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The BH3-only protein BID promotes hepatocarcinogenesis by inhibiting the p38-mediated oxidative stress response

Johanna Orlik¹, Sven Schüngel¹, Laura Elisa Buitrago-Molina¹, Robert Geffers², Michael P. Manns¹, Atan Gross³ and Arndt Vogel¹

¹Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

²Department of Cell Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany

³Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel

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Contact:

Prof. Dr. Arndt Vogel, MD

Medical School Hannover

Phone: +49 511 532 9590

Fax: +49 511 532 8392

email: vogel.arndt@mh-hannover.de

Summary

Apoptosis is critical for maintaining tissue homeostasis and apoptosis evasion is considered as a hallmark of cancer. Increasing evidence however also suggests that proapoptotic molecules can contribute to the development of cancer including liver cancer. The aim of this study was to further clarify the role of the proapoptotic BH3-only protein BID for chronic liver injury and hepatocarcinogenesis.

Loss of BID significantly delayed tumor development in two mouse models of FAH-mediated and HBsTg-driven hepatocarcinogenesis suggesting a tumor-promoting effect of BID. The degree of liver injury as well as basal and mitogen-stimulated hepatocyte proliferation was not modulated by BID. Moreover, there were no *in vivo* or *in vitro* evidence that BID was in the DNA damage response in hepatocytes and hepatoma cells. Our data revealed that chronic liver injury was associated with strong activation of the oxidative stress response. BID impaired full activation of the MAPK p38 pathway following oxidative stress thereby facilitating the malignant transformation of hepatocytes. *Conclusion:* We provide evidence that the tumor-promoting function of BID in chronic liver injury is not related to enhanced proliferation or an impaired DNA damage response. In contrast, BID promotes malignant transformation by suppressing p38 activity following oxidative stress.

Introduction

Liver cancer is the fifth most common cancer in men and the ninth in women worldwide (1). Among all cancer types, it is the second leading cause of cancer (1) and therefore a considerable health problem. Patients with hepatocellular carcinoma (HCC), the predominant form of liver cancer (2), are confronted with limited therapeutic options (3). In order to develop new innovative approaches to treat and to prevent tumor development in the liver, a better understanding of the underlying cellular mechanisms and molecular alterations is required.

Apoptosis, the best studied form of programmed cell death, is critical for maintaining tissue homeostasis and its deregulation is recognized as an important factor for tumorigenesis (4, 5). The decision whether a cell should live or die is largely mediated by the BCL-2 family of anti- and proapoptotic proteins. The antiapoptotic proteins BCL-2, BCL-XL and MCL-1 are frequently overexpressed in HCC (6-11) supporting the hypothesis that apoptosis resistance could contribute to HCC development and progression (5). However, there is also evidence that increased apoptosis and proapoptotic molecules such as CD95 and BCL-2 like proteins promote hepatocarcinogenesis. In mouse models of ovary and liver cancer, loss of CD95 reduced cancer incidence and tumor size, demonstrating a growth-promoting role of CD95 during hepatocarcinogenesis (12). In the murine model of DEN-mediated hepatocarcinogenesis, loss of PUMA or overexpression of BCL-2 delayed tumor development (13, 14). In addition, hepatocyte-specific deletion of antiapoptotic MCL-1 enhanced proliferation and hepatocarcinogenesis in mice (15). Based on these data, apoptosis can no longer be considered as a tightly sealed barrier against malignant disease, but as a mechanism that is also capable of causing tumor formation (16).

The proapoptotic BCL-2 family member BID is an important mediator of apoptosis by connecting the receptor- and the mitochondria-mediated pathways of apoptosis (17). Beyond its major function for apoptosis, BID has also been described to be critical for the onset of tumors. Treatment with the carcinogen diethylnitrosamine (DEN) significantly delayed tumor development, which was attributed to impaired hepatocyte proliferation (18). Moreover, loss of BID delayed T-cell leukemogenesis in *Atm*^{-/-} mice (19).

The aim of this work was to further clarify the role of proapoptotic BID during hepatocarcinogenesis. Mice lacking an important enzyme of the tyrosine degradation pathway, the fumarylacetoacetate hydrolase (FAH), served as a murine HCC model. FAH deficiency leads to accumulation of toxic metabolites that cause liver damage (21, 22). Whereas treatment with the drug NTBC produces a healthy phenotype, reduced medication induces chronic liver injury and finally the development of HCC (23). *Fah*^{-/-} mice were crossbred with *Bid*^{-/-} mice to study the role of BID during chronic liver injury and hepatocarcinogenesis.

Materials and Methods

Mice: All mouse experiments were performed according to the guidelines and with approval of the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Germany). Mice were kept in individually ventilated cages under pathogen-free conditions and exposed to a 12 h light-dark cycle at 20-24°C and 50-60% relative humidity. Usually, mice received an autoclaved standard diet (Altromin 1320) and water ad libitum. C57BL/6-*Fah*^{tm1Mgo} (*Fah*^{-/-}) mice and C57BL/6-*Fah*^{tm1Mgo} C57BL/6-*Bid*^{tm153SJK} (*Fah*^{-/-} *Bid*^{-/-}) double knockout mice received the standard diet mixed with the drug NTBC (15 mg/kg). To induce chronic liver injury, 6- to 8-week-old *Fah*-deficient mice received the standard diet combined with 2.5% NTBC in the drinking water (187,5 ng/ml NTBC). Tg(Alb1HBV)44Bri (*HBsTg*) mice expressing the HBV surface antigen (24) were crossed with C57BL/6-*Bid*^{tm153SJK} (*Bid*^{-/-}) mice to obtain Tg(Alb1HBV)44Bri C57BL/6-*Bid*^{tm153SJK} (*HBsTg Bid*^{-/-}). The generation of *Bid*^{S61A/S78A} mice is described in the supplementary methods. Nude mice (CrI:NMRI-Foxn1^{nu}) were purchased from Charles River (Sulzfeld, Germany). Mice designated for analysis of hepatocyte proliferation received water containing 0.8 mg/ml BrdU four days before sacrifice.

Cell Culture: The murine hepatoma cell line Hepa1c1c7 (CRL-2026) and the helper cell line HEK293T (ATTC-3216) were obtained from ATTC (Manassa, Virginia, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under a 5% CO₂ humidified environment.

Treatment with DNA Damaging Agents: Hepa1c1c7 cells were treated with the cytostatic drugs cisplatin and doxorubicin, obtained from Hannover Medical School Therapeutic Drug Center, and with the chemical H₂O₂ (Sigma-Aldrich) to induce DNA damage and oxidative stress, respectively.

Cell Viability Assay: Viability was determined using the alarmBlue Cell Viability Assay according to the manufacturer's protocol (Thermo Scientific). Fluorescence was measured at 544 nm excitation and 590 nm absorbance by a fluorescence reader (SpectraMAX Gemini EM) and calculated according to the alarmBlue assay protocol.

Cell Cycle Analysis: Hepa1c1c7 cells were treated with 50µM cisplatin, 0.5µM doxorubicin or 100µM H₂O₂. Eight hours after treatment, cells were washed with PBS, detached with 200 µl Accutase (PAA Laboratories) at 37°C. After washing with PBS, cells were fixed in 1 ml 70% ice cold ethanol for 24 hours at -20°C. Fixed cells were washed with ice cold PBS, centrifuged at 500 x g for 10 min and resuspended in 200 µl PBS + 2mM EDTA. RNase was added at 12.5 µg/ml and incubated at 37°C for 30 min in the dark. After addition of PI (Sigma-Aldrich) at 25 µg/ml, the suspension was measured by a flow cytometer (LSRII, BD).

Immunoprecipitation: Immunoprecipitation was carried out with Dynabeads Protein G (Life Technologies) according to the manufacturer's protocol.

Statistical Analysis Data are represented as mean \pm SD. Data were analyzed by analysis of variance followed by Student's t-test to determine significance. p-values were considered statistically significant when $p < 0.05$.

Results

Loss of Bid Delays Tumor Formation in Chronically Injured *Fah*^{-/-} Mice

To study the role of BID in hepatocarcinogenesis, *Fah*^{-/-} and *Fah/BID*^{-/-} mice in the C57BL/6 background were generated. Next, NTBC was reduced to 2,5% of the normal dose to induce chronic liver injury. *Fah*^{-/-} and *Fah*^{-/-} *Bid*^{-/-} mice were phenotypically indistinguishable from each other and there was no overt morphologic phenotype. At 3, 6, 9 and 12 months of treatment, mice were analyzed for the onset of liver tumors. At each time point, *Fah*^{-/-} *Bid*^{-/-} mice displayed fewer liver tumors compared to *Fah*^{-/-} mice (Figure 1A, B). The cumulative tumor incidence and number were significantly lower in *Fah*^{-/-} *Bid*^{-/-} mice (Figure 1 A, C), whereas the mean maximum tumor size remained equal between both genotypes (Figure 1D). Since first liver tumors were detectable at six months of 2.5% NTBC treatment, three-month-treated mice were subsequently used to analyze the molecular mechanisms that contribute to the delayed hepatocarcinogenesis in *Fah*^{-/-} *Bid*^{-/-} mice.

Induction of Oxidative Stress-Responsive Proteins Indicates Moderate Liver Damage at Early Stages of Chronic Liver Injury

First, we were interested to determine whether BID modulates the degree of chronic FAA-induced liver injury. Markers of liver injury were investigated with histological and biochemical markers. Histological examination revealed only mild acinar inflammation without a significant difference between the two groups (Figure 2A). Accordingly, bilirubin and transaminase levels as biochemical markers of liver injury were similar between *Fah*^{-/-} and *Fah*^{-/-} *Bid*^{-/-} mice (Figure Suppl. 1). TUNEL-positive cells were hardly detected in any group (Fig. 2A). We have previously shown that accumulating FAA induces oxidative stress and an oxidative stress response mediated by NRF2 (27). Protein expression of two well-known targets of NRF2, NQO1 and HO-1, were strongly induced in chronically injured *Fah*^{-/-} mice (Figure 2B). Loss of BID led to a considerably weaker induction of HO-1 during chronic liver injury. Together, these data indicate that loss of BID does not significantly affect chronic liver injury induced by FAA. Expression of BID, however, was required for full activation of HO-1.

BID Is Dispensable for Hepatocyte Proliferation *In Vivo*

Several recent studies have shown that Bcl-2 like proteins do not only regulate apoptosis but also cell proliferation. Moreover, it has been shown that BID may impair hepatocyte proliferation during DEN-induced hepatocarcinogenesis (18) and progression through S-phase following DNA damage (28, 29). To elucidate whether BID and phosphorylation of BID play a role in S-phase transition of hepatocytes, we first analyzed liver regeneration in *Bid*^{S61A/S78A} knock-in, XXX and *Bid*^{-/-} mice and their respective littermate controls following partial hepatectomy (PH). Ki67 positive cells were readily detectable 37 hours following PH in

WT mice reaching a maximum at around 42 hours and decreasing thereafter (Fig. 3). *Bid*^{S61A/S78A} knock-in mice displayed essentially the same proliferation rate as the WT type controls, indicating that phosphorylation of BID is not required for S-phase transition of hepatocytes (Fig. 3B,C). Moreover, there was also not difference in the proliferation rate of hepatocytes between *Bid*^{-/-} mice and their littermate controls (Fig. 3E,F). To further elucidate the role of BID for hepatocyte proliferation, a panel of cell cycle related proteins was analyzed following partial hepatectomy. In agreement with the immunohistochemistry data, a similar expression of proteins involved in the priming phase of liver regeneration such as STAT3, PCNA and c-JUN but also of cyclin D, E, A and B was evident in *Bid*^{-/-} and *Bid*^{S61A/S78A} mice and their respective controls (Suppl Fig. 2). Together, these data suggest that BID itself and its phosphorylation status do not play an essential role for liver regeneration of healthy mice.

To test whether the delay of tumor development in *Fah*^{-/-} *Bid*^{-/-} mice was accompanied by impaired hepatocytes proliferation in mice with chronic liver injury, mice were analyzed for basal hepatocyte proliferation. With about 1-4% proliferating hepatocytes, the basal proliferation rate was low in healthy mice and increased only slightly upon chronic liver injury (Fig. 2C). Microarray analysis identified 22 cell cycle-associated genes whose expression was regulated by at least 2-fold in comparison with healthy *Fah*^{-/-} mice. Only three genes, cyclin D1, p15 and Igf2, were stronger up-regulated in *Fah*^{-/-} mice compared to *Fah*^{-/-} *Bid*^{-/-} mice (Suppl. Fig. 3). In line with the microarray results, protein levels of p21 and cyclin D1 were higher in mice with chronic liver injury, which was more prominent in *Fah*^{-/-} mice. Hence, in agreement with the proliferation rate of hepatocytes an induction of cell cycle-associated genes was detectable during chronic liver injury, which was however similar between the two groups. Differences in proliferation may be low under basal conditions but more evident after mitogen stimulation. Therefore, chronically injured mice were also subjected to 2/3 PH. The proliferative capacity of chronically injured mice was strongly impaired after PH (Fig. 2D), which is in line with our previous observation (23). However, loss of BID had no significant influence on PH-induced hepatocyte proliferation in chronically injured *Fah*-deficient mice.

Taken together, these results indicate that BID does not significantly impair baseline hepatocyte proliferation during FAA-induced liver injury and following PH in wild type and *Fah*-deficient mice. Moreover, phosphorylation of BID was dispensable for mitogen-stimulated hepatocyte proliferation.

Delayed tumor development is accompanied with a reduced expression of Cancer-Related Genes and Proteins in Chronically Injured BID-Deficient Mice

Microarray analysis was performed to identify molecular pathways that contribute to the tumor-promoting function of BID during hepatocarcinogenesis. 50 cancer-associated genes

were identified whose expression was induced in tumor-prone mice compared with control mice (Fig. 4). Among them, 16 genes showed more than 10-fold regulation. Alpha-fetoprotein (*Afp*) and *H19*, which are known tumor markers in HCC (30, 31), were up-regulated by 15-fold in 2.5% NTBC *Fah*^{-/-} mice, but only by 2.5- and 3-fold, respectively, in 2.5% NTBC *Fah*^{-/-} *Bid*^{-/-} mice in agreement with the delayed tumor development. Of note, *Fah*^{-/-} mice exhibited a stronger up-regulation of four genes involved in the oxidative stress response, *Gpx2* (74.6-/24.4-fold), *Srxn1* (10.6-/4.7-fold), *Ho1* (5.2-/1.8-fold) and *Maoa* (3.4-/1.7-fold) compared with *Fah*^{-/-} *Bid*^{-/-} mice, suggesting that BID might modulate the oxidative stress response in the *Fah* model. In a second approach, genes with at least 10-fold regulation were subjected to Ingenuity Pathway Analysis (Suppl. Figure 4). Chronic liver injury induced the expression of genes involved in molecular transport, cell growth and cell proliferation in both *Fah*^{-/-} and *Fah*^{-/-} *Bid*^{-/-} mice. However, genes related to cancer were only induced in *Fah Bid*^{-/-} mice.

The PI3K/AKT/mTOR pathway is frequently deregulated in cancer and contributes to tumor development in the *Fah* model (Buitrago et al.)(Suppl. Fig. 5). AKT showed elevated phosphorylation only in chronically injured *Fah*^{-/-} mice. However, protein expression of the upstream and downstream signaling molecule mTOR remained unchanged. S6 kinase (S6K) and 4E-BP1 are downstream targets of mTOR. Expression of 4E-BP1 and its phosphorylated form were similarly induced upon chronic injury. In contrast, phosphorylation of the ribosomal protein S6 (S6) was significantly reduced in chronically injured *Fah*^{-/-} *Bid*^{-/-} mice.

BID Is Dispensable for Hepatocyte Proliferation and the DNA Damage Response *In Vitro*

Previous studies favored DNA damage as a crucial mechanism for *Fah*-mediated carcinogenesis (22) and have identified BID as an effector of the DNA damage response (28, 29). DNA damage was not detectable by γ -H2AX staining in *Fah*-deficient livers on low dose NTBC (data not shown), suggesting that FAA induces only minor, if any DNA damage *in vivo*. To determine the role of BID in the DNA damage response in murine hepatoma cells, an *in vitro* approach was applied using Hepa1c1c7 cells that were stably transduced with BID shRNA. Two BID shRNAs, BID #1 shRNA and BID #5 shRNA, were chosen for subsequent experiments (Fig. 5A).

In line with the *Fah* model, reduced expression of BID had no significant impact on cell proliferation in Hepa1c1c7 cells, as determined by the crystal violet method (Fig. 5B). To test whether impaired proliferation becomes evident *in vivo*, BID-silenced cells were injected into the flank of nude mice and monitored for tumor growth. As a result, tumors grew equally among all groups (Fig. 5C,D). *In vitro* studies with stable BID knockdown cells support the *in vivo* findings that BID is dispensable for proliferation of hepatocytes and hepatoma cells.

Hepa1c1c7 cells expressing BID shRNA were treated with different concentrations of doxorubicin, cisplatin and H₂O₂ and analyzed for p53 protein expression as a read-out for the DNA damage response (Fig. 6A). All three substances impaired cell viability independently of BID (Fig. 6B). One concentration was chosen to analyze p53 and HO-1 expression at the protein level. Whereas p53 protein expression was similarly induced after treatment with all three damaging agents, HO-1 protein was slightly upregulated in H₂O₂-treated BID knockdown cells (Fig. 6C).

DNA damaging agents stop cell cycle progression to enable repair of damaged DNA, usually by arresting cells in the G2/M phase. Therefore, cell cycle phase distribution was analyzed 8 hours after treatment (Fig. 6D). Cisplatin, doxorubicin and H₂O₂ treatment increased the amount of cells in the G2/M phase. However, Hepa1c1c7 cells with reduced BID levels did not differ from control cells regarding cell cycle distribution, suggesting that BID is dispensable for the DNA damage response.

Loss of BID Delays Hepatocarcinogenesis by Reactivating the p38 Cellular Stress Response

Neither *in vivo* nor *in vitro* analyses revealed a major impact of BID on proliferation or the DNA damage response. Due to strong activation of the oxidative stress response in chronically injured mice, subsequent experiments were performed to clarify the role of BID during cellular stress. Environmental changes of the cell are sensed by the mitogen-activated protein kinase (MAPK) family, which mediates proliferation, differentiation, survival and/or apoptosis (32, 33). In contrast to the extracellular signal-regulated kinase (ERK) MAPK pathway and the c-JUN NH2-terminal kinase (JNK) pathway (data not shown), the p38 MAPK, implicated in suppression of tumorigenesis (32), showed a consistent genotype-specific protein expression pattern *in vivo* and *in vitro* (Fig. 7).

Chronic liver injury was accompanied by a reduced activation and phosphorylation of p38, which was significantly stronger in tumor prone *Fah*^{-/-} mice (Figure 7A). To investigate whether BID enters into direct interaction with p38 to suppress its phosphorylation, BID was immunoprecipitated from the protein lysate of chronically injured *Fah*^{-/-} mice, where strongest p38 suppression was observed. Immunoprecipitation analysis however did not reveal a direct interaction between BID and p38 (Fig. 7B).

To confirm the important relationship between BID and p38 during oxidative stress, a second mouse model was used, the HBV surface antigen transgenic mice (*HBsTg*) strain. *HBsTg* mice suffer from extensive oxidative DNA damage and develop chronic liver injury that subsequently proceeds to HCCs, mimicking the human disease (24). After ten months, first liver tumors were detected in *HBsTg* mice and, in accordance with the *Fah* model, tumor development was significantly delayed in *HBsTg Bid*^{-/-} mice (Fig. 7C). Moreover, loss of BID

resulted in increased levels of phosphorylated p38 compared with WT and *HBsTg* mice (Fig. 7D).

To confirm the interrelationship between BID and p38 *in vitro*, we analyzed Hepa1c1c7 cells after BID siRNA treatment and BID shRNA-transduced Hepa1c1c7 cells after H₂O₂ treatment. Compared with control cells, BID-silenced Hepa1c1c7 cells showed elevated levels of phosphorylated p38 72 hours after siRNA treatment and 24 hours after H₂O₂ treatment (Fig. 7E). Collectively, *in vivo* and *in vitro* experiments suggest a suppressive role of BID on p38 activity in the context of oxidative stress. In the absence of BID, p38 activity is reactivated, facilitating a protective response to cellular stress.

Discussion

Apoptosis resistance is one of the hallmarks of cancer cells (5). Consequently, proapoptotic molecules are supposed to induce apoptosis and to prevent tumor development. Contrary to this expectation, two previous studies with independent mouse models showed that loss of proapoptotic BID delays tumor development (18, 19). In line with these findings, loss of BID significantly delayed tumor development following *Fah*^{-/-}-mediated and *HBsTg*-driven chronic injury. However, the underlying mechanisms how BID contributes to tumor formation are not fully understood, ranging from modulation of proliferation to involvement of the DNA damage response. Our attempt was therefore to examine more precisely which cellular mechanisms and signaling pathways are influenced by BID during chronic liver injury and hepatocarcinogenesis.

In DEN-treated *Bid*^{-/-} mice, delayed hepatocarcinogenesis was attributed to reduced hepatocyte proliferation (18). To test whether this correlation is also valid in the *Fah* model, we analyzed basal and mitogen-stimulated proliferation in healthy mice and in mice with chronic liver injury. The basal proliferation rates were comparable among healthy and chronically injured mice and independent of BID. Accordingly, expression of cell cycle-related genes was similarly induced upon chronic liver injury without genotype specificity. Mice were also subjected to partial hepatectomy to stimulate proliferation and both genotypes showed similar proliferation rates. It was striking that *Fah*-mediated moderate injury substantially impaired hepatocyte proliferation, indicating that hepatocytes are disturbed to a degree that excludes proliferation as a possible compensatory mechanism to chronic liver damage. Likewise, mitogen-stimulated proliferation was similar between *Bid*^{S61A/S78A} knock-in mice and wild type controls and underlines that also BID phosphorylation has no impact on hepatocyte proliferation *in vivo*. Furthermore, shRNA-mediated silencing of BID in the hepatoma cell line Hep1c1c7 did not affect proliferation *in vitro* and tumor growth *in vivo*. Collectively, our data indicate that BID is not required for hepatocyte in our mouse models and for hepatoma cell proliferation.

The *Fah* model is characterized by accumulation of toxic metabolites, which are predicted to alkylate DNA and proteins, leading to excessive oxidative stress and genomic instability (34). Due to the assigned role of BID in the DNA damage response (28, 29), chronically injured *Fah*^{-/-} and *Fah*^{-/-} *Bid*^{-/-} mice were analyzed for γ -H2AX, a common indicator of DNA double strand breaks. However, immunohistochemical stainings of chronically injured mice were negative for γ -H2AX. Hence, the role of BID in the DNA damage response was further delineated *in vitro* by treating Hepa1c1c7 cells with doxorubicin, cisplatin and H₂O₂. In our experimental setting, knockdown of BID had no effect on cell viability, cell cycle arrest or the responsiveness of p53 protein expression, which argues against an important role of BID for the DNA damage response in hepatocytes and hepatoma cells. In line with this finding,

analysis of primary mouse embryonic fibroblasts showed that cell cycle arrest and apoptosis were indistinguishable between *Bid*^{-/-} and wild type cells (35). Collectively, our data indicate that BID is not involved in the DNA damage response of hepatocytes and hepatoma cells.

Our results show that the oxidative stress response is significantly activated in *Fah*- and *HBsTg*-driven liver disease. Recently, BID has been associated with oxidative stress. Loss of BID phosphorylation, ATM knockout or exposing mice to irradiation increased mitochondrial BID in hematopoietic stem cells and correlated with an increase in mitochondrial oxidative stress (36). We therefore analyzed the effect of BID on the cellular stress kinases ERK, c-JUN and p38 *in vivo* and *in vitro* and found compelling evidence that BID suppressed p38 activity. The fact that healthy *Fah*^{-/-} and *Fah*^{-/-} *Bid*^{-/-} mice and untreated Hepa1c1c7 cells with or without a BID knockdown express equal levels of phosphorylated p38 indicate that BID does not inhibit p38 phosphorylation in general. p38 activity is only suppressed in the context of an oxidative stress response, as shown by upregulation of Ho1, Nqo1 or Srxn1 in the *Fah* model. siRNA and H₂O₂ treatment of Hepa1c1c7 cells was associated with suppression of p38 activity, which could be reactivated upon BID silencing. Both siRNA and H₂O₂ treatment are potent inducers of the NRF2-mediated stress response (37) (38), which confirms the link between BID and p38 inhibition during oxidative stress. Activation of the p38 MAPK pathway inhibits proliferation or mediates apoptosis to protect cells from oxidative stress (32). Transformation of fibroblasts from *p38α*^{-/-} mice with *HRas12* was associated with increased ROS production, increased proliferation, increased ability to form colonies in soft agar, decreased apoptosis and accelerated subcutaneous tumor formation (39). The fact that ectopic expression of p38α rescued these effects suggested that full activation of p38 suppresses tumorigenesis (40). Dolado et al. proposed a model in which tumor cells uncouple ROS production from p38 activation to overcome the tumor-suppressive function of p38 (39). We therefore hypothesize that BID overrides p38 activity by indirect interaction, leading to sustained ROS levels and consequently to malignant transformation (Fig. 8). Loss of BID reactivates p38 activity thereby delaying tumor formation.

Together, we provide evidence in two independent mouse models that BID contributes to hepatocarcinogenesis. The tumor-promoting function of BID was not attributed to enhanced proliferation or modulation of the DNA damage response, but promotes malignant transformation of hepatocytes by suppressing p38 activity during oxidative stress induced liver injury.

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Figure 1

Loss of BID Delays *Fah*-Mediated Hepatocarcinogenesis

Fah and *Fah Bid* mice were treated with 2.5% NTBC for 6, 9 and 12 months and analyzed for liver tumors. (A) Tumor incidence, (B) representative macroscopic pictures, (C) tumor number and (D) tumor size are shown for each time point. (C-D) The lines within the scatter plots indicate the mean value for each group. (A, C, D) A cumulative mean value was generated for both genotypes. Statistically significant differences with a p-value greater than 0.05 between both genotypes are indicated with an asterisk.

Figure 2

BID Is Dispensable for the Degree of Liver Damage as well as Basal and Mitogen-Stimulated Liver Regeneration during *Fah*-Mediated Chronic Injury

Healthy (100% NTBC) and chronically injured (2.5% NTBC) *Fah*^{-/-} (F) and *Fah*^{-/-} *Bid*^{-/-} (FB) mice were analyzed after three months of NTBC treatment. (A) Representative microscopic pictures of liver tissues stained with hematoxylin and eosin (H&E) or the TUNEL method. (B) Western blot analysis of total liver lysates from pooled samples (n=8). (C-D) Representative microscopic pictures and quantification of (C) unstimulated and (D) mitogen-stimulated livers. Hepatocyte proliferation was determined using the proliferation markers Ki67 and BrdU. TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling, BrdU: bromodeoxyuridine, PH: partial hepatectomy.

Figure 3

The Presence and Phosphorylation Status of BID Are Dispensable for Mitogen-Stimulated Liver Regeneration in C57BL/6 Mice

(A-C) *Bid*^{-/-} mice, (D-F) *Bid*^{S61A/S78A} knock-in (KI) mice and their littermate controls were subjected to partial hepatectomy and analyzed for BID protein expression and Ki67-positive cells at indicated time points. Whereas *Bid*^{-/-} mice and their wild type (WT) control mice were bred in a C57BL/6 background, *Bid*^{S61A/S78A} KI mice and their wild type (WT*) control mice were bred in a mixed background.

Figure 4

Loss of BID Attenuates Expression of Cancer-Related Genes during *Fah*-Mediated Chronic Liver Injury

Gene expression of cancer-related genes in healthy (100% NTBC) and chronically injured (2.5% NTBC) *Fah*^{-/-} and *Fah*^{-/-} *Bid*^{-/-} mice after three months of NTBC treatment. Gene expression is displayed as fold change compared with healthy *Fah*^{-/-} mice. Genes are labeled with the cellular processes for which they play an important role.

Figure 5

BID Is Dispensable for Hepatocyte Proliferation of Hepa1c1c7 Cells

(A-D) Hepa1c1c7 cells were subjected to lentiviral-mediated gene silencing of BID. (A) Six different shRNA plasmids were tested for silencing their silencing efficiency by western blot analysis. (B) Proliferation determined by the crystal violet method. (C-D) Quantification and representative pictures of tumor size two weeks after injection of 2 million Hepa1c1c7 cells into the flank of nude mice.

Figure 6

BID Is not Involved in the DNA Damage Response in Hepa1c1c7 Cells

(A-D) Treatment of Hepa1c1c7 cell lines with doxorubicin, cisplatin and H₂O₂. BID-silenced cells (BID 1 and BID 5) were compared with untransduced control cells and cells silenced with a nontargeting shRNA. (A) Western blot analysis of untransduced (ut) Hepa1c1c7 cells 6 hours after treatment with different compound concentrations using p53 as a read out for DNA damage. (B) Cell viability determined by the alarmBlue method after treatment with DNA damaging agents. (C) Western blot analysis after treatment with 0.5 μM doxorubicin, 50 μM cisplatin and 100 μM H₂O₂. p53 and HO-1 were used as a read out for DNA damage and oxidative stress, respectively. (D) Cell cycle analysis 8h after treatment with 0.5 μM doxorubicin, 50 μM cisplatin and 100 μM H₂O₂. Histograms display the amount of cells with a distinct DNA content as measured by propidium iodide (PI) incorporation. The DNA content predicted the respective cell cycle phase and enabled quantification.

Figure 7

Loss of BID Reactivates p38 Phosphorylation in the Context of Oxidative Stress

(A) Western blot analysis of three-month-treated healthy (100% NTBC) and chronically injured (2.5% NTBC) *Fah*^{-/-} (F) and *Fah*^{-/-} *Bid*^{-/-} (FB) mice using total liver lysates pooled from 8 samples. Four representative samples within the group of chronic injury were quantified separately and as a mean value. (B) Western blot analysis after immunoprecipitation (IP) of BID from the total liver lysate pool of chronically injured *Fah*^{-/-} mice. (C) Tumor incidence in the model of HBV-mediated chronic hepatitis (*HBsTg*). (D) Western blot analysis of three-month-old *HBsTg* (H) and *HBsTg Bid*^{-/-} (HB) mice using total liver lysates pooled from 4 samples. The samples were quantified separately and as a mean value. (E) Western blot analysis of siRNA-treated Hepa1c1c7 cells and H₂O₂-treated shRNA-expressing Hepa1c1c7 cells. Statistically significant differences with a p-value greater than 0.05 are indicated with an asterisk.

Figure 8**Proposed Model for the Interaction between BID and p38 during Cellular Stress**

HO-1 is a common cellular stress sensor that responds to various kinds of stress, including DNA damage and oxidative stress. Upon cellular stress, HO-1 and/or other factors activate p38 by phosphorylation (P). If necessary, activated p38 inhibits proliferation and induces apoptosis. Under certain circumstances, e.g. in the presence of high H₂O₂ levels, BID stimulates other, yet unknown factors that suppress p38 activity. Inhibition of p38 activity can lead to malignant transformation. In the absence of BID, p38 is activated and can execute its protective function in the cell, which impedes or delays malignant transformation.