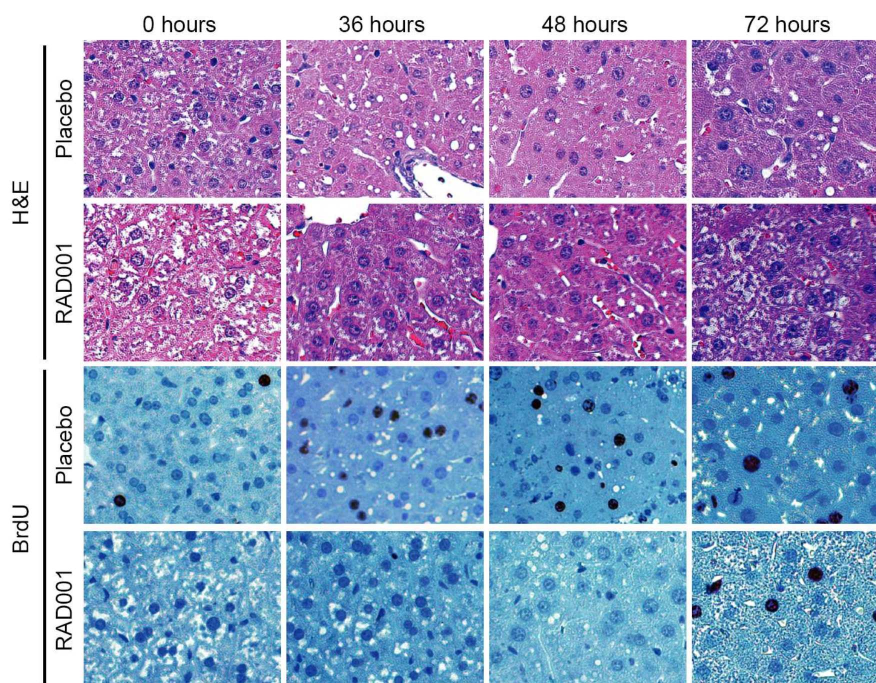

Figure 24 RAD001 only delay proliferation of healthy hepatocytes after partial hepatectomy
Partial hepatectomy was performed as described in Materials and Methods. RAD001 or placebo was administered to mice two days before surgery until harvesting. Histological sections of paraffin-embedded livers are shown. Proliferating cells were detected by BrdU staining. Treatment with RAD001 significantly delayed entry into the S-phase of C57BL/6 hepatocytes after PH.

4.5 RAD001 affects global protein translation and expression of several cell cycle-related proteins

Previous studies have shown that rapamycin and its analogues inhibit protein translation (Fingar and Blenis, 2004; Tang et al., 2001). In order to analyze the effect of

RAD001 on overall protein translation, nutrient-induced growth after a period of fasting was determined. Fasting triggers a 40-50% reduction in liver weight, total protein and ribosome content (Volarevic et al., 2000). To determine weather RAD001 inhibits hepatocyte proliferation by affecting protein translation, C57BL/6 mice treated either with RAD001 or placebo were deprived of food for 48h before re-feeding for 24h.

First, overall protein content was determined in both groups; however, no significant differences were found (figure 25). Next, to specifically analyze the effect of RAD001 on protein synthesis, RAD001 was given at the beginning of the re-feeding phase to the C57BL/6 mice. In this second approach, treatment with RAD001 significantly suppressed overall protein content (figure 25).

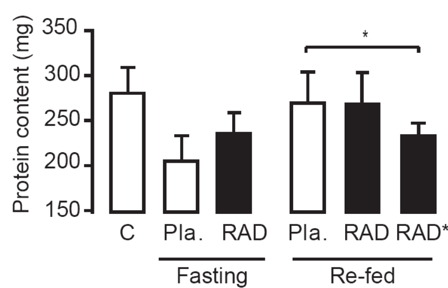

Figure 25 RAD001 affects the protein content in liver
C57BL/6 mice were fasted for 48h and re-fed for 24h. RAD001 or placebo was administered during both the fasting and re-feeding period. Protein content was determined in livers of RAD001 and placebo-treated mice (*$p \leq 0.0001$, C: control group; Pla.: Placebo; RAD: RAD001; RAD*: RAD001 treatment only during re-feeding phase).

Interestingly, phosphorylation levels of 4E-BP1 and Akt plus the expression of eIF-4E were not altered during starvation and re-feeding with RAD001 treatment. Phosphorylation of S6 was inhibited only in RAD001-treated mice during re-feeding period (figure 26). Together, these data show that inhibition of mTOR by RAD001 significantly inhibits overall protein synthesis in hepatocytes.

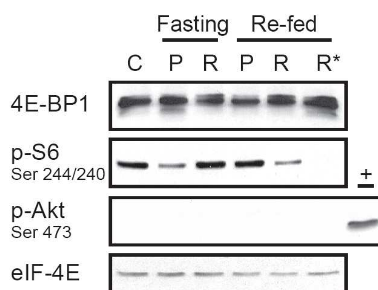

Figure 26 Immunoblots showing protein expression of 4E-BP1 and eIF-4E and phosphorylation of S6 and Akt in fasted and refed livers
Positive control for phospho-Akt: Total liver lysate from insulin-injected mice.

To understand better how RAD001 inhibits proliferation of hepatocytes, the expression of cell cycle-regulating proteins was analyzed in Fah-deficient mice that were on and off NTBC and in C57BL/6 mice following partial hepatectomy. In *Fah*$^{-/-}$ mice that were on NTBC, levels of cyclin E and B remain unchanged after RAD001 and placebo treatment (figure 27). No expression of phospho-Rb, cyclin D1 and A, and c-jun was detected in those mice. However, after NTBC withdrawal, an increase in those proteins was seen in placebo treated-mice (figure 27). Interestingly, RAD001 treatment reduced the phosphorylation of Rb and the expression of cyclin D1 and c-jun. No changes of levels of cyclin E and B were seen in mice that were taken off NTBC (figure 27).

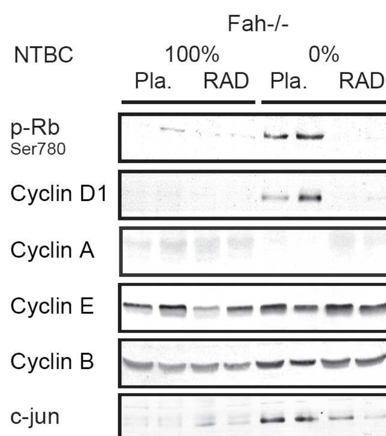

Figure 27 Expression levels of several cell cycle related protein in *Fah*$^{-/-}$ mice
RAD001 or placebo was administered to *Fah*$^{-/-}$ mice for 14 days after NTBC withdrawal. Immunoblots showing the expression of designed proteins.

In *Fah/p21*$^{-/-}$ mice that were on NTBC, the expression of cyclin D1, E and B was detected in placebo-treated mice, and no significant changes were seen after RAD001

treatment (figure 28). No phosphorylation of Rb and expression cyclin A and c-jun was observed in those mice. After NTBC withdrawal, however, the expression was increased in most of the protein analyzed with the exception of cyclin A. Similar to the *Fah*$^{-/-}$ mice, RAD001 treatment suppressed the phosphorylation of Rb and the expression of cyclin D and c-jun (figure 28). No significant changes in levels of cyclin E and B were seen in mice that were taken off NTBC.

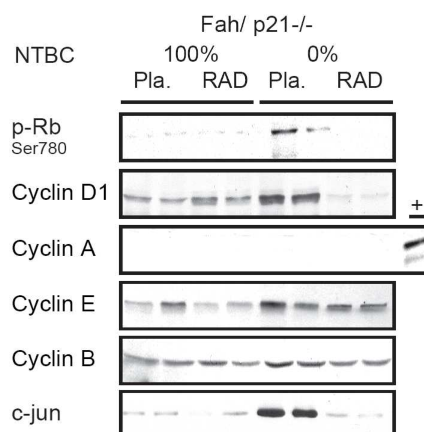

Figure 28 Levels of several cell-cycle related proteins in *Fah/p21*$^{-/}$ mice RAD001 or placebo was administered to *Fah/p21* $^{-/-}$ mice for 14 days after NTBC withdrawal. Immunoblots showing the expression of designed proteins. Positive control for cyclin A: Total liver lysate from C57BL/6 mice after 48h partial hepatectomy.

In *Fah/p53*$^{-/-}$ mice that were on NTBC, the expression of phospho-Rb, cyclin E and B was detected in placebo-treated mice; after RAD001 treatment, levels of phospho-Rb and cyclin E were slightly reduced (figure 29). Interestingly, all proteins analyzed were significantly induced after NTBC withdrawal. RAD001 treatment notably reduced the phosphorylation of Rb, cyclin D1 and A. No significant changes were seen in cyclin E and B levels. Interestingly, RAD001 suppressed the expression of c-jun in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice that were off NTBC, in which the proliferation was inhibited. In contrast, c-jun levels were markedly higher in RAD001-treated *Fah/p53*$^{-/-}$ mice that were off NTBC, in which proliferation was not completely inhibited (figure 29).

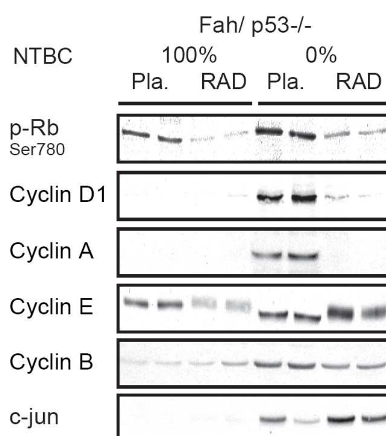

Figure 29 Levels of several cell cycle-related proteins in *Fah/p53*$^{-/-}$ mice RAD001 or placebo was administered to *Fah/p53* $^{-/-}$ mice for 14 days after NTBC withdrawal. Immunoblots showing the expression of designed proteins.

To mediate proliferation, Rb activity is regulated during cell-cycle progression. Mitogenic signaling cascades stimulate the activity of CDK complexes that phosphorylate Rb. This phosphorylation attenuates the interaction of Rb with E2F family members. Thus, as cells progress through cell cycle, Rb-mediated repression is alleviated. In Fah-deficient mice taken off NTBC, Rb was activated. Interestingly, treatment with RAD001 significantly reduced phosphorylation of Rb in those mice (figures 27-29).

Next, cell cycle related proteins were analyzed in RAD001 and placebo-treated mice following partial hepatectomy. Levels of cyclin D1 increased 24h after partial hepatectomy in placebo-treated mice. However, the expression of cyclin D1 was significantly delayed in RAD001-treated mice. Additionally, cyclin E and A protein levels were diminished in RAD001-treated mice when compared with placebo-treated C57BL/6 mice. Similar to Fah-deficient mice, no significant changes were seen in cyclin B levels (figure 30). Expression of c-jun, which peaked at 6h in placebo-treated mice, was significantly delayed in RAD001-treated mice (figure 30) where the hepatocyte proliferation was also delayed.

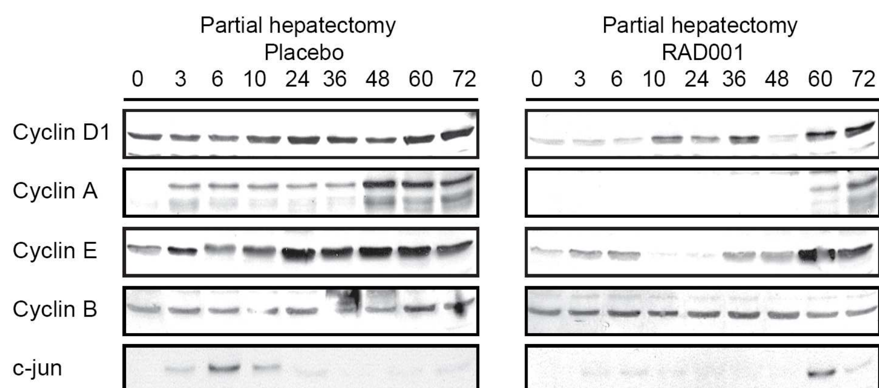

Figure 30 RAD001 affects the expression levels of several proteins related to cell cycle
Liver extracts from C57BL/6 mice after partial hepatectomy were prepared and representative samples were subjected to Western Blotting using the indicated antibodies.

As showed above, p21 expression is reduced by treatment with RAD001 in contrast to p27 levels that were only moderately affected by RAD001. In order to analyze the effect of RAD001 on p27 and CDK-cyclin complexes, immunoprecipitation studies were performed with Fah-deficient mice.

Immunobloting of p27 and cyclin E immunocomplexes showed high levels of CDK-cyclin complexes in *Fah*$^{-/-}$, *Fah/p21*$^{-/-}$and *Fah/p53*$^{-/-}$ mice that were taken off NTBC. However, treatment with RAD001 considerably disturbed the interaction of p27 with CDK2, CDK4 and cyclin E in those mice (figure 31), suggesting that RAD001 inhibits factors involved in transition from the G0 phase to the G1 phase in the cell cycle.

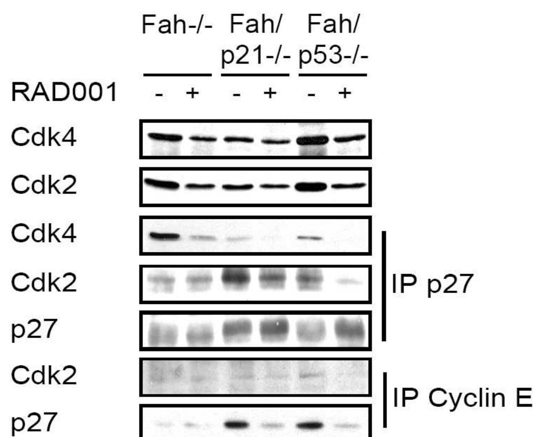

Figure 31 Immunobloting of p27 and cyclin E immunocomplexes in Fah-deficient mice
Analysis of the expression level of CDK2 and CDK4 *in Fah*$^{-/-}$*, Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ mice that were taken off NTBC. p27 and cyclin E were immunoprecipitated from liver extracts, and the amount of bound CDK4, CDK2 or p27 was determined by Western Blotting.

Additionally, when RAD001 was given 10, 20 or 30 hours after partial hepatectomy to wild-type mice, it only showed an antiproliferative effect 10 hours after surgery. Hepatocytes that already passed the early G0/G1 restriction point were no longer responsive to RAD001, and similar to placebo-treated mice, livers displayed many BrdU and Ki67 positive hepatocytes (figure 32). Taken together, RAD001 treatment regulates the interaction of p27 with CDK-cyclin complexes, preventing cell-cycle progression from the G0/G1 phase into the S-phase.

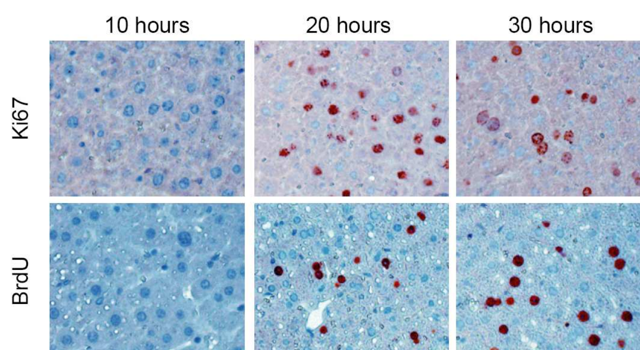

Figure 32 Hepatocytes that already passed the early G0/G1 restriction point were no longer responsive to RAD001
RAD001 was given to C57BL/6 mice at 10, 20 or 30h and harvested at 36h after partial hepatectomy. Proliferation rates were analyzed by Ki67 and BrdU labelling.

4.6 RAD001 more effectively inhibits proliferation of hepatocytes with DNA damage

To confirm the observation that RAD001 more effectively prevents proliferation of damaged instead of healthy hepatocytes, wild type hepatocytes were transplanted into *Fah/p21*$^{-/-}$ mice after NTBC withdrawal and treated either with RAD001 or placebo. This approach permit to analyze the effect of RAD001 on healthy, transplanted WT hepatocytes and on damaged *Fah/ p21*$^{-/-}$ recipient hepatocytes in same mouse.

Three months after transplantation, the livers were normal in shape and size. Then, cell proliferation and apoptosis were analysed by BrdU incorporation and TUNEL staining respectively. BrdU staining revealed several positive cells, but no TUNEL-positive hepatocytes were detectable in livers of transplanted RAD001-treated *Fah/p21*$^{-/-}$ mice (figure 33). Fah immunostaining showed that more than 60% of the liver parenchyma was re-populated with WT Fah-positive transplanted hepatocytes (figure 33). Interestingly, however, almost all BrdU positive cells were also positive for Fah in RAD001-treated mice in contrast with placebo-treated mice, in which BrdU positive cells were both Fah-negative and Fah-positive (figure 33).

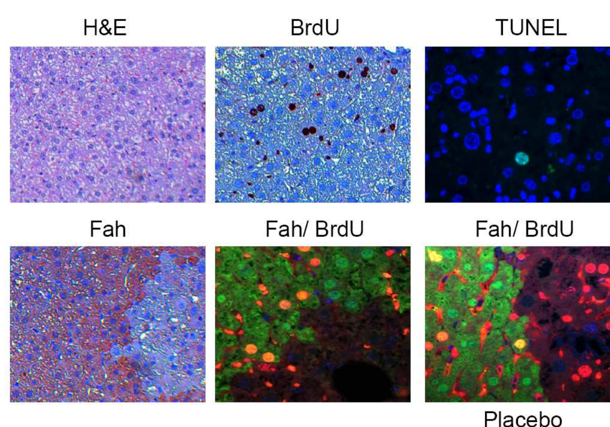

Figure 33 RAD001 inhibits, more effectively, the proliferation of hepatocytes with DNA damage
Wild-type hepatocytes were transplanted to *Fah/p21*$^{-/-}$ mice and treated for 3 months with RAD001 after NTBC withdrawal. Representative H&E staining and immunohistochemical detection of Fah are shown. Proliferation and apoptosis were determined by BrdU and TUNEL staining. Proliferating hepatocytes in RAD001 treated livers are Fah-positive.

Subsequently, phosphorylation levels of S6, 4E-BP1 and Akt were determined. In *Fah/p21*$^{-/-}$ mice that were on NTBC, only 4E-BP1 phosphorylation was detected. After NTBC withdrawal, S6 phosphorylation was significantly increased, which was totally suppressed after RAD001 treatment in both groups, transplanted and control mice; contrary, no changes in 4E-BP1 levels were seen (figure 34).

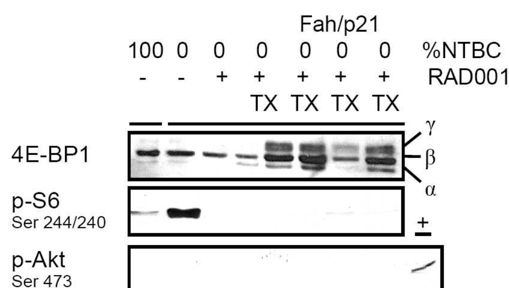

Figure 34 4E-BP1 and phosphorylation of S6 and Akt levels in *Fah/p21*$^{-/-}$-transplanted mice
Immunoblots showing the protein levels of 4E-BP1, phospho-S6 and phospho-Akt from total liver lysates. Positive control for phospho-Akt: Total liver lysate from insulin-injected mice. TX: transplanted mice.

Additionally, levels of cyclin D1 were high in RAD001-treated transplanted mice; in contrast, c-jun was not detectable (figure 35). Altogether, these data confirm that RAD001 treatment more effectively inhibits proliferation of hepatocytes with DNA damage.

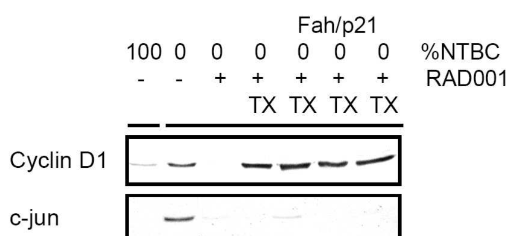

Figure 35 Cyclin D1 and c-jun levels in *Fah/p21*$^{-/-}$-transplanted mice
Immunoblots showing the protein levels of cyclin D1 and c-jun from liver lysates. TX: transplanted mice.

4.7 RAD001 sustains apoptosis sensitivity in hepatocytes during chronic liver injury

Previously, it has been shown that Fah-deficient mice develop a profound resistance against apoptosis following NTBC withdrawal (Vogel et al., 2004). On the other hand, several reports have shown that rapamycin and its analogues enhance apoptosis sensitivity in tumor cells (Beuvink et al., 2005). In this study, apoptosis was analysed by TUNEL staining. As expected, almost no apoptotic hepatocytes were detected in *Fah*$^{-/-}$ mice that were taken off NTBC. In contrast, *Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ mice exhibited an increased number of apoptotic hepatocytes, which was not significantly changed in mice that were treated with RAD001 (figure 36A,B).

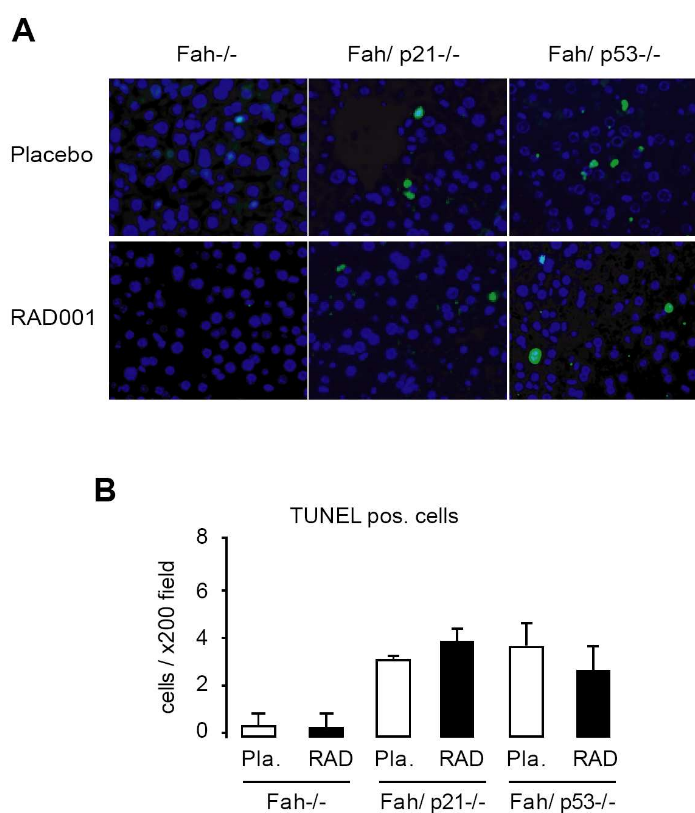

Figure 36 TUNEL staining in *Fah*$^{-/-}$, *Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ mice after NTBC withdrawal
Fah$^{-/-}$, *Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ mice were treated either with RAD001 or placebo for 14 days after NTBC withdrawal. TUNEL staining was performed in liver sections. Positive cells were counted in an x200 magnification. Average ± SD is shown.

To investigate in more detail weather mTOR plays a role for the regulation of apoptosis during chronic liver injury, *Fah*$^{-/-}$ mice were taken off NTBC, treated with either RAD001 or placebo for 14 days and then challenged with the mAb Fas to induce hepatocellular apoptosis. Liver tissue from *Fah*$^{-/-}$ mice that were on NTBC and treated with mAb Fas displayed severe hepatocyte destruction and massive hemorrhage. Additionally, TUNEL staining revealed multiple positive cells (figure 37). In contrast, *Fah*$^{-/-}$ mice that were off NTBC and treated with mAb Fas, although some tissue damage is revealed, no TUNEL positive cells were detectable (figure 37). When *Fah*$^{-/-}$ mice that were off NTBC treated with RAD001 were challenged with mAb Fas, a fulminant liver failure occurred similar to *Fah*$^{-/-}$ mice that were on NTBC (figure 37).

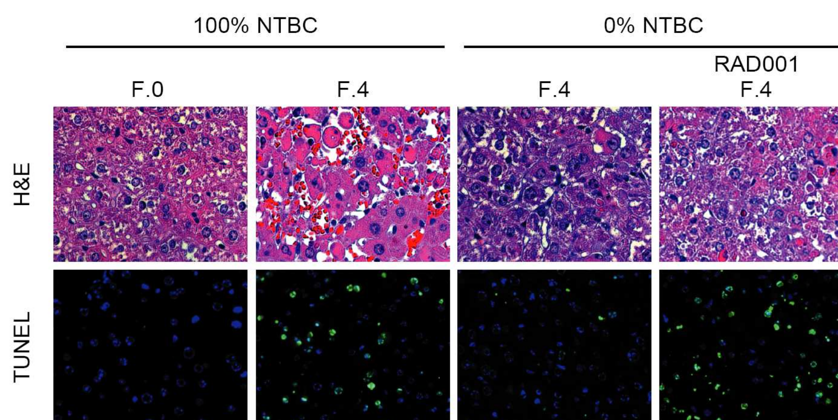

Figure 37 RAD001 sustains apoptosis sensitivity in hepatocytes during liver injury
Hepatocellular apoptosis was induced by mAb Fas injection in *Fah*$^{-/-}$ mice after 14 days of RAD001 treatment and NTBC withdrawal. H&E staining showed disruption of liver tissue in mAb Fas-injected mice. TUNEL positive hepatocytes were observed in RAD001-treated livers in contrast to the placebo treated mice.

According to the results showed above, there was no caspase-3 activity, and very strong activation was seen in *Fah*$^{-/-}$ mice that were on NTBC before and after induction of apoptosis by mAB Fas injection (figure 38); as expected, caspase-3 activity was diminished after NTBC withdrawal. Additionally, treatment with RAD001 sustained the apoptosis sensitivity in *Fah*$^{-/-}$ mice that were taken off NTBC.

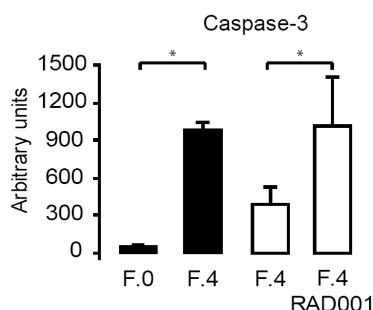

Figure 38 Measurement of caspase-3 activity in *Fah*$^{-/-}$ mice
Caspase activity was determined in control and mAb Fas-challenged mice (closed bars: on NTBC; open bars: off NTBC). (F.0: control, F.4: mAb Fas injected mice and scarified after 4h; *p≤0.0001).

In concordance with the data from histology and caspase-3 activity, cleavage of Bid and caspase-9 were seen in *Fah*$^{-/-}$ mice that were on NTBC and RAD001-treated *Fah*$^{-/-}$ mice that were off NTBC, after mAb Fas injection (figure 39). Together these data indicate that treatment with RAD001 sustains the apoptosis sensitivity in hepatocytes with DNA damage.

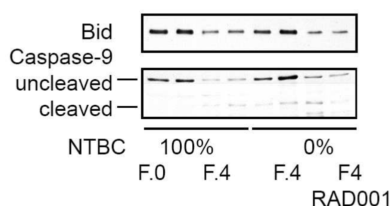

Figure 39 Cleavage of caspase-9 and Bid expression
Cleavage of caspase-9 and Bid were measured in liver extracts by Western blotting. (F.0: control, F.4: mAb Fas-injected mice and scarified after 4h).

To better understand the mechanism by which RAD001 sustains apoptosis sensitivity in Fah-deficient mice, some apoptosis-related proteins were analyzed by Western Blotting. In *Fah*$^{-/-}$ mice that were on NTBC, no significant changes were seen in the most analyzed proteins in RAD001 and placebo-treated mice; Bim was not detected in those mice. After NTBC withdrawal, levels of Fas receptor were slightly increased in RAD001-treated *Fah*$^{-/-}$ mice that were off NTBC when compared with placebo-treated mice. Expression of the inhibitor of caspase-8, Flip-s, was reduced in apoptosis resistant *Fah*$^{-/-}$ mice that were off NTBC; however, levels were restored under RAD001 treatment. An increased expression of anti-apoptotic members of the Bcl-2 family, Bcl-xL and Mcl-1, was seen in both *Fah*$^{-/-}$ RAD001 and placebo-

treated mice after NTBC withdrawal. Bim and Bid levels, pro-apoptotic proteins, were also increased in *Fah*$^{-/-}$ mice after NTBC withdrawal. Treatment with RAD001 reduced the levels of Bim in *Fah*$^{-/-}$ mice that were taken off NTBC but not Bid levels. Expression levels of cIap1, an endogenous inhibitor of caspases, were not significantly changed (figure 40).

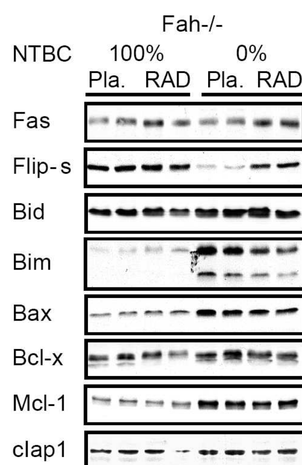

Figure 40 Analysis of pro- and anti-apoptotic proteins in RAD001 or placebo treated *Fah*$^{-/-}$ mice
Liver extracts were isolated, and representative samples (2 mice per group) were subjected to Western Blotting using the indicated antibodies. (Pla.:Placebo, RAD: RAD001).

Furthermore, absence of p21 and p53 did not influence the expression of the analyzed proteins in comparison to *Fah*$^{-/-}$ mice; only the expression of Flip-s was changed: reduced in *Fah/p21*$^{-/-}$ and increased in *Fah/p53*$^{-/-}$ mice, and taken off NTBC (figure 41). The most consistent effect of RAD001 treatment on apoptosis-related proteins was the reduced expression of pro-apoptotic protein Bim in all three mouse lines following NTBC withdrawal. However, none of the analyzed proteins correlated with the sensitivity/resistance to apoptosis in *Fah*$^{-/-}$ mice.

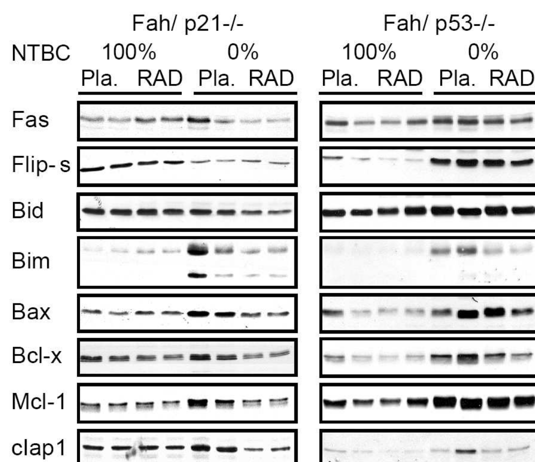

Figure 41 Analysis of pro- and anti-apoptotic proteins in RAD001 or placebo-treated *Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ mice
Liver extracts were isolated, and representative samples (2 mice per group) were subjected to Western Blotting using the indicated antibodies. (Pla.:Placebo, RAD: RAD001).

4.8 Long-term treatment with RAD001 delays liver tumor development

All data presented above show that treatment with RAD001 specifically inhibits proliferation of damaged hepatocytes and sustains the apoptosis hepatocyte sensitivity during chronic liver injury. The next question to address was whether long-term treatment with RAD001 delays tumor development in the liver. Therefore, *Fah/p21*$^{-/-}$ mice were taken off NTBC and treated daily with either RAD001 or placebo for 4 months. *Fah/p21*$^{-/-}$ mice were used because they have an accelerated hepatocyte proliferation and develop liver tumors within 3 months, which is significantly faster than in *Fah*$^{-/-}$ mice (Willenbring et al., 2008b). At the end of the experiment, body weight was similar in RAD001 and placebo-treated mice (figure 42A). In contrast, liver weight of placebo-treated mice was almost two times higher than livers from RAD001-treated mice; this difference was due to the presence of multiple tumors in the placebo-treated mice (figure 42B).

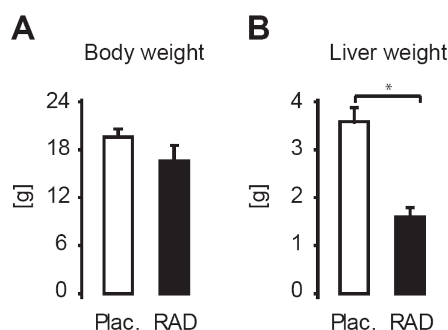

Figure 42 ***Fah/p21$^{-/-}$*** **mice were treated with RAD001 or placebo for 4 months after NTBC withdrawal**
A Body weights of RAD001 and placebo-treated mice. **B** Livers from placebo-treated mice were significantly bigger than those from RAD001-treated mice (*p≤0.0001).

In addition, transaminase levels were also significantly higher in RAD001-treated mice (figure 43), suggesting that treatment with RAD001 did not ameliorate overall liver injury in *Fah/p21$^{-/-}$* mice that were taken off NTBC.

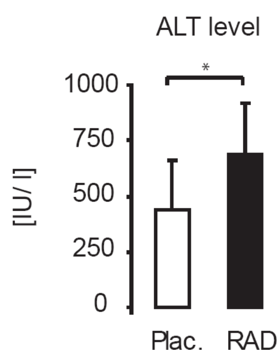

Figure 43 Transaminase levels of long term RAD001 and placebo-treated ***Fah/p21$^{-/-}$*** **mice**
Increased levels of ALT were found in RAD001-treated mice (*p≤0.0001).

All animals treated with placebo developed multiple and large tumors. Treatment with RAD001 significantly delayed tumor onset and progression (figure 44). In 40% of RAD001-treated mice, no macroscopic visible tumors were detected, 30% displayed a granular liver surface and 30% minimal tumor nodules were visible. Analysis of liver histology showed a severe hepatitis in all mice that is also confirmed by ALT levels, which are higher in RAD001-treated mice (figures 43, 45).

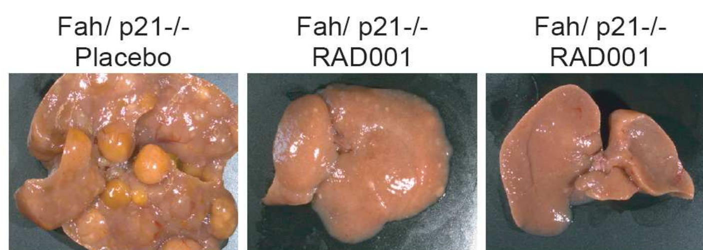

Figure 44 Long-term treatment with RAD001 significantly delays tumor development in the liver
Fah/p21$^{-/-}$ mice were treated with either RAD001 or placebo for 4 months after NTBC withdrawal. Liver pictures showing the anti-proliferative effect of a long-term RAD001 treatment.

In order to investigate the mechanism by which RAD001 delays tumor formation in the liver, analysis of proliferation and apoptosis was performed. BrdU incorporation was used to determined cell proliferation. BrdU immunohistochemistry revealed multiple positive hepatocytes in *Fah/p21*$^{-/-}$ placebo-treated mice in contrast with RAD001-treated mice where hepatocyte proliferation was markedly inhibited (figure 45). However, many small- and oval-shaped cells were BrdU-positive in RAD001-treated livers. When the ability of hepatocyte regeneration is compromised, a subpopulation of liver cells is induced to proliferate. These oval cells --termed so due to their oval shape, ovoid nucleus and small size (compared with hepatocytes)-- proliferate in the periportal region of the liver and, as liver damage progresses, they infiltrate into the parenchyma along the bile canaliculi between the hepatic cords. In this study, immunodetection of oval cells by A6 staining revealed a significantly higher number of positive cells in *Fah/p21*$^{-/-}$ RAD001-treated mice than in placebo-treated mice. Additionally, TUNEL staining was used to determine apoptosis. Several TUNEL-positive cells were seen in both groups (figure 45), indicating that RAD001 treatment did not alter apoptosis in *Fah/p21*$^{-/-}$ mice that were taken off NTBC.

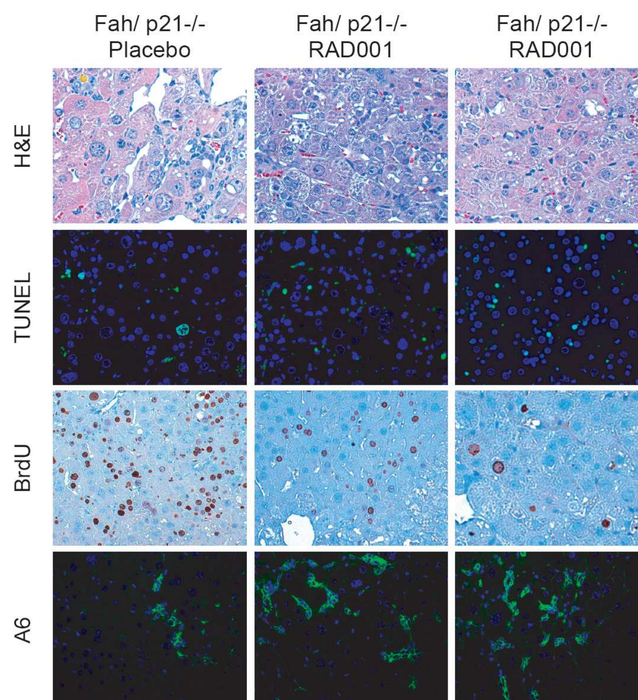

Figure 45 Liver histology of long-term RAD001-treated *Fah/p21$^{-/-}$* mice
Representative H&E staining, analysis of apoptosis, proliferation and immunohistochemical detection of the levels of A6 are shown.

At the end of the experiment, placebo-treated *Fah/p21$^{-/-}$* mice displayed low levels of 4E-BP1, S6 and Akt phosphorylation (figure 46). However, long-term treatment with RAD001 suppressed phosphorylation of S6, did not induce a sustaining activation of Akt and did not affect the expression of eIF-4E.

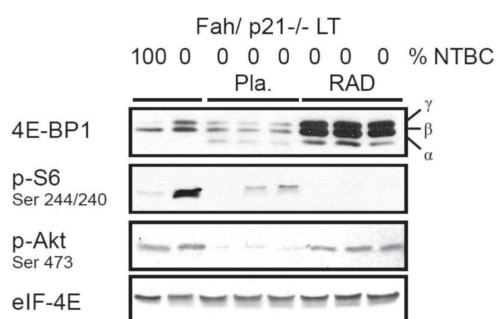

Figure 46 Expression of 4E-BP1 and eIF-4E and phosphorylation levels of S6 and Akt in long-term RAD001-treated *Fah/p21$^{-/-}$* mice
Fah/p21$^{-/-}$ mice were treated with RAD001 or placebo for 4 months after NTBC withdrawal. Liver extracts were isolated, and representative samples were subjected to Western Blotting using the indicated antibodies (The first two lanes are protein samples from 15 days RAD001-treated mice; Pla.:Placebo, RAD: RAD001).

Additionally, cyclin A, E and c-jun were almost not detectable in placebo-treated *Fah/p21*$^{-/-}$ mice. In contrast, the expression of cyclin D1 and eIF-4E was seen in those mice (figures 46, 48). Interestingly, RAD001-treated mice presented higher levels of cyclin D1, cyclin A, cyclin E and c-jun than in placebo-treated mice (figure 47). Altogether, these data show that RAD001 delays tumorigenesis mainly by inhibiting proliferation of damaged hepatocytes rather than by inducing apoptosis. Surprisingly, several cell-cycle related proteins were up-regulated in mice that were treated for 4 months in comparison with mice that were treated for 14 days, with RAD001.

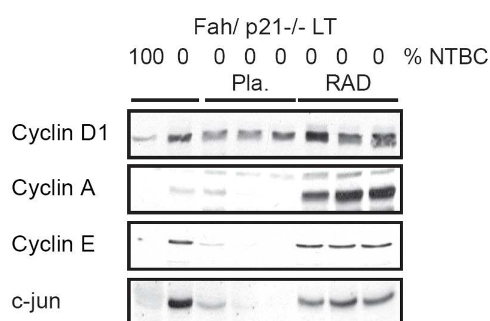

Figure 47 Expression of several cell-cycle related proteins in long-term RAD001-treated *Fah/p21*$^{-/-}$ mice
Fah/p21$^{-/-}$ mice were treated with RAD001 or placebo for 4 months after NTBC withdrawal. Liver extracts were isolated, and representative samples were subjected to Western Blotting using the indicated antibodies (The first two lanes are protein samples from 15 days RAD001-treated mice; Pla.:Placebo, RAD: RAD001).

5 Discussion

Rapamycin was approved as an immunosuppressive drug in 1999 and, since then, has been used for preventing allograft rejection. Although rapamycin was shown to possess anti-tumor properties over 20 years ago (Eng et al., 1984), it was never taken forward for cancer patients. However, as more information on the role of mTORC1 in human tumors, including hepatic tumors, has emerged, the application of rapamycin for this indication is being reevaluated. Modified versions of rapamycin, such as RAD001, are currently in various stages of clinical trials. Therefore, the purpose of the present study was to determine how RAD001 affects proliferation and apoptosis of non-transformed hepatocytes during chronic liver disease. The findings show that treatment with RAD001 did not significantly reduce FAA-induced hepatocyte injury. Additionally, RAD001 treatment effectively inhibits proliferation of hepatocytes with DNA damage and sustains their apoptosis sensitivity during chronic liver injury. Interestingly, long-term treatment with RAD001 markedly delays liver tumor development in *Fah/p21*$^{-/-}$ mice.

5.1 Effect of RAD001 on hepatocyte growth and proliferation

mTOR is involved in several pathways including control of cell proliferation, regulation of ribosomal biogenesis and induction of the protein translation apparatus. *In vitro* studies have shown that rapamycin suppresses tumor growth by arresting the cell cycle in the G1 phase (Decker et al., 2009; Wendel et al., 2004). To better understand the role of mTOR in hepatocarcinogenesis and on hepatocyte proliferation and apoptosis, the effect of RAD001 in the mouse model for HT1 was analyzed. HT1 is characterized by an extremely high susceptibility for liver cancer. After RAD001 treatment, the baseline proliferation of healthy hepatocytes was not affected, but the proliferation of hepatocytes with DNA damage was markedly suppressed. In addition, RAD001 induced cell-cycle arrest in *Fah/p21*$^{-/-}$ mice that were taken off NTBC where many proliferating hepatocytes were seen; in contrast,

RAD001 did not completely arrest the cell cycle in *Fah/p53*$^{-/-}$ mice that were taken off NTBC.

For a cell to proliferate, it must up-regulate the biosynthesis apparatus needed to support cell growth. Several studies have shown that the mRNA transcripts for all ribosomal protein and protein synthesis elongation factors contain an unusual oligopyrimidine tract, referred to as 5'TOP. The translation of these mRNA transcripts is dependent on S6K1 activation and mediated by an increase of S6 phosphorylation (Jefferies et al., 1994). *In vitro*, it has been demonstrated that the activation either of S6K1 or 4E-BP1/eIF-4E pathways partially rescues the inhibitory effect of rapamycin on G0 to S phase cell-cycle progression. Additionally, S6K1 and 4E-BP1/eIF-4E pathways independently regulate mTOR-dependent cell-cycle control in parallel, and a simultaneous downregulation of these two pathways result in an additive arrest of cell-cycle progression in the G1 phase compared to downregulation of the pathways individually, indicating in some sense that these represent the two major pathways that control cell cycle in an mTOR-dependent manner (Fingar et al., 2004).

In rats, it has been shown that partial hepatectomy induces a transient increase of S6K1 activity in the liver, when compared with sham-operated animals. The amount of S6K1 does not increase, but the active kinase shows high phosphorylation levels. Additionally, rapamycin blocks the activation of S6K1 in response to partial hepatectomy in a dose-dependent manner (Jiang et al., 2001). Several studies have shown that the effect of rapamycin in hepatocyte proliferation is comparable to inhibition of S6K1 and S6 phosphorylation. A research group has shown that adult mouse livers that have a conditionally deleted S6 gene failed to regenerate after two-thirds partial hepatectomy; this lesion in cell proliferation does not seems to result from a lack of translational capacity, because no difference in the abundance or rate of accumulation of p21 or cyclin D1 protein in S6-deficient versus wild-type liver cells was observed. Moreover, they showed that S6-deficient liver cells have the ability to synthesize proteins and grow. The failure to progress to the S phase in the cell cycle appeared to be due to a block in expression of cyclin E mRNA (Volarevic et al., 2000). According to these findings, in this study, it was seen that treatment with RAD001 strongly suppressed S6 phosphorylation in livers from *Fah*$^{-/-}$, *Fah/p21*$^{-/-}$ and *Fah/p53*$^{-/-}$ and WT mouse livers. However, this effect showed a dissociation between S6 phosphorylation and proliferation of hepatocytes. Several BrdU-positive

hepatocytes were seen in *Fah/p53*$^{-/-}$ and long-term RAD001-treated *Fah/p21*$^{-/-}$ mice and in WT livers after 72h partial hepatectomy and RAD001 treatment despite the sustained inhibition of S6 phosphorylation. Thus, these results show that S6 is a good biomarker to control mTOR inhibition but it is not reliable in controlling hepatocyte proliferation.

It has been shown that phosphorylation of S6 and hepatocyte proliferation does not always match. In rats, the basal hepatic S6K1 activity in the late gestation-fetal phase is lower than that in adult animals. However, this period is characterized by a high rate of hepatocyte proliferation, showing a discrepancy between cell proliferation and activity of this enzyme. Additionally, when rapamycin was administrated to fetal hepatocytes, cell proliferation could proceed even though S6 phosphorylation was markedly decreased, suggesting that fetal hepatocytes utilize an alternative, rapamycin-resistant pathway for DNA synthesis and proliferation (Boylan et al., 2001). Previous studies have shown the existence of S6-independent mechanisms for 5'TOP mRNA translation: S6K1 knockout murine embryonic stem cells do not show any S6 phosphorylation; however, 5'TOP mRNAs were detected in polysomal fractions, indicating active eukaryotic elongation factors. Consistent with these results, RAD001-inhibited phosphorylation of S6 in hepatic progenitor cells, failed to inhibit proliferation of these cells in long-term treated *Fah/p21*$^{-/-}$ mice, suggesting that mTOR inhibition specifically affects proliferation of adult hepatocytes, independent of S6 phosphorylation.

In contrast to inhibition of S6 phosphorylation in Fah-deficient and WT mice, treatment with RAD001 moderately affected the phosphorylation of 4E-BP1. It has been shown that partial hepatectomy leads to a substantial increase in 4E-BP1 phosphorylation: from the ß specie to the most phosphorylated form, γ. However, and in agreement with this study, when rapamycin was given to the mice that underwent partial hepatectomy, the phosphorylation of 4E-BP1 was not significantly changed. Another study showed that 4E-BP1 phosphorylation was not reduced in rapamycin-treated rats as compared with the controls after partial hepatectomy; the ratio of the γ band to the total amount of 4E-BP1 appeared to slightly increase at higher doses of rapamycin, indicating that 4E-BP1 might be more phosphorylated in rapamycin-treated animals than in controls (Jiang et al., 2001). Additionally, eIF-4E/eIF-4G complex assembly was induced in the regenerating mouse livers; however, this complex was disturbed after RAD001 treatment (Goggin et al., 2004). This sug-

gests that phosphorylation of 4E-BP1 plays a minor role in mediating the effects of mTOR on mitogen-stimulated hepatocyte proliferation. Thus, additional downstream targets of mTOR are probably involved in regulation of proliferation of hepatocytes.

It has been shown that a negative feedback loop exists between S6 kinase and insulin receptor substrate-1 (IRS1) that activates Akt kinase following suppression of mTORC1 (Guertin and Sabatini, 2007). In contrast, no activation of Akt kinase was seen in Fah-deficient mice after RAD001 administration. However, when *Fah/p21*$^{-/-}$ mice were treated for a long time, an activation of Akt kinase was detected. It has been demonstrated that rapamycin can induce Akt phosphorylation in tumors, implying that its potential antitumor activity is attenuated by the release of feedback inhibition of growth signaling pathways. This suggests that combined inhibition of constitutively activated oncoproteins and of normal pathways that are down-regulated by oncoprotein-inhibition may be much more effective combined than alone. For example, combination therapy with an mTOR inhibitor and an inhibitor of the growth factor receptor, such as IGF-IR, that normally drives PI3K activity in that tumor, might also be a promising combination therapy (O'Reilly et al., 2006).

Increased protein synthesis is one of the major anabolic events required for the growth response. Studies suggest that one of the key points upon which signaling pathways converge to regulate growth is the mTOR signaling pathway: mTOR is an important regulator of translational initiation (Fingar et al., 2002; Gingras et al., 2001b; Schmelzle and Hall, 2000; Thomas and Hall, 1997). This pathway controls phosphorylation of 4E-BP1, releasing its inhibitory interaction with eIF-4E, allowing eIF-4E to associate with eIF-4G to form the active eIF-4F complex, a necessary component of the 40S initiation complex. eIF-4E activity appears to be particularly important for the translation of mRNAs containing a highly structured 5' untranslated region, such as transcripts encoding many proteins associated with growth and proliferation control (Gingras et al., 2001a; Gingras et al., 2001b; Sonenberg and Gingras, 1998; West et al., 1998). Additionally, eIF-4E levels are limited for cap-dependent translation in most systems, and over-expression of eIF-4E in rodent cells causes malignant transformation (Lazaris-Karatzas et al., 1990; Mamane et al., 2004). Several studies demonstrated that eIF-4E acts as an oncogene (Mamane et al., 2004). Therefore, increased eIF-4E expression in cancer cells is thought to enhance eIF-4F complex formation, and as a consequence, the translation of a subset of mRNAs that contains highly structured 5' untranslated regions,

such as vascular endothelial growth factor (VEGF). Indeed, eIF-4E is elevated in numerous types of cancers, including HCC (De Benedetti and Graff, 2004). Furthermore, the importance of eIF-4E's role in tumorigenesis is reinforced by the finding that the eIF-4F complex is necessary for maintaining tumor cell growth (Avdulov et al., 2004). Importantly, siRNA treatment to reduce eIF-4E expression inhibits the growth of several cell lines including those of head and neck squamous carcinoma cells (Oridate et al., 2005). To better understand how mTOR regulates hepatocytes proliferation, the effect of RAD001 on protein translation was analyzed in three different experimental systems: mitogen-stimulated proliferation of damaged hepatocytes during liver injury, mitogen-stimulated proliferation of healthy hepatocytes after partial hepatectomy and nutrient-induced growth after a period of fasting. Levels of eIF-4E were slightly upregulated in Fah-deficient mice. However, treatment with RAD001 did not significantly affect its expression. In fact, RAD001 fails to inhibit eIF-4F assembly and to reduce the translation-dependent accumulation of oncogenic proteins in mammalian cells (Beretta et al., 1996; Jefferies et al., 1994; Tamburini et al., 2009). Additionally, no significant changes were seen in this study in the expression of the translation initiation factors following RAD001 treatment. Interestingly however, treatment of mice during re-feeding after a period of starvation revealed that RAD001 slightly, but significantly, inhibited global protein synthesis in the liver. Similarly, treatment with the drug results in a small decrease in overall protein synthesis in most cell lines, which might contribute to the inhibition of proliferation.

Most cells are arrested specifically in the G1 phase of the cell cycle following rapamycin treatment. Therefore, several cell-cycle related proteins and their inhibitors were analyzed. As showed in previous studies, p21 levels were increased after NTBC withdrawal (Willenbring et al., 2008a) and were reduced after RAD001 treatment (Beuvink et al., 2005). However, in S6-deficient hepatocytes, the expression of cyclin D, p21 and p27 does not change after partial hepatectomy when compared with their control counterparts. Here, expression of p27 was not significantly affected in RAD001-treated mice. Additionally, S6-deficient hepatocytes show no induction of cyclin E and A after partial hepatectomy (Volarevic et al., 2000). In Fah-deficient mice, RAD001 treatment not only affected the expression of cyclin E and A but also the expression of cyclin D and c-jun. However, RAD001 did not affect the cyclin A expression in *Fah/p53*$^{-/-}$ mice. Similarly, previously, it has been shown that

cyclin A levels were reduced to a very low level by rapamycin in wild-type cells but remained high in cells lacking p53 (Huang et al., 2001).

Several studies have shown that cyclin D is overexpressed in different cancer types, including HCC, and that its overexpression could contribute to tumor development. However, cyclin D expression is downregulated by rapamycin in some cell types. Nevertheless, when cyclin D is constitutively expressed, it contributes to malignant transformation by reducing the dependence on extracellular signals that normally control proliferation, leading to a reversion of rapamycin-induced cell-cycle arrest, suggesting that cyclin D is an important mediator of proliferation, downstream of mTOR (Nelsen et al., 2003).

However, cyclin D levels are not reduced in all cells that display inhibition of proliferation. Some studies show that rapamycin induces cell-cycle arrest in the G1 phase, but the levels of cyclin D were not affected in human cell lines. Contrary to the findings from this study, it has also been shown that rapamycin failed to inhibit cell-cycle progression in cells lacking p21, indicating that rather than cyclin D, p21 is most important in cell-cycle regulation through mTOR (Huang et al., 2001). Another study showed that rapamycin inhibits cell proliferation by destabilizing the CDK2-cyclin D-p21-PCNA complexes in cell cycle suggesting that cyclin D and p21 function together in order to inhibit cell-cycle progression (Law et al., 2006). In this study, it was seen a correlation between downregulation of cyclin D expression and inhibition of hepatocyte proliferation in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ after RAD001 treatment and NTBC withdrawal. Contrary to these observations, expression of cyclin D was reduced in *Fah/p53*$^{-/-}$ mice that were off NTBC in which the hepatocyte proliferation was not completely suppressed.

Interestingly, fetal hepatocytes in late gestation demonstrate a high rate of proliferation that is not inhibited by rapamycin or dependent on S6 phosphorylation (Boylan et al., 2001). The fetal hepatocytes express significant amounts of cyclin D and appear to proliferate in a mitogen-independent manner (Awad et al., 2000; Awad and Gruppuso, 2000), suggesting that the expression of cyclin D, which is induced in fetal hepatocytes through pathways distinct from those observed in adult cells (Awad and Gruppuso, 2000), promotes rapamycin resistance (Nelsen et al., 2003). In agreement with these observations, increased levels of cyclin D were seen in *Fah/p21*$^{-/-}$ mice after long-term treatment with RAD001. Therefore, estimation of

cyclin D levels is not a reliable marker for predicting cellular response under RAD001 treatment. Additionally, the results in *Fah/p21*$^{-/-}$ mice clearly indicate that the effect of RAD001 on hepatocyte proliferation is independent of p21.

Some studies have show that c-jun is a critical regulator of hepatocyte proliferation and survival during liver regeneration (Behrens et al., 2002; Eferl et al., 2003). Here, expression levels of c-jun were downregulated in RAD001-treated *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice after NTBC withdrawal in which hepatocyte proliferation was inhibited. Nevertheless, a high expression of c-jun in RAD001-treated *Fah/p53*$^{-/-}$ mice that were taken off NTBC was seen in which hepatocyte proliferation was reduced but not totally inhibited. Furthermore, *Fah/p21*$^{-/-}$ mice --that were treated long-term treated with RAD001-- showed high levels of c-jun, correlating with the fact that proliferation was only attenuated in those mice. Additionally, c-jun expression was delayed in RAD001-treated mice after partial hepatectomy in which hepatocyte proliferation was also delayed. Moreover, RAD001 and placebo-treated *Fah*$^{-/-}$*/c-jun*$^{fl/fl}$*/mx-cre* mice, in which c-jun was conditionally deleted in hepatocytes, displayed a similar number of proliferating hepatocytes 72 hours after partial hepatectomy, suggesting that cell-cycle progression occurs independently of c-jun (data not shown). However, the data from this study suggest that mTOR regulates c-jun expression during hepatocyte proliferation.

Several studies have shown that the mTOR and p53 pathway communicate with each other. Activation of p53 by a physiological relevant stress signal, DNA damage, inhibits mTOR activity in normal cells, the primary MEFs. A similar inhibition of mTOR activity in human cancer cells was observed after the activation of a temperature-sensitive mutant of p53. This regulation of mTOR by p53 is independent of the cell-cycle regulation by p53, because p53 regulates mTOR in *p21*$^{-/-}$ MEFs as well as it does in WT MEFs (Feng et al., 2005). A strong activation of the p53/p21 pathway occurs in Fah-deficient mice after NTBC withdrawal (Willenbring et al., 2008a). After RAD001 treatment, levels of p53 and p21 were significantly lower in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice than in the controls, suggesting that activation of mTOR during chronic liver injury contributes to p53 accumulation in hepatocytes. Additionally, it has been shown that cells with constitutive mTOR activation decreased p53 levels after treatment with rapamycin and following DNA damage (Lee et al., 2007).

Studies suggested that rapamycin inhibits cell-cycle progression independently of p53 (Metcalfe et al., 1997). Additionally, other studies suggested that rapamycin slowed but could not stop cell-cycle progression in p53 mutant tumor cell, leading to apoptosis (Huang et al., 2001). However, the effect of RAD001 on hepatocyte proliferation and on c-jun protein expression was markedly attenuated in p53 null mice. Therefore, these data suggest that loss of p53 decreases the ability of hepatocytes to arrest before the S-phase in response to RAD001 treatment. However, the exact mechanism by which cells arrest in G1 due to activation of mTOR and p53 pathways remains to be explored.

p21 and p27 serve as assembly factors for CDK complexes. Rapamycin downregulates p21 levels; however, no effect of rapamycin in the levels of p27 was seen. This would suggest that rapamycin selectively disrupts CDK complexes that require p21 as an assembly factor but not those that require p27 as an assembly factor. *In vitro*, disruption of CDK2-cyclin D1-p21-PCNA complexes correlates with rapamycin-induced cell-cycle arrest, and this disruption correlated best with downregulation of cellular p21 levels. Additionally, CDK2-cyclin D complexes are inactivated by rapamycin treatment in a relatively selective manner through p21 downregulation in cells *in vivo* (Law et al., 2006). It has been recently shown that rapamycin does not affect the amount of either CDK2 or cyclin E protein in the complex and that the amount of p27 coimmunoprecipitated with cyclin E increases in response to rapamycin in tumorigenic hepatic cell lines (Jimenez et al., 2009). In T cells, rapamycin inhibits CDK2-cyclin E kinase activity and increases the amount of p27 coimmunoprecipitated with cyclin E. In this study, RAD001 treatment substantially inhibited phosphorylation of Rb in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice that were off NTBC indicating that the drug affected activation of CDK-cyclin complexes. Interestingly however, the immunoprecipitation studies presented here showed that treatment with RAD001 did not induce CDK2-cyclin E-p27 complexes but rather inhibited the formation of CDK-cyclin complexes. Altogether, these data suggest that RAD001 may inhibit Rb inactivation by multiple pathways and therefore inhibit cell-cycle progression. Importantly, it has been published that Rb plays a key role in tumorigenesis in the liver, compromising hepatocyte proliferation and genome integrity. Evaluation of Rb status may be a useful prognostic factor in human HCC (Mayhew et al., 2007).

The data presented above showed that RAD001 specifically inhibits factors facilitating the transition of cells from the G0 phase to the G1 phase of the cell cycle. It has

been shown that rapamycin fails to inhibit the proliferation of the cells that already pass G1/S transition or are growing exponentially, suggesting that the antiproliferative effect of rapamycin might be cell-cycle phase-dependent (Kawamata et al., 1998). Accordingly, when WT mice, 20h after PH are treated with RAD001, the drug fails to inhibit cell cycle, indicating once more that RAD001 prevents progression into the S-phase only in cells that have not passed the G0/G1 restriction point in the cell cycle.

5.2 Effect of RAD001 on hepatocytes apoptosis

Dysregulation of the apoptosis pathways is a hallmark of cancer because mutations in apoptotic regulators invariably accompany tumorigenesis. The mTOR pathway is involved in key events during apoptosis in cancer, and consequently, rapamycin has been associated with apoptosis induction in different tumor systems. Previously, it has been shown that treatment with rapamycin affected only moderate activity in inducing apoptosis of cancer cells (Tam et al., 2009). However, it has been shown that rapamycin specifically induced apoptosis independently of p53 in human rhabdomyosarcoma cells. In cells lacking p53, rapamycin induces rapid and sustained activation of apoptosis signal-regulating kinase 1 (ASK1), c-jun N-terminal kinase (JNK), and elevation of phosphorylated c-jun that results in apoptosis. This effect is abrogated by ectopic overexpression of p21, with p21 binding to ASK1 and blocking JNK activation (Hosoi et al., 1999; Huang et al., 2003; Huang et al., 2004). In this study, treatment with RAD001 induced only modestly hepatocellular apoptosis in Fah-deficient mice after NTBC withdrawal.

In contrast, in combination therapy, rapamycin sensitizes cancer cells to apoptosis. It has been shown that rapamycin promotes cisplatin-induced apoptosis in wild-type p53, but not mutant p53 tumor cells, due to its ability to block p53-induced p21 expression (Beuvink et al., 2005). In human ovarian cancer cell lines, rapamycin and carboplatin additively enhance apoptosis (Schlosshauer et al., 2009). Moreover, when Ras and mTOR pathways are blocked by administration of sorafenib and rapamycin to human HCC cell lines, apoptosis is significantly enhanced when compared to sorafenib alone (Newell et al., 2009). In additional to the combination therapy, rapamycin reverses the chemoresistance in Akt-induced tumorigenesis by dis-

rupting the Akt signaling and sustaining apoptosis sensitivity in cancer cells (Wendel et al., 2004). In this study, interestingly however, RAD001 treatment markedly sustained the apoptosis sensitivity against Fas-induced apoptosis in Fah-deficient mice that were off NTBC.

Previous studies show that rapamycin can affect the apoptosis sensitivity of tumor cells by regulating several pro- and anti-apoptotic proteins. In a mouse model for lung cancer, it has been shown that rapamycin promotes apoptosis by up-regulating the pro-apoptotic protein Bid and Bax and down-regulating Bcl-xL (Yang et al., 2009). Furthermore, it has been shown that mTOR transcriptionally regulates FLIPs levels and controls the sensitivity of human glioma cells to apoptosis induced by the death ligand TRAIL. The accumulation of FLIPs mRNA and FLIPs protein expression, which is overexpressed in many tumors, confers apoptosis resistance to cancer cells. TRAIL-induced apoptosis can be suppressed by activation of either the S6K1 or 4E-BP1 arms of the mTOR pathway, and inhibition of either arm sensitizes cells to TRAIL-induced apoptosis as effectively as mTOR inhibition by rapamycin (Panner et al., 2005). Also, rapamycin can modulate expression of Mcl-1, an important anti-apoptotic regulator (Mills et al., 2008). However, the expression of several apoptosis-related proteins, analyzed in this study, was not regulated after RAD001 treatment, revealing no consistent changes in apoptosis-sensitive and apoptosis-resistance mice; only Bim, which is a major regulator of apoptosis in the lymphoid and liver, was slightly upregulated following NTBC withdrawal.

In response to chemotherapy, p53 protein is stabilized and mediates apoptosis and cell cycle-arrest. The mechanism of p53-dependent cell-cycle arrest is primarily mediated by p21. However, there is evidence that p21 is a major inhibitor of p53-dependent apoptosis. It is unclear how a cell decides between apoptosis and p21-dependent cell-cycle arrest after DNA damage and stabilization of p53. It has been shown that high levels of p21 expression often mediate cell-cycle arrest and protect from p53-dependent apoptosis. Additionally, p21 blocks apoptosis by interacting with proapoptotic molecules such as procaspase-3, caspase-8 and the kinase apoptosis signal-regulating kinase 1 (ASK1) (Gartel and Tyner, 2002). Therefore, the sustained sensitivity against Fas-induced apoptosis might be related to the loss of p21 in Fah-deficient RAD001-treated mice that were taken off NTBC.

6 Conclusion

Rapamycin and its analogues are tested as therapeutic agents for the treatment of different cancers due to their capacity to inhibit cell growth and proliferation. Currently, RAD001 is in phase I study for dose escalation and in phase II study for intent to treat, as well as pharmacokinetic, pharmacogenetic and surrogate marker study. Prevention is the only realistic approach for reducing mortality rates associated with hepatocellular carcinoma worldwide. Preventing the development of HCC is referred to as primary prevention. Fortunately, the natural history of HCC provides many opportunities for an impact to be made on the occurrence of primary liver cancer because of its known association with chronic liver disease and cirrhosis. The first opportunity involves the use of measures to avoid risk factors for HCC including viral infections and toxins. The second is early recognition and treatment of acute and inherited liver diseases, which may block the transition to chronic disease. The third is to prevent progression of chronic disease to cirrhosis. Finally, once cirrhosis is established, chemoprevention can interfere with the molecular events leading to HCC. This study suggests that RAD001 should be clinically used as an effective chemopreventive agent in the liver. The data presented here show that RAD001 specifically inhibits the proliferation of damaged hepatocytes and sustains their apoptosis sensitivity during chronic liver injury. Altogether, the mTOR inhibition might be recommendable as an effective strategy to delay hepatocarcinogenesis in patients at risk.

7 References

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EDUCATION

September 2005 - March 2010
PhD in Biology
Hannover Medical School, Germany
Department of Gastroenterology, Hepatology and Endocrinology
Thesis: mTOR inhibition impairs proliferation of hepatocytes with DNA damage during chronic liver injury thereby delaying liver tumor development, ***summa cum laude***

October 2002 - September 2004
MSc in Horticulture
Hannover University, Germany
Major: Genetics, Plant Breeding and Bioinformatics
Thesis: Reverse genetic approach to investigate protein functions related to plant mitochondria, ***magna cum laude***

September 1995 - April 2002
BSc in Biology
Valley University, Colombia
Major: Genetics
Thesis: Parent-offspring analysis using molecular markers to identify putative fathers of Cassava hybrids

August 1984 - May 1995
High School
Bethlemitas Catholic School Palmira-Valle, Colombia

INTERNATIONAL RESEARCH EXPERIENCE

Since September 2005
Medical School Hannover, Germany
Department of Gastroenterology, Hepatology and Endocrinology
Project: Role of Chk2 in Hepatocellular Carcinoma
mTOR inhibition in the prevention of liver tumor development

November – December 2007
Weizmann Institute of Science, Rehovot, Israel
Department of Biological Regulation
Project: Role of Bid in apoptosis and proliferation

November 2004 - August 2005

Leibniz Hannover University, Hannover, Germany
Department of Natural Sciences, Institute of Applied Genetics
Project: Transcriptome analysis of Manganese toxicity and Manganese tolerance in Cowpea (*Vigna unguiculata* (L.) Walp)

March 2003

Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany
Department of Molecular Cell Biology, Applied Biochemistry
Internship: Biochemical characterization of Arabidopsis trichomes; western blot analysis of proteins involved in defence reactions

February – June 2002

Leibniz Hannover University, Hannover, Germany
Department of Horticulture, Institute of Plant Nutrition
Organization and planning of experiments; statistical analysis of data

January – October 2001

International Centre of Tropical Agriculture (CIAT), Cali, Colombia
Department of Biotechnology, Molecular Marker Laboratory
Supervision and handling of Cassava genomic library (*Manihot esculenta* Crantz)

PUBLICATIONS

Schüngel S, **Buitrago-Molina LE**, Nalapareddy PD, Lebofsky M, Manns MP, Jaeschke H, Gross A, Vogel A. (2009). The strength of the Fas ligand signal determines whether hepatocytes act as type 1 or type 2 cells in murine livers. *Hepatology* 50(5):1558-66

Buitrago-Molina LE, Pothiraju D, Lamlé J, Marhenke S, Kossatz U, Breuhahn K, Manns MP, Malek N, Vogel A. (2009). mTOR inhibition impairs proliferation of hepatocytes with DNA damage during chronic liver injury thereby delaying liver tumor development. *Hepatology* 50(2):500-9.

Marhenke S, Lamlé J, **Buitrago-Molina LE**, Cañón JM, Geffers R, Finegold M, Sporn M, Yamamoto M, Manns MP, Grompe M, Vogel A. (2008). Activation of nuclear factor E2-related factor 2 in hereditary tyrosinemia type 1 and its role in survival and tumor development. *Hepatology* 48(2):487-96.

Führs H, Hartwig M, **Buitrago-Molina LE**, Heintz D, Van Dorsselaer A, Braun HP, Horst WJ (2008). Early manganese-toxicity stress-response in *Vigna unguiculata* L. – a proteomic and transcriptomic study. *Proteomics* 8, 149-59.

CONGRESSES

June 2010, Dubrovnik, Croatia

European Association for the Study of the Liver (EASL)
Special Conference - Hepatocellular carcinoma from genomics to treatment
Poster: Role of p21 in proliferation and apoptosis of hepatocytes

April 2009, Copenhagen, Denmark

44ª Annual Meeting of the European Association for the Study of the Liver (EASL)
Poster: Rapamycin prevents proliferation of hepatocytes with DNA damage during liver chronic disease delaying the tumor formation in the liver

October 2006, Boston, US

American Association of the Study of Liver Diseases (AASLD)
Poster: Rapamycin as a chemoprevention against Hepatocellular Carcinoma

September 2004, Braunschweig, Germany

Botanikertagung 2004
Poster: Reverse genetic approach to investigate protein functions related to plant mitochondria

PRIZE

May 1992

XI Colombian Mathematical Olympiad
First place

LANGUAJE SKILLS

Spanish	First language
English	Advanced
German	Intermediate
French	Basic

Hannover, 28th May 2010