p21 ablation in liver enhances DNA damage, cholestasis and carcinogenesis

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Abstract

Genetic mouse studies suggest that the NF-κB pathway regulator NEMO (also known as IKKγ) controls chronic inflammation and carcinogenesis in the liver. However, the molecular mechanisms explaining the function of NEMO are not well defined. Here we report that overexpression of the cell cycle regulator p21 is a critical feature of liver inflammation and carcinogenesis caused by the loss of NEMO. NEMOΔhepa mice develop chronic hepatitis characterized by increased hepatocyte apoptosis and proliferation that causes the development of fibrosis and hepatocellular carcinoma, similar to the situation in human liver disease. Having identified p21 overexpression in this model, we evaluated its role in disease progression and LPS-mediated liver injury in double mutant NEMOΔhepa/p21−/− mice. Eight week-old NEMOΔhepa/p21−/− animals displayed accelerated liver damage that was not associated with alterations in cell cycle progression or the inflammatory response. However, livers from NEMOΔhepa/p21−/− mice displayed more severe DNA damage that was further characterized by LPS administration correlating with higher lethality of the animals. This phenotype was attenuated by genetic ablation of the TNF receptor TNF-R1 in NEMOΔhepa/p21−/− mice, demonstrating that DNA damage is induced via TNF. One year old NEMOΔhepa/p21−/− mice displayed greater numbers of hepatocellular carcinoma and severe cholestasis compared to NEMOΔhepa animals. Therefore, p21 overexpression in NEMOΔhepa animals protects against DNA damage, acceleration of hepatocarcinogenesis and cholestasis. Taken together, our findings illustrate how loss of NEMO promotes chronic liver inflammation and carcinogenesis, and they identify a novel protective role for p21 against the generation of DNA damage.
Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and represents the third leading cause of cancer-related mortality (1). HCC develops when the balance between cell proliferation and cell death is disrupted, and the subsequent aberrant proliferation leads to tumor growth. Cell proliferation is controlled by protein kinase complexes consisting of cyclins and cyclin-dependent kinases (Cdks), which regulate distinct phases of the cell cycle.

p21 is involved in numerous growth-inhibitory pathways as shown in cell culture systems and in vivo (2). For example embryonic fibroblasts lacking p21 expression are significantly impaired in their ability to arrest in G1 phase in response to DNA damage (3), and in vivo experiments with p21 knockout mice (p21−/−) revealed earlier hepatocyte DNA synthesis, cyclin/CDK kinase activation and S phase gene expression compared to wild-type mice in the partial hepatectomy (PH) model (4). In contrast, after PH mice with hepatic p21 overexpression displayed impaired hepatocyte proliferation (5).

p21 has been considered as a tumor suppressor because p21−/− mice display spontaneous tumor formation after 16 months and additionally these mice are more sensitive to chemically induced carcinogenesis (6)(7).

However, deletion of p21 does not necessarily promote tumor growth and a potential function as an oncogene has also been described. These assumptions are based on human studies as it has been shown that p21 is upregulated during inflammation and fibrosis in chronic liver diseases, and high p21 expression is linked to hepatocarcinogenesis in cirrhotic patients (8)(9). An oncogenic function for p21 has also been proposed in animal models. Mice deficient for p53 spontaneously develop multiple tumors while additional deletion of p21 in p53-deficient mice
increases the survival of these mice from 6 to 9 months and for instance leads to a significant reduction of thymic lymphomas (10).

From these data at present the role of p21 in tumorigenesis is not clearly defined. In our present study we thus used the NEMO<sup>Δhepa</sup> model to investigate the role of p21 during inflammation driven liver carcinogenesis. The NEMO<sup>Δhepa</sup> model is of specific interest as loss of NEMO immunoreactivity in liver cells is also found in a substantial proportion of human HCCs and in the mouse model it resembles stage dependent evolution of chronic liver disease as also found in humans leading to fibrosis progression and the occurrence of HCC (11)(12)(1).

Our group showed that deletion of NEMO in hepatocytes triggers increased p21 expression (13). In the present study we aimed to investigate whether p21 overexpression in NEMO<sup>Δhepa</sup> livers has a tumor suppressive or oncogenic function. We demonstrate here that p21 overexpression is protective in this model, as hepatocarcinogenesis and cholestasis is significantly enhanced upon deletion of p21 in NEMO<sup>Δhepa</sup> mice. Therefore our study defines p21 as a tumor suppressor in this inflammation triggered model for liver carcinogenesis.
Material and Methods

Animal approach.

Mice were housed and treated in accordance with the guidelines of the National Academy of Sciences (NIH publication 86-23 revised 1985) and breed in the animal facility of the University Hospital RWTH Aachen. Animal studies were approved by the regional authorities for nature, environment and consumer protection of the state North Rhine-Westphalia (LANUV, Germany).

NEMO<sup>hepa</sup> and p21<sup>-/-</sup> mice were generated as previously described (12)(3). Double knockout mice (NEMO<sup>hepa</sup>/p21<sup>-/-</sup> mice) were generated by crossing NEMO<sup>hepa</sup> mice with p21<sup>-/-</sup> mice. NEMO<sup>hepa</sup>/TNF-R1<sup>-/-</sup> mice were generated as previously described (14) and crossed with NEMO<sup>hepa</sup>/p21<sup>-/-</sup> mice in order to generate triple knockout mice (NEMO<sup>hepa</sup>/p21<sup>-/-</sup>/TNF-R1<sup>-/-</sup> mice). All strains were kept in a C57BL/6/ background.

Examinations were performed at the age of 8, 26 and 52 week. Liver weight and body weight were recorded; serum and liver tissue were collected.

Liver injury model.

Experiments were performed on 8 week-old male mice. Lipopolysaccharide (LPS) (Sigma) was injected i.p. at a concentration of 25 µg/10 g body weight and mice were sacrificed 7 h later. For the survival experiments female mice at the age of 8 to 10 weeks were used and observed for 12 h.

Immunofluorescence stainings.
Immunofluorescence staining was performed on frozen liver sections. For BrdU staining mice had to be injected with BrdU (AppliChem, Cheshire, CT) two hours before sacrificing.

Hepatocyte proliferation, cell death and DNA double-strand breaks (DSB) were quantified by counting the number of nuclei positive for either Ki67, BrdU, TUNEL or phospho-H2AX relative to the total nuclei per power field (200x) stained with 4’,6-diamidino-2-phenylindole (DAPI). Stained microscopic images were acquired with a Zeiss Axio Imager.Z1 microscope, AxioCam MRm or AxioCam ICc3 camera using Axiovision 4.8 software (all from Carl Zeiss, Inc., Oberkochen, Germany).

Microarray analysis.

One hundred nanogram of total RNA was labelled with the Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands) and hybridized on Affymetrix GeneChip Mouse Gene 1.0 ST arrays (Santa Clara, CA) according to manufacturers’ instructions. Data was normalized with the Robust Multi-Array Average (RMA) method and probe sets were annotated to Entrez ID’s according to Dai et al. (15)(16)(17). Genes were filtered on an intensity level of at least 20 in at least two samples and an interquartile range > 0.2 (log2 scale). Intensity based-moderated t-statistics were used to calculate significant differences (18). All data were analyzed using an in-house developed system (19). Data is available in the Gene Expression Omnibus (GEO accession number: GSE61100).

Statistical Analysis.

Data are presented as mean ± standard deviation of the mean. Statistical significance was determined by Students t test.
Results

P21 overexpression protects NEMO<sup>Δhepa</sup> mice from severe liver injury.

Deletion of NEMO in hepatocytes triggers apoptosis and compensatory proliferation in the liver. Unexpectedly, the Cdk inhibitor p21, known to inhibit cell cycle proliferation, is overexpressed under these conditions (Fig. 1A). In order to characterise the role of p21 for progression of chronic liver injury, we generated NEMO<sup>Δhepa</sup>/p21<sup>−/−</sup> double knockout mice (Fig. 1A and supplementary Fig. 1A).

p21 overexpression in NEMO<sup>Δhepa</sup> livers is protective as its loss leads to an increase in serum aspartate-aminotransferase (AST) levels in NEMO<sup>Δhepa</sup>/p21<sup>−/−</sup> compared to NEMO<sup>Δhepa</sup> animals (Fig. 1B).

Due to its known role in cell cycle control, we next investigated the impact on hepatocyte proliferation by studying the expression of proteins involved in cell cycle regulation. Quantitative Real Time PCR analysis revealed increased mRNA expression levels of cell cycle mediators controlling late G1- and S-phase progression such as Cyclin A2 (CcnA2), Cyclin E2 (CcnE2), E2F1 and E2F2 in NEMO<sup>Δhepa</sup>/p21<sup>−/−</sup> livers (supplementary Fig. 2A-E). Overexpression of CcnA2 and E2F1 was confirmed by Western blot analysis, which also showed a downregulation of the mitogen inducible Cyclin D (CcnD) in p21-deleted NEMO<sup>Δhepa</sup> livers (Fig. 1C).

Unpredicted, elevation of the S-phase marker CcnA2 was not accompanied by an increase in hepatocyte proliferation, as evidenced by quantification of BrdU incorporation (Fig. 1D) and Ki67 staining (supplementary Fig. 2F), showing no significant differences between NEMO<sup>Δhepa</sup> and NEMO<sup>Δhepa</sup>/p21<sup>−/−</sup> livers. To further explore this finding, we measured kinase activity of Cdk2, which is the binding partner of CcnA2 and CcnE2. Cdk2 kinase activity was only slightly detectable in
NEMO and p21\textsuperscript{−/−} controls. In contrast, we detected increased kinase activity in both NEMO\textsuperscript{Δhepa} and NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers to approximately the same extend thereby confirming that loss p21 did not affect basal hepatocyte proliferation in NEMO-deficient livers (Fig. 1E). PCNA is a master regulator of DNA-synthesis-associated processes like DNA replication and nucleotide excision repair. Western blot analysis for PCNA did not show any significant differences in its expression between NEMO\textsuperscript{Δhepa} and NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers (supplementary Fig. 2G).

As deletion of p21 had no impact on DNA replication, we examined potential mechanisms, which might explain the lack of an increase in DNA replication in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers. We thus investigated the protein expression of the Cdk inhibitors p27 and p18 by Western blot analysis. NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers revealed slightly increased p27 phosphorylation and additionally p18 was upregulated (Fig. 1F). Thus, loss of p21 is likely compensated by modulation of alternate cell cycle inhibitors which best explains the unchanged basal cell cycle activity in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers.

**Lack of p21 expression in NEMO\textsuperscript{Δhepa} mice triggers increased DNA damage.**

DNA damage induces p53 phosphorylation, which consecutively increases p21 transcription, thereby blocking G\textsubscript{1}-S-phase transition and replication of damaged DNA. Thus we next investigated if this mechanism might be activated in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers. We found an upregulation of p53 phosphorylation in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} compared to NEMO\textsuperscript{Δhepa} livers. Additionally, the phosphorylation of c-Jun N-terminale Kinases (JNK) was increased most likely as a response to increased genotoxic stress (Fig. 2A).
We previously demonstrated that NEMO\textsuperscript{Δhepa} livers display mild DNA double strand breakage (13). Based on the shown differences in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers we next focussed more specifically on the relevance of p21 deletion for DNA damage in these mice. Loss of p21 expression strongly and significantly enhanced DNA damage in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} compared to NEMO\textsuperscript{Δhepa} livers as evidenced by pH2AX staining (Fig. 2B). To better characterise these findings, we performed array analysis to detect changes in the expression of genes involved in DNA damage. Interestingly, we found an upregulation of the DNA checkpoint kinase Chk2 and further genes involved in DNA repair such as the transcription factor FoxM1 and the recombinase Rad51 in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} compared to NEMO\textsuperscript{Δhepa} livers (Fig. 2C). FoxM1 controls the expression of the DNA repair gene Rad51, and Rad51 in turn interacts with the tumor suppressor BRCA1. A strong and significant mRNA upregulation of the DNA damage marker BRCA1 was observed in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers (Fig. 2D). Hence our results demonstrated that after loss of p21 expression in NEMO\textsuperscript{Δhepa} livers the DNA damage as well as the expression of genes involved in DNA repair were increased. The enhanced DNA damage in the double knockout mice also explains the increased liver damage (Fig. 1B).

**Inflammation is ameliorated in the absence of p21 in the NEMO\textsuperscript{Δhepa} liver.**

The inflammatory response leading to chronic hepatitis is important to drive liver disease progression in NEMO\textsuperscript{Δhepa} mice. CD45 immunohistochemistry pointed to a strong inflammatory response in NEMO\textsuperscript{Δhepa} livers. Unexpectedly, p21 deletion reduced CD45 staining in NEMO\textsuperscript{Δhepa}/p21\textsuperscript{−/−} livers (Fig. 3A). This finding was further confirmed by Q-RTPCR (supplementary Fig. 3). The infiltrating cells were also positive for the macrophage marker F4/80 (Fig. 3B). Additionally, these findings were
associated with a reduced expression of TNF and upregulation of its negative regulator TIMP3 (Fig. 3C,D). Together these findings suggest that the inflammatory response is reduced in NEMO\textsuperscript{hepa}/p21\textsuperscript{−/−} livers arguing that the changes in DNA damage might be relevant to explain the phenotype of NEMO\textsuperscript{hepa}/p21\textsuperscript{−/−} livers.

**P21 deletion increases the sensitivity towards DNA damage.**

Lipopolysaccharide (LPS) has been shown to induce DNA strand breakage (20). Therefore we tested if loss of p21 expression increases sensitivity against LPS-driven DNA damage. In wt animals (NEMO\textsuperscript{ff}) minor DNA double strand breakage, as detected by pH2AX staining, was observed after LPS stimulation (Fig. 4A). In p21\textsuperscript{−/−} livers the amount of pH2AX-positive cells was significantly increased, indicating that p21 is involved in mediating protection against DNA damage. In NEMO\textsuperscript{hepa} livers LPS triggered strong DNA damage, which was significantly enhanced in NEMO\textsuperscript{hepa}/p21\textsuperscript{−/−} livers. These findings further support our idea that p21 counter-acts DNA damage in NEMO-deficient hepatocytes.

Next, we tested the impact of LPS stimulation on liver injury. In agreement with our previous studies, NEMO\textsuperscript{hepa} mice showed a severe increase in ALT and AST levels after LPS treatment, which was nearly doubled in NEMO\textsuperscript{hepa}/p21\textsuperscript{−/−} animals (Fig 4B, supplementary Fig. 5A). This observation was also reflected in liver histology as determined by H&E staining. Here NEMO\textsuperscript{hepa}/p21\textsuperscript{−/−} animals showed severe haemorrhages within the liver tissue, which was not evident in NEMO\textsuperscript{hepa} livers and the respective controls (Fig 4C). To further characterise the mode of cell death, we performed TUNEL-staining and found that the number of TUNEL-positive areas was significantly highest in NEMO\textsuperscript{hepa}/p21\textsuperscript{−/−} livers suggesting that the increase in DNA damage and higher liver injury was associated with increased apoptosis (Fig 4D).
In order to characterize the inflammatory response after LPS stimulation, we performed Q-RTPCR analysis for IL-1β expression. Interestingly, we found significant IL-1β downregulation in p21 single knockout mice after LPS stimulation. However, no significant difference was found between NEMO\textsuperscript{hepa} single and double knockout mice showing a comparable reactivity after LPS stimulation (supplementary Fig. 4A). Furthermore we analyzed IL-6 expression levels by Q-RTPCR and found that NEMO\textsuperscript{hepa} and NEMO\textsuperscript{hepa}/p21\textsuperscript{-/-} livers also showed comparable IL-6 levels (supplementary Figure 4B). As IL-1β and IL-6 regulation are not different between both mouse strains after LPS stimulation, these results indicate that the higher sensitivity of NEMO\textsuperscript{hepa}/p21\textsuperscript{-/-} hepatocytes also explain the higher mortality of these animals.

The oxidative stress response in the liver of LPS treated mice was measured by CM-H\textsubscript{2}DCFDA staining to detect reactive oxygen species (ROS) (supplementary Fig. 4C). The presence of oxidative stress in NEMO\textsuperscript{hepa} livers has been already described in our previous studies (11)(13). Our present result shows that p21 deletion itself does not cause ROS accumulation. We did observe by CM-H\textsubscript{2}DCFDA staining that NEMO\textsuperscript{hepa} and NEMO\textsuperscript{hepa}/p21\textsuperscript{-/-} mice have elevated ROS levels in comparison to control mice (NEMO\textsuperscript{hepa}\textsuperscript{ff} and p21\textsuperscript{-/-}). However deleting p21 in NEMO\textsuperscript{hepa} mice did not enhance ROS production in the liver. This has been observed in LPS treated (supplementary Fig. 4C) and untreated mice (data not shown). The microscopic pictures of the LPS treated group are included in the supplementary Figure 4. Therefore we conclude that oxidative stress does not account for higher DNA damage in NEMO\textsuperscript{hepa}/p21\textsuperscript{-/-} livers.

Recently we have described that TNF-R1 deletion in NEMO\textsuperscript{hepa} livers rescues hepatocyte injury and apoptosis (14). Therefore we crossed NEMO\textsuperscript{hepa}/p21\textsuperscript{-/-} with
TNF-R1\textsuperscript{−/−} animals in order to generate NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}/TNF-R1\textsuperscript{−/−}} mice (supplementary Fig. 1B).

We stimulated NEMO\textsuperscript{Δhepa}, NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}}, NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}/TNF-R1\textsuperscript{−/−}} and respective controls with LPS. 7 h after LPS stimulation TNF-R1\textsuperscript{−/−} deletion resulted in a substantial improvement in liver injury compared to NEMO\textsuperscript{Δhepa} and NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} mice (supplementary Fig. 5B). H&E staining demonstrated that acute liver haemorrhage was completely prevented in triple knockout mice (supplementary Fig. 5C). Additionally, DNA double strand breakage was significantly reduced after TNF-R1 deletion in NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} livers (supplementary Fig. 5D). Importantly, 57\% of NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} mice did not survive the first 7 hours after LPS stimulation, while none of the WT mice and only 14\% of the NEMO\textsuperscript{Δhepa} animals died during this observation period. Interestingly, deletion of TNF-R1 was highly beneficial as all NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}/TNF-R1\textsuperscript{−/−}} animals survived the challenge (supplementary Fig. 5E). This suggests that the LPS-hypersensitivity due to lack of p21 is mediated via TNF-dependent signaling.

NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} livers show increased cholestasis associated with changes in biliary architecture.

Macroscopic analysis of livers from 52 week-old NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} animals revealed an overall yellowish appearance (Fig. 5A). As a result we studied markers of cholestasis and found that alkaline phosphatase (AP) (Fig. 5B), total and direct bilirubin levels (supplementary Fig. 6A,B) were significantly increased in the serum of NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} animals. Hence, NEMO\textsuperscript{Δhepa/p21\textsuperscript{−/−}} mice suffered from severe cholestasis. Histological examination of NEMO\textsuperscript{Δhepa} livers revealed enhanced
steatotic lesion whereas NEMO<sup>±hepa</sup>/p21<sup>−/−</sup> livers showed large necrotic areas frequently located around bile ducts (Fig. 5C,D).

These significant differences prompted us to study the impact on CK19-positive cells and we thus performed immunofluorescence staining. This staining revealed that CK19 positive cells in NEMO<sup>±hepa</sup> livers are located in HCC samples around bile ducts, while in NEMO<sup>±hepa</sup>/p21<sup>−/+</sup> HCCs single CK19 positive cells were equally distributed in the tissue (Fig. 5E). In contrast, no differences in CK 19 staining were found in non-tumorous areas of NEMO<sup>±hepa</sup> and NEMO<sup>±hepa</sup>/p21<sup>−/+</sup> livers (Fig. 5F).

**p21 deletion promotes hepatocarcinogenesis.**

The long term consequences of increased injury in NEMO<sup>±hepa</sup>/p21<sup>−/−</sup> livers were assessed in 52 week-old animals. Macroscopically NEMO<sup>±hepa</sup>/p21<sup>−/−</sup> livers showed more tumor nodules compared to NEMO<sup>±hepa</sup> mice (Fig 6A). The tumors displayed broadening of liver cell cords and loss of the reticulin network. In order to microscopically classify the observed nodules we performed stainings with established HCC markers like Golgi protein-73 (GP73) and glutamine synthetase (GS) (Fig. 6B) demonstrating that tumors in both strains classified as HCCs.

Liver/body weight ratio was significantly enhanced in NEMO<sup>±hepa</sup>/p21<sup>−/−</sup> livers (Fig. 6C) as well as liver injury markers like ALT and AST (Fig. 6D, supplementary Fig. 7). Quantification of hepatocarcinogenesis revealed a significantly higher number of tumors per liver in NEMO<sup>±hepa</sup>/p21<sup>−/−</sup> compared to NEMO<sup>±hepa</sup> mice (Fig. 6E), while the average size of these tumors was similar between the groups (Fig. 6F).

**Earlier tumor onset in 26 week old double knockout mice.**
After we found increased tumor burden in 52 weeks old NEMO<sup>Δhpepa</sup>/p21<sup>−/−</sup> livers, we studied if changes in tumor growth and cell proliferation were evident at earlier time points. 26 week-old NEMO<sup>Δhpepa</sup>/p21<sup>−/−</sup> livers showed already macroscopically significant differences compared with NEMO<sup>Δhpepa</sup> mice. The liver surface of NEMO<sup>Δhpepa</sup>/p21<sup>−/−</sup> animals showed a more irregular surface and small nodules could be detected on the hepatic surface (Fig. 7A). These changes were associated with a higher liver/body weight ratio (Fig. 7B). Additionally, at this time point more Ki67 positive cells were found in the liver of NEMO<sup>Δhpepa</sup>/p21<sup>−/−</sup> animals suggesting higher proliferation in 26 week-old livers (Fig. 7C).

In order to better characterize the 26 week tumors, we performed staining for CK19, Gp73 and GS. Our result shows that in NEMO<sup>Δhpepa</sup> as well as in NEMO<sup>Δhpepa</sup>/p21<sup>−/−</sup> livers GP73 positive cells are partially present in the tumor area, whereas the tumors are in both cases negative for GS (supplementary figure 8). As shown in supplementary Figure 8 we did not observe any signs of malignant growth at this age. Therefore these tumors have been classified as adenomas.
Discussion

The role of p21 for tumorigenesis is not fully understood. Convincing studies demonstrated that p21 can act as an oncogene as well as a tumor suppressor. In the present study we aimed to investigate the relevance of p21 for hepatocarcinogenesis in the NEMO\textsuperscript{\textregistered}\textregistered\textsuperscript{-}\textsuperscript{hepa} mouse model. This model is of clinical interest as these animals develop a cascade of events as also found in humans, which leads from chronic hepatitis to liver cirrhosis and growth of hepatocellular carcinomas (HCC). Interestingly, we found in human HCC that NEMO is downregulated thus indicating that results found in NEMO\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{hepa} livers might have a direct relevance for human liver diseases (1).

In former studies we found in NEMO\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{hepa} livers an overexpression of the cell cycle inhibitor p21 (13). To study its relevance for disease progression, we generated NEMO\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{hepa}/p21\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{/-} double knockout mice and examined the relevance of p21 deletion for disease progression and its sensitivity towards LPS-induced liver injury.

Because p21 is a cell cycle inhibitor we first investigated the impact on cell proliferation in NEMO\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{hepa}/p21\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{/-} livers. Interestingly in 8 week-old animals p21 deletion had no influence on the proliferation of hepatocytes. This was an unexpected result since p21 binds to CcnE/cdk2 and CcnA/cdk2 complexes thereby preventing progression from G\textsubscript{1} to S-phase. At present we propose that loss of p21 expression is compensated by the increased activity of other cell cycle inhibitors such as p-p27 and p18, which is supported by our present data.

Furthermore, we found that deletion of p21 in NEMO\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{hepa} mice was beneficial in terms of inflammation as there was a clear amelioration of inflammatory cells and cytokines (TNF) in NEMO\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{hepa}/p21\textsuperscript{\textregistered}\textsuperscript{-}\textsuperscript{/-} livers. Reduced TNF expression is most likely caused by increased TIMP3 levels in the livers. TIMP3 is able to inhibit TACE, a protease that generates soluble TNF from the cell surface form of the cytokine (21).
Hence, TIMP3 is an important negative TNF regulator (22). As a consequence of reduced cytokine production the mitogenic stimulation of CcnD was decreased in 8 week-old NEMO^{h pca}/p21^{-/} livers.

Despite a reduced inflammatory response the loss of p21 expression triggered increased liver injury in NEMO^{h pca} mice, which was reflected by elevated serum transaminases (AST). Further analysis revealed that increased liver injury in the double knockout mice is mediated through enhanced DNA damage. As a consequence of higher DNA damage the expression of important mediators for DNA repair (BRCA1, RAD51 and FoxM1) were enhanced in NEMO^{h pca}/p21^{-/} livers. However, several recent studies suggested that overexpression of DNA repair proteins is associated with a disadvantage for the treatment of breast cancer, glioblastoma multiforme and human soft tissue sarcoma cells, as they mediate resistance to chemotherapy. This has been nicely shown for FoxM1 and RAD51 mediating resistance against chemotherapeutics like Doxorubicin (23)(24)(25)(26). Thus these findings indicated that loss of p21 in NEMO^{h pca} liver may lead to more aggressive disease progression and likely a higher rate of HCCs.

A responsible mechanism for exacerbation of DNA damage in p21-deficient NEMO^{h pca} mice could be the elevated expression of CcnA2 and CcnE2 which did not cause enhanced hepatocyte proliferation in 8 week-old animals. Recent publications showed that ectopic overexpression of CcnA or CcnE in mouse embryonic fibroblast (MEFs) lead to an increase in DNA double strand breakage (27). The observed down-regulation of CcnD, a protein also known to be involved in DNA-repair mechanisms (28), might additionally contribute to enhanced DNA damage in NEMO^{h pca}/p21^{-/} livers (28).
Hepatocyte-specific deletion of NEMO inhibits activation of NF-κB and causes substantial apoptosis in the liver after LPS-injection (11). p21 has an additional function in the suppression of autoimmunity and in the inhibition of apoptosis (29)(30). We therefore investigated if p21 overexpression in NEMO<sup>hepa</sup> livers can provide protection after challenging the animals with LPS.

Our LPS experiments strongly suggest that p21 has a yet unknown function in protecting from DNA damage. As LPS-dependent cytotoxicity is mainly mediated through TNF, we tested if the p21-dependent effect on DNA damage is also related to this pathway and deleted TNF-R1 in NEMO<sup>hepa</sup>/p21<sup>−/−</sup> mice. The triple knockout mice (NEMO<sup>hepa</sup>/p21<sup>−/−</sup>/TNF-R1<sup>−/−</sup>) show a massive reduction in DNA damage and cell death. Additionally, AST serum values and liver histology was significantly improved. Finally, all triple knockout mice survived the LPS challenge suggesting that the LPS-dependent effect on DNA damage in p21 deleted animals is mediated via TNF.

As p21 deletion triggered higher DNA damage resulting in higher liver injury we examined the relevance for spontaneous hepatocarcinogenesis in aging mice. Here, the protective role of p21 was already visible in NEMO<sup>hepa</sup> mice at the age of 26 weeks. Double knockout mice at this age had already a higher liver weight/body weight ratio and more frequently showed small tumors. At this age p21 deletion also resulted in enhanced hepatocyte proliferation as evidenced and quantified by Ki67 staining. Finally, loss of p21 overexpression caused exacerbation of hepatocarcinogenesis which resulted in a significantly increased number of HCCs in 52 week-old animals. Interestingly, only the number of HCC nodules was increased and not the size of the tumors itself suggesting that the loss of p21 has more impact on tumor initiation than on tumor progression.
In a study of Maeda et al. (31) was shown that a brief oral administration of an antioxidant (BHA, butylated hydroxyanisole) around the time of DEN exposure prevented excessive DEN-induced carcinogenesis in Ikkß<sup>−/−</sup> mice. In our chronic model ROS production is not the cause for enhanced hepatocarcinogenesis in NEMO<sup>−/−</sup> p21<sup>−/−</sup> mice, since we did not observe enhanced ROS levels compared to NEMO<sup>−/−</sup> single knockout mice.

p21 has been recently shown to promote hepatocarcinogenesis in chronic cholestatic liver injury, as loss of p21 in Mdr2<sup>−/−</sup> mice significantly delayed tumor development (32). Mdr2<sup>−/−</sup> mice are lacking the Abc4 protein which is encoded by the multidrug resistance-2 gene, as a consequence they develop chronic periductular inflammation and cholestatic liver disease resulting in the development of hepatocellular carcinoma (HCC). Inhibition of NF-κB by expression of an IκB super-repressor (IκBαSR) transgene in hepatocytes has been shown to strongly reduce HCC development in Mdr2<sup>−/−</sup> mice, suggesting that NF-κB acts as a tumor promoter in the Mdr2<sup>−/−</sup> model (33). Whereas in the NEMO<sup>−/−</sup> model used in our study the lack of NFκB activation in hepatocytes leads to HCC development. This suggests that the role of p21 to act as an oncogene or tumor suppressor depends on NFκB.

Additionally, disease progression in NEMO<sup>−/−</sup>/p21<sup>−/−</sup> livers was associated with the occurrence of a cholestatic phenotype. As oval cells can differentiate into hepatocytes and cholangiocytes we analysed CK19 positive cells. Analysis of HCCs obtained from NEMO single and double knockout mice showed an expression of CK19 positive cells, which in NEMO<sup>−/−</sup> HCCs were located around bile ducts, while was spread diffusely throughout the tissue in HCCs of NEMO<sup>−/−</sup>/p21<sup>−/−</sup> livers. CK19 positive HCCs are known to be more malignant than CK19 negative HCCs, due to the high recurrence frequency after operative resection in patients (34). Especially
primary liver cancers with a more biliary phenotype are known to have a poorer prognosis after surgical resection (35). Furthermore it is assumed that activation of the oval cell compartment in a setting of chronic injury initiates or promotes HCC development (36). Strong oval cell activation has been found in pre-neoplastic livers of Mdr2\(^{-/-}\) mice, which was severely impaired in Mdr2\(^{-/-}\)/Rage\(^{-/-}\) livers and results in reduced HCC formation (36).

In summary our results demonstrate that p21 overexpression in NEMO\(^{\Delta\text{hepa}}\) mice reduces liver disease progression. Importantly our results show that p21 is involved in protecting from DNA damage triggered by the inflammatory stress found in these livers, which was further supported by our LPS experiments. As evidenced in the aging experiments these changes are associated with increased tumor initiation but not increased tumor progression. Thus we describe for the first time an additional protective function of p21 against DNA damage.
References


Figure Legends

Figure 1. Increased injury in livers of NEMOΔhepa/p21⁻/⁻ mice is not associated with changes in hepatocyte proliferation. (A) Western blot analysis of whole liver extracts was performed using p21 antibody. GAPDH was used as loading control. (B) AST serum levels and (C) Western blot analysis of whole liver extracts using E2F1, CcnA and CcnD antibody are shown. GAPDH was used as loading control. (D) BrdU staining are presented (red: BrdU-positive cells, blue: nuclei which are stained with DAPI, magnification: x200). (E) Cdk2 kinase Assay is depicted. (F) Western blot analysis of whole liver extracts using p-p27 and p18 antibody were performed. GAPDH was used as loading control. (Values are mean ± SD. n = 5 animals/time point; *P <0.05 [NEMOfff versus p21⁻/⁻, NEMOΔhepa versus NEMOΔhepa/p21⁻/⁻]). For immunofluorescence staining 6-10 sections were quantified.

Figure 2. DNA damage is enhanced in the absence of p21 in NEMOΔhepa mice. (A) Western blot analysis of whole liver extracts using p-p53 and p-SAPK/JNK (p-p54/p-p46) antibody were performed. GAPDH was used as loading control. (B) pH2AX staining are shown (red: pH2AX-positive cells, blue: nuclei which are stained with DAPI, magnification: x200). (C) Microarray analysis shows upregulation of DNA repair genes (Rad51, FOXM1, Check2 and Brca2) mainly in p21-deficient NEMOΔhepa mice. (D) BRCA1 qRT-PCR of liver mRNA was determined (Values are mean ± SD. n = 5 animals/time point; **P <0.01, ***P <0.001 [NEMOofff versus p21⁻/⁻, NEMOΔhepa versus NEMOΔhepa/p21⁻/⁻]). For immunofluorescence staining 6-10 sections were quantified.
Figure 3. Inflammation is attenuated in p21-deficient NEMOΔhepa mice. (A) CD45-staining was performed (brown: CD45-positive cells, blue: nuclei, magnification: x100). (B) F4/80 staining (red: F4/80-positive cells, blue: nuclei which are stained with DAPI, magnification: x200). qRT-PCR of liver mRNA was determined for (C) TNF and (D) TIMP3 (Values are mean ± SD. n = 5 animals/time point; *P <0.05 , **P <0.01 [NEMOff versus p21−/−, NEMOΔhepa versus NEMOΔhepa/p21−/−]). For immunofluorescence staining 6-10 sections were quantified.

Figure 4. p21 overexpression is beneficial to reduce LPS mediated DNA damage in NEMOΔhepa mice. (A) pH2AX staining are depicted (red: pH2AX-positive cells, blue: nuclei which are stained with DAPI, magnification: x100) of frozen liver sections from mice 7h after LPS treatment. (B) ALT serum levels were evaluated. (C) H&E staining from liver sections of LPS treated mice are shown (magnification: x200). (D) TUNEL staining (green: TUNEL-positive cells, blue: nuclei which are stained with DAPI, magnification: x100) of frozen liver sections from mice 7h after LPS treatment are presented. (Values are mean ± SD. n = 5 animals/time point; **P <0.01, ***P <0.001 [NEMOoff versus p21−/−, NEMOΔhepa versus NEMOΔhepa/p21−/−]). For immunofluorescence staining 6-10 sections were quantified.

Figure 5. Development of severe cholestasis is dependent on p21 expression. (A) Macroscopic images of a cholestatic liver obtained from NEMOΔhepa/p21−/− mice are shown. (B) Serum levels of AP are depicted. (C) H&E staining (magnification: x100); error bars in the NEMOΔhepa liver indicate areas of steatosis and in the NEMOΔhepa/p21−/− liver areas of necrosis. (D) H&E staining showing large areas of necrosis and cell death in double knockout livers (Magnification: x400). (E) CK19
staining of HCC areas are presented (green: CK19-positive cells, blue: nuclei which are stained with DAPI, magnification: x200). (F) CK19 staining of non-tumor areas are shown (green: CK19-positive cells, blue: nuclei which are stained with DAPI, magnification: x200). (Values are mean ± SD. n = 5-10 animals/time point; **P <0.01 [NEMO^{0f} versus p21^{−/−}, NEMO^{Δhepa} versus NEMO^{Δhepa/p21^{−/−}}]).

Figure 6. Enhanced hepatocarcinogenesis due to loss of p21 expression. (A) Macroscopic images of livers from 52 week-old mice are shown. (B) Paraffin embedded liver tumor sections were stained for H&E, GP73 and GS. (C) Liver weight/Body weight ratio was calculated. (D) ALT serum levels were determined. (E) Tumor number per mice is shown. (F) Largest tumor per mice is shown. (Values are mean ± SD. n = 5-10 animals/time point; *P <0.05, **P <0.01, ***P <0.001 [NEMO^{0f} versus p21^{−/−}, NEMO^{Δhepa} versus NEMO^{Δhepa/p21^{−/−}}]).

Figure 7. 26 week-old double knockout mice show earlier tumor formation. (A) Macroscopic images of livers from 26 week-old mice are depicted. (B) Liver weight/Body weight ratio has been calculated. (C) Ki67 stainings are presented (green: Ki67-positive cells, blue: nuclei which are stained with DAPI, magnification: x200). (Values are mean ± SD. n = 5 animals/time point; **P <0.01, ***P <0.001 [NEMO^{0f} versus p21^{−/−}, NEMO^{Δhepa} versus NEMO^{Δhepa/p21^{−/−}}]). For immunofluorescence staining 6-10 sections were quantified.

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Abbreviations:

Cdk: cyclin-dependent kinase; NEMO: NF-κB essential modulator; TNF-R1: Tumor Necrosis Factor Receptor 1; CK19: cytokeratin 19; HCC: hepatocellular carcinoma; LPS: Lipopolysaccharide; BrdU: Bromodeoxyuridine; DAPI: 4’,6-diamidino-2-phenylindole; Ccn: cyclin; PCNA: Proliferating cell nuclear antigen; SAPK/JNK: stress-activated protein kinase/Jun-N-terminal kinase; Chk2: checkpoint kinase2; FoxM1: forkhead box M1; BRCA1: breast cancer 1; TIMP3: Tissue inhibitor of metalloproteinase 3; ALT: alanine aminotransferase; AST: aspartate-aminotransferase; ROS: reactive oxygen species; AP: alkaline phosphatase; GP73: Golgi protein-73; GS: glutamine synthetase; TACE: Tumor Necrosis Factor-α converting enzyme; qRT-PCR: quantitative Real-Time PCR; wt: wildtype
Figure 1.
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Figure 7.
p21 ablation in liver enhances DNA damage, cholestasis and carcinogenesis

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