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Liver-specific *Ldb1* deletion results in enhanced liver cancer development

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Abstract

Background & Aims—LIM-domain-binding (Ldb) proteins have been demonstrated to be essential not only to key embryonic developmental processes but also to carcinogenesis. We have previously demonstrated Ldb1 to be of high biological and developmental relevance, as a targeted deletion of the *Ldb1* gene in mice results in an embryonic lethal and pleiotropic phenotype.

Methods—We have now established a liver-specific *Ldb1* knock out to investigate the role of Ldb1 in carcinogenesis, in particular in hepatocellular carcinoma (HCC) development, *in vivo*.

Results—These mice demonstrated a significantly enhanced growth of liver cancer by means of tumor size and number, advocating for an essential role of Ldb1 in HCC development. In addition, proliferation and resistance against apoptosis were increased. In order to identify the functional disturbances due to a lack of Ldb1, we performed a 15 k mouse gene microarray expression analysis. We found the *Myc* oncogene to be regulated in the microarray analysis and were able to further confirm this regulation by demonstrating an over-expression of its downstream target *Cyclin D1*. Furthermore, we were able to demonstrate a down-regulation of the tumor suppressor *p21*. Finally, the liver stem cell marker EpCAM was also identified to be over expressed in $Ldb1^{-/-}$ knock out mice.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Conclusions—We have established a significant role of Ldb1 in cancer development. Furthermore, we provided evidence for a myc/cyclin D1, p21, and EpCAM-dependent signalling to be key downstream regulators of this novel concept in HCC development.

Introduction

LIM-domain-binding (Ldb) proteins are highly conserved among species from worm to man. These proteins have been demonstrated to be expressed in a wide range of embryonic and adult tissues and to interact with multiple LIM-homeodomain (LIMHD) and LIM-only (LMO) proteins through their LIM interacting domain (LID) [1-3]. LIM proteins are characterized by a consensus domain consisting of several cysteine and histidine which bind zinc ions and are responsible for protein–protein interactions. The protein family was named after the first three family members *Lin-11, Isl-1*, and *Mec-3*. The LIM-HD and LMO proteins had previously been demonstrated to be essential not only to key embryonic developmental processes such as cell fate determination, cytoskeletal organization, and tissue development but also (as many embryological relevant genes are “re-activated” in cancer development) in cancer de-differentiation [4-7].

Ldb1 itself is thought to be unable to directly interfere with DNA and therefore to directly be involved in transcriptional regulation. However, by binding to LIM-homeodomain proteins, Ldb1 may very well be able to regulate transcription. It has been demonstrated that the LIM domains in LIM-HD transcriptional regulators can inhibit the DNA-binding activity of the HD [8], but can also increase the transcriptional activity of LIM-HDs in synergy with other classes of transcription factors [9,10]. Finally, Ldb1 itself is subject to regulation. RLIM is able to interact with and ubiquitinate Ldb cofactors bound to LIM-HD proteins, and thus target Ldb1 for degradation by the 26S proteosome [11].

We have previously demonstrated that all these regulatory effects of Ldb1 are of high biological relevance as a targeted deletion of the *Ldb1* gene in mice resulted in an embryonic lethal, pleiotropic phenotype. There was no heart anlage, and head structures are truncated anterior to the hindbrain. In about 40% of the mutants, posterior axis duplication was observed. Furthermore, the expression of several Wnt-inhibitors was curtailed in the mutant, suggesting that Wnt pathways may be involved in axial patterning regulated by Ldb1 [12].

With Ldb1 interacting with LIM and other regulatory proteins, potentially being involved not only in embryonic but also in cancer development, [13], we speculated about a role for Ldb1 in cancer development. However, at present, only very limited data were available on Ldb1 and cancer development. Lately, Setogawa et al. [14] had demonstrated that the tumor suppressor LKB1/STK11 (Liver kinase B1, also known as Serine–threonine kinase 11, STK 11) induces p21 expression through collaboration with an Ldb1-containing protein complex further including LMO4 (Lim only protein 4) and GATA6 (GATA binding protein 6). In addition, Johnsen et al. reported that Ldb1 is involved in regulation of the biological activity of the estrogen receptor alpha during the development of human breast cancer [15].

We now report for the first time on *in vivo* evidence by means of conditional mouse knock out experiments that Ldb1 is essential to cancer development and plays a critical role in the

development of hepatocellular carcinoma (HCC). We furthermore enlight some of the major regulated pathways during HCC development in *Ldb1* deleted mice.

Materials and methods

Animals

To generate liver-specific L*db1*-knock out mice ($Ldb1^{-/-}$) conditional ldb1-floxed animals (ldb1 fl/fl, [16]) were crossbred with albumin-cre (alb-cre) animals. Experimental animals were homozygous for $Ldb1^{-/-}$ and heterozygous for albcre on a C57Bl/6 background. Genotyping was performed as described before [12,17,18]. Age-matched wild-type (wt) littermates were used as controls. Animal care was in accordance with the governmental and institutional guidelines and all experiments were performed under the written approval of the state animal care commission.

Induction of carcinogenesis

To induce liver carcinogenesis a single diethylnitrosamine (DEN) i.p. injection (0.05 mg per animal) was performed at day 7 *post partum*. Promotion of carcinogenesis was achieved by continuously adding phenobarbital (0.05% w/v) to the drinking water. Mice were sacrificed after 9 months and analyzed for liver cancer development.

Initial analysis of livers

Livers were assessed visually and the amount of appearing tumor nodules at the surface of these livers was counted and their size measured. To investigate liver structure and tumor histology, formalin-fixed and paraffin-embedded sections (5 μm thick) were stained with hematoxylin and eosin (H&E).

Immunohistochemistry

Immunohistochemistry was performed on frozen sections (6 lm thick). Ki-67 staining was performed on acetone-fixed sections by using 1:50 rat-anti-mouse Ki-67 (DakoCytomation, Glostrup, Denmark) as primary antibody. Signal detection was performed using ‘Vectastain ABC Kit’ (Vector Laboratories, Burlingame, USA) and ‘Fuchsin Substrate-Chromogen System’ (DakoCytomation, Glostrup, Denmark). As a measurement of proliferation the total number of Ki-67-positive and -negative cells in the liver tissue was counted. In total, more than 5000 cells were counted for both $Ldb1^{-/-}$ and WT liver sections.

Analysis of apoptosis

Primary hepatocytes were isolated by a two-step collagenase perfusion as described [18]. After cultivation of the isolated cells for 3 h in DMEM + 10% FC medium, the cells were incubated with 0.2 μg/ml Jo2-Antibody (BD Biosciences, Heidelberg, Germany) for 16 h. After splitting these cells into 96-well plates (8000 cells per well), viable cells were detected using ‘CellTiter-Glo Luminiscent Cell Viability Assay’ (Promega, Mannheim, Germany) according to the manufacturer’s instructions.

Microarray analysis

Microarray analysis was performed on a genome-wide expression profiling Ldb1$^{-/-}$ and wild-type using a 15,000 mouse developmental cDNA microarray [19]. After hybridization and scanning of expression, raw data were subject to Z normalization: Z(raw data) = [ln(raw data) – avg(ln(raw data))]/[std dev(ln (raw data))], where ln is natural logarithm, avg is the average over all genes of an array, and std dev is the standard deviation over all genes of an array.

In comparing differential expression of genes between Ldb1$^{-/-}$ and wild-type animals three factors of significance were computed. Firstly, only genes, whose average intensity between the two treatments was greater than 0, were considered. This eliminates spurious observations of high fold changes from genes of low intensities comparable to the background. The filtered genes were then tested for *p* values and *z*-ratios. The *p* values are a measure of the repeatability of a gene's intensity between replicate arrays. The *z*-ratios are a measure of fold change between treatments. Z ratio (between treatment A and B) = z(A)-z(B)/std dev. Finally, we divided by the standard deviation over all genes on an array to determine whether a given ratio was statistically significant considering the array as a whole.

RealTime-PCR

Total RNA was isolated using 'Tri Reagent' (Sigma–Aldrich, Taufenkirchen, Germany) and cDNA was applied from 0.5 μg total RNA with oligo-dt-primers by using the 'First Strand cDNA Synthesis Kit' for RT-PCR (AMV), (Roche, Mannheim, Germany) both according to the manufacturer's instructions.

Specific mRNA transcripts were quantified with a LightCycler (Roche, Mannheim, Germany) by using 'LightCycler Fast Start DNA Master SYBR Green I' (Roche, Mannheim, Germany) and the following primers: *GAPDH* (GenBank Acc. No. NM_001303; for: GGCATTGCTCTCAATGACAA; rev: TGTGAGGGAGATGCTCAGTG) and rS6 (GenBank Acc. No. NM_009096; for: GTCCGCCAGTATGTTGTCAG; rev: GTTGCAGGACACGAGGAGTA) as housekeeping-genes; *c-myc* (GenBank Acc. No. NM_010849; for: TCCTGTACCTCGTCCGATTC; rev: GGTTTGCCTCTTCTCCACAG), *Cyclin D1* (GenBank Acc. No. NM_007631; for: CACAACGCACTTTCTTTCCA; rev: ACCAGCCTCTTCCTCCACTT), *p21* (GenBank Acc. No. NM_007669; for: TCTTGCACTCTGGTGTCTGA; rev: TTCAGGGTTTTCTCTTGCAG), and EpCAM (Tacstd1, GenBank Acc. No. NM_008532, for: 5′-TGTGGTGGTGTCATTAGCAG-3′, rev: 5′-GGATCTCACCCATCTCCTTT-3′) according to the manufacturer's instructions. Determination of gene expression was performed by using the LightCycler software package. Relative gene expression was given as *x*-fold expression of the used housekeeping gene *GAPDH*.

Statistical analysis

Mean ± standard error of the mean was given. For comparison of experimental groups, the nonparametric Mann–Whitney-*U*-test was applied. A $p \leq 0.05$ was considered to be significant and a $p \leq 0.01$ was considered to be highly significant.

Results

Characterization of Ldb1$^{-/-}$ livers

After crossing the conditional Ldb1^{floxed} mice to Alb-Cre mice allowing for liver-specific deletion of *Ldb1* in liver, mice homozygous for the *Ldb1* deletion survived and did not exhibit any obvious pathological phenotype. In initial descriptions of the Alb-Cre mouse, Postic et al. had reported that recombination appeared to be complete by 6 weeks of age [20]. To further investigate both liver structure and function in particular, we initially looked at hematoxylin and eosin (H&E)-stained sections of *Ldb1*-deleted livers. On these sections we found a regular liver parenchyma without any signs of necrosis, inflammation, or irregular cell shapes. As for the liver function of liver specifically *Ldb1*-deleted mice, we measured common liver tests for screening of the adult liver function. ALT, AST, and bilirubin were demonstrated to be comparable to wild-type mice suggesting a generally normal liver function.

Increased cell proliferation in Ldb1$^{-/-}$-deleted livers

Ki-67 staining was performed to investigate proliferation in *Ldb1*$^{-/-}$-deleted livers in comparison to WT livers. In *Ldb1*$^{-/-}$-deleted livers a significantly increased proliferation was demonstrated (Fig. 1).

Ldb1$^{-/-}$ deletion leads to an enhanced development of liver cancer

Liver cancer development was induced by the commonly used DEN/Phenobarbital tumor model. After 9 months, mice were sacrificed and livers were analyzed for tumor development. In a first step, the number of visible tumor nodules on the surface was counted. Comparing the tumor counts of *Ldb1*$^{-/-}$-deleted mice to those of wild-type mice demonstrated a significantly increased incidence of developing liver cancer nodules. Next, we analyzed these livers for the size of developed tumor nodules after 9 months. In comparison to wild-type mice, the *Ldb1*$^{-/-}$ mice exhibited a highly significant increased volume of tumor nodules. On average, the tumor nodules of the *Ldb1*$^{-/-}$ livers were of 214 mm^3 volume (n = 25), whereas the wild-type nodules measured, on average, only 25 mm^3 (n = 7) (Fig. 2).

Finally, H&E staining of these livers confirmed the developing tumors to be hepatocellular carcinoma (Fig. 3).

Thus, in our model, the development of HCC was not accompanied by a previous development of liver fibrosis/cirrhosis usually present in humans with HCC.

Gene expression analysis of Ldb1$^{-/-}$ livers

In order to identify the molecular changes due to *Ldb1* deletion resulting in an increased tumor development, we performed microarray analyses on *Ldb1*-deleted livers in comparison to wild-type animals. These analyses were performed in three biological replicates. Genes were considered to be differentially expressed with either a z-ratio >1.5 or a p value <0.05 and an average intensity >0. This group contained 309 gene IDs, of which 237 coded for known and characterized genes (Supplementary material).

Among the differentially regulated genes known to initiate tumor growths were the *Myc* and *Cyclin D1* oncogenes. The identification of the *Myc* oncogene being regulated in our microarray data (RT-PCR also demonstrated a clear trend wt: 1.000 ± 1.087, $Ldb1^{-/-}$: 2.241 ± 1.145; $p = 0.07$. However, statistical significance was marginally missed due to a higher variance in these samples. Data not shown) provided a potential mechanism leading to tumor development. To further evaluate this, we investigated the regulation of additional downstream targets by means of RT-PCR. Cyclin D1 has been demonstrated to function as an oncogene driving cell cycle progression by acting as a growth factor sensor to integrate extra cellular signals into the cell cycle machinery, although it may also promote apoptosis [21]. Cyclin D1 or its upstream activators not only accelerate tumor formation but also may drive tumor progression to a more aggressive phenotype. Thus, we investigated the regulation of *Cyclin D1* expression. By means of RT-PCR, we were able to demonstrate a significant difference in *Cyclin D1* expression that depended on *Ldb1* deletion. In livers of $Ldb1^{-/-}$-deleted mice expression was 4.5-fold higher (wt: 1.000 ± 0.071, $Ldb1^{-/-}$: 4.485 ± 1.021; $p = 0.001$) (Fig. 4).

P21 encodes a potent cyclin-dependent kinase inhibitor. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli [22]. It may be instrumental in the execution of apoptosis following caspase activation [23]. Thus, *p21* expression was of interest in the search for commonly cancer-related molecules and pathways regulated by deletion of *Ldb1*. Again, by means of RT-PCR, we were able to demonstrate a significant difference in *p21* expression that depended on *Ldb1* deletion. In livers of $Ldb1^{-/-}$-deleted mice expression was decreased 3.8-fold (wt: 1.000 ± 0.176; $Lbd1^{-/-}$: 0.264 ± 0.040; $p = 0.050$) (Fig. 5).

Epithelial cell adhesion molecule (EpCAM, Tacstd1)-positive HCC cells possess a greater ability to form liver cancer *in vivo* [24] and are discussed to have cancer stem cells/progenitor cell characteristics [25]. Since *EpCAM* was found to be highly differentially expressed in microarray analysis, we further confirmed this change in expression by means of RT-PCR. In livers of $Ldb1^{-/-}$-deleted mice, expression was 5.5-fold increased compared to WT animals (wt: 1.000 ± 0.114; $Lbd1^{-/-}$: 5496 ± 0.605; $p<0.001$) (Fig. 6). This significant increase in *EpCAM* expression was also found in DEN tumor-induced mice where *EpCAM* was found to be 4-fold over expressed (wt: 5.117 ± 1.973; $Lbd1^{-/-}$: 21.053 ± 5.808; $p = 0.02$).

Sensitivity to apoptosis

Apoptosis is one of the major types of programmed cell death. A deficiency in apoptosis results in uncontrolled cell proliferation and subsequently cancer development.

The Jo2 anti-mouse CD95 monoclonal antibody induces lethality in mice characterized by hepatocyte death and liver haemorrhage through CD95-mediated apoptosis. Thus, we investigated a possible difference in response to apoptosis stimuli potentially being involved in an increased cancer development. Homozygous loss of *Ldb1* leads to a prominent decrease in hepatocyte sensitivity to CD95-mediated apoptosis *in vivo*. After 16 h in the presence of 5 to 200 ng/ml Jo2, $Ldb1^{-/-}$ hepatocytes in primary culture were significantly

less responsive to Jo2-induced death than wild-type animals. Under 5, 10, 50, 100, and 200 ng/ml Jo2, we observed a significantly increased resistance to apoptosis, suggestive of a higher sensitivity for tumor development (Fig. 7).

Discussion

Ldb1 represents a ubiquitously expressed gene involved in key regulatory embryonic developmental processes. These functions of *Ldb1* have been attributed to the interaction of the protein with multiple LIM-, LIM-Homeodomain-, and other regulatory proteins [1-3]. These LIM-HD and LMO proteins had previously been demonstrated to be essential not only for key embryonic developmental processes but also in cancer development [4-7]. Furthermore, we had previously demonstrated several Wntinhibitors to be down regulated in the constitutive $Ldb1^{-/-}$ knock out and suggested these changes to be critical to severe disturbances during embryonic development in these mice [12]. Since these interactions have been reported to be involved in the development of multiple cancers and, in addition, since many embryonic regulatory mechanisms are re-activated during cancer development, we speculated about a role of Ldb1 in cancer development.

Investigating a conditional $Ldb1^{-/-}$ knock out crossed for a liver-specific *Ldb1* deletion utilizing Albumin-Cre-mice, we were able to show, for the first time *in vivo*, a significant involvement of Ldb1 in HCC development. After induction of HCC by means of DEN/Phenobarbital, we found a significant increase in the development of HCC in $Ldb1^{-/-}$ mice in both number and volume of tumor nodules. These increases in tumor size and number were highly significant.

Deregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human hepatocarcinogenesis [26]. Both an activation of proliferation signals and an inhibition of death process, leading to survival and then proliferation, may contribute to the development of HCC in affected cells. Thus, we further investigated a pathological regulation of these two physiological conditions to further narrow down the underlying pathological mechanism in Ldb1-dependent HCC development.

Proliferation in $Ldb1^{-/-}$-deleted livers was investigated by means of Ki-67 immunostaining. Comparing the $Ldb1^{-/-}$-deleted liver tissue to the wild-type liver tissue, we were able to demonstrate a substantial increase in cell proliferation upon *Ldb1* deletion. Thus, an increased HCC development in $Ldb1^{-/-}$-deleted mice may be attributed to an increase in cancer cell proliferation.

On the other tip of the scale of cancer development is the physiological mechanism of apoptosis. Apoptotic events in hepatocytes can be regulated by several different stimuli that bind to death receptors in the cell membrane, such as Fas ligand (FasL), tumor necrosis factor-alpha (TNF-α), or TNF-related apoptosisinducing ligand (TRAIL), which activate the extrinsic pathway. Among these, the CD95 receptor is widely expressed in tissues, and the dysfunction of the regulatory mechanisms in CD95 receptor signalling has been reported in several diseases, including cancer [27]. CD95 death receptor-mediated apoptosis and thus, also resistance towards it, may be investigated by means of the CD95 monoclonal antibody

Jo2, previously shown to exhibit massive apoptosis of liver cells [28]. Applying the Jo2 antibody to *Ldb1*$^{-/-}$-deleted and wild-type hepatocytes in primary culture, we demonstrated a significantly increased resistance to apoptosis. Since an inhibition of the programmed cell death subsequently leads to an increased survival, proliferation, and thus ultimately to cancer development or growth, the increased resistance towards apoptosis may also support an increased tumor growth.

Thus, reviewing the results of our investigation on the tissue proliferation and resistance towards apoptosis, we found that both tissue proliferation and apoptosis were deregulated, thus simultaneously leading to a complete deregulation of the physiological balance between these two mechanisms.

Having established a significant role of *Ldb1* in (liver) cancer development and having demonstrated that *Ldb1* deletion leads to an increased proliferation and resistance towards apoptosis, we next turned our attention to the underlying genetic mechanisms leading to this significant phenotype. Among the differentially regulated genes in our microarray analysis of *Ldb1*$^{-/-}$ versus WT animals, the *Myc* oncogene was found to be up regulated. To further confirm this pathological signalling, we investigated the expression of one of its common downstream effectors *Cyclin D1* by means of RT-PCR. Various regulators of cell cycle were frequently involved in the carcinogenesis of many human cancer types. Cyclins are thought to be essential proteins in cell cycle regulation and the binding of the cyclin family with cyclin-dependent kinases regulates their activity and contributes to cell cycle regulation especially during G1 phase progression. In particular, amplification of the *Cyclin D1* gene and over-expression of this protein were found in 13% of HCC [29]. Furthermore, cyclin D1 transgenic mice were demonstrated to exhibit an increased tumor development, proving, *in vivo*, that a *Cyclin D1* activation is a key feature of HCC tumor development [30]. Coordination of c-myc with cyclin D1 accelerates tumor formation. Thus, we investigated *Cyclin D1* expression in *Ldb1*$^{-/-}$-deleted mice by means of RT-PCR in order to find out whether cell cycle regulation, mediated by cyclin D1, and therefore ultimately tumor growth, was essential for *Ldb1*-dependent HCC development. *Cyclin D1* was significantly over expressed in *Ldb1*$^{-/-}$-deleted and wild-type mice, arguing for an activation of cyclin D1-dependent signalling in *Ldb1*$^{-/-}$-deleted mice.

The LKB1/STK11 serine/threonine kinase was previously reported to be mutated in Peutz–Jeghers syndrome and various sporadic cancers [31]. *In vivo* proof of an essential role of the tumor suppressor in HCC came from *Lkb1* gene knock out mice where loss of heterozygosity was shown to be associated with an increased HCC development [32]. The tumor suppressor LKB1 has recently been demonstrated to transmit its downstream signal via the cell cycle regulator p21 [14]. In order to regulate p21, this tumor suppressor needs the assembly of a protein complex consisting of Lkb1, Ldb1, GATA6, and LMO2 [14]. Thus, we speculated that the deletion of *Ldb1* inhibited the assembly of this tumor suppressor-containing complex, subsequently leading to an increased tumor development. By means of RT-PCR, we were able to demonstrate the down-regulation of p21. However, the down-regulation of p21 expression is not specific for this *Lkb1* cascade, as it is known to be a negative target of myc [23].

Finally, the stem cell/progenitor marker EpCAM was found to be significantly upregulated in *Ldb1*$^{-/-}$ mice in microarray data and this result was confirmed by RT-PCR. Accumulating evidence suggested an involvement of cancer stem/progenitor cells in tumor development. Stem cells were generally defined by the ability to differentiate into multiple cell lineages and to selfrenew. Initially, in leukaemia but subsequently also in multiple solid tumors, a small subset of tumorigenic cells have been shown to generate new tumors in xenograft transplantation. This observation, among others, has led to an increasing discussion about cancer stem cells and the hypothesis that a small subset of cancer cells bearing stem cell/progenitor features are not only indispensable for tumor development and proliferation but also help to escape conventional chemotherapy [33].

Although a definite proof of a cancer stem cell concept in HCC is still missing, the evidence is accumulating [25]. Yamashita et al. have demonstrated that epithelial cell adhesion molecule (EpCAM, Tacstd1)-positive HCC cells have a greater ability to form liver cancer *in vivo* [24]. Since in normal liver as well as in other tumors, EpCAM has been proven to have stem cell/progenitor cell characteristics [34], the authors concluded that HCC growth and invasiveness are dictated by a subset of EpCAM-positive cells *in vivo* [24]. Since EpCAM expression was significantly increased in microarray analysis, we confirmed these results by RT-PCR and demonstrated a significantly higher expression of *EpCAM* in *Ldb1*$^{-/-}$ mice. Thus, from our results, one may speculate that a higher expression of *EpCAM* may lead to the enrichment in cell/progenitor cell features, resulting in an increased tumor growth.

Since EpCAM, Cyclin D1, and c-myc are all known targets of Wnt pathway, *Ldb1* gene knock out mice may have activation of the Wnt pathway and future studies will be necessary to characterize this.

In conclusion, we have added *Ldb1*, a gene not previously known to be related to tumor development, to the list of factors involved in the known genetic networks of HCC development. Furthermore, the increased tumor development in *Ldb1*$^{-/-}$ deleted mice was suggested to be due to the deregulation of myc/cyclin D1 signalling, a decreased expression of the tumor suppressor p21, and an increased expression of the stem cell/progenitor marker EpCAM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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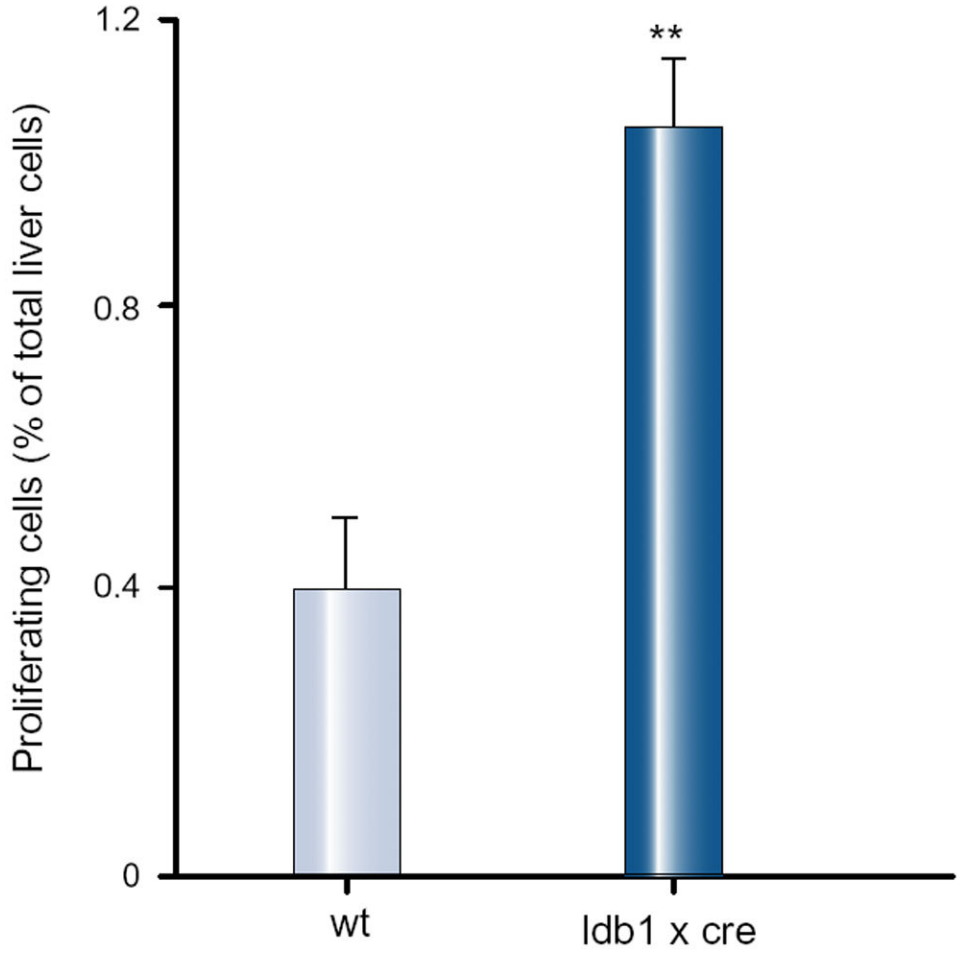

Fig. 1. *Ldb1*$^{-/-}$ KO mice exhibited a significantly increased cell proliferation compared to wild-type animals

Proliferation was measured by Ki-67 staining.

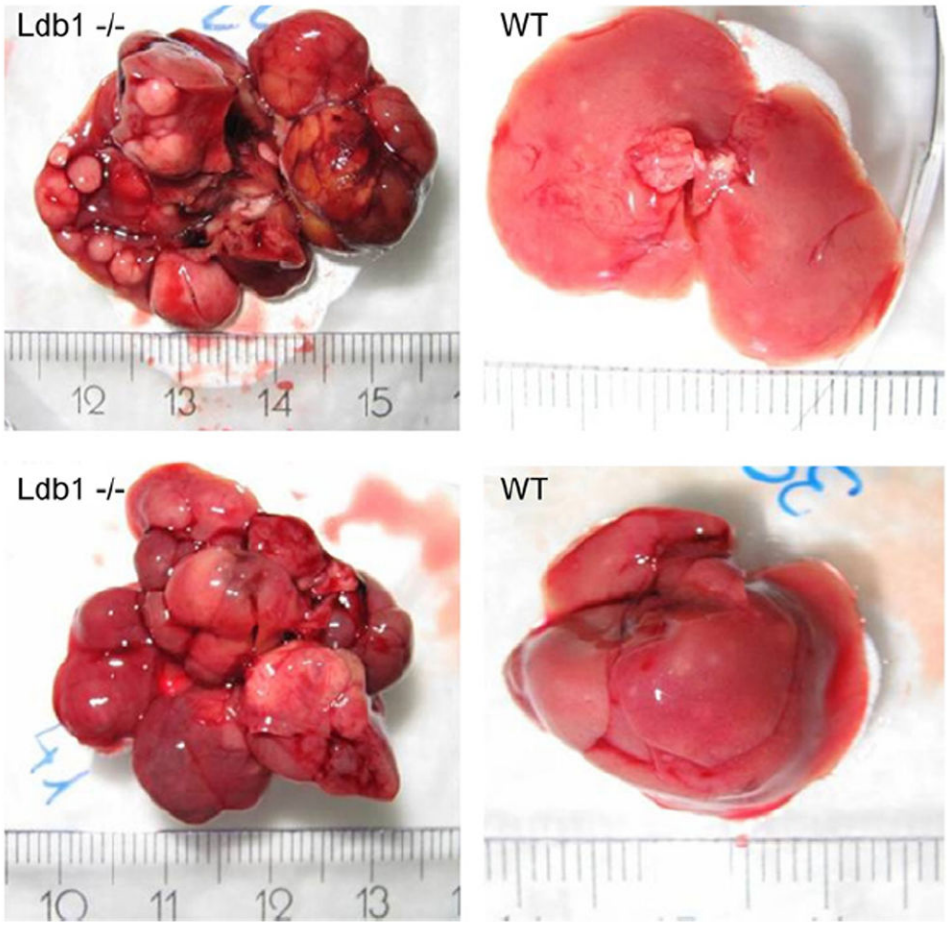

Fig. 2. Liver cancer development after induction with DEN/Phenobarbital over 9 months

Liver-specific *Ldb1*$^{-/-}$ KO mice developed significantly larger and more tumors (left side) compared to wild-type mice (right side). Selected livers of *Ldb1*$^{-/-}$ KO mice and wild-type animals are shown above.

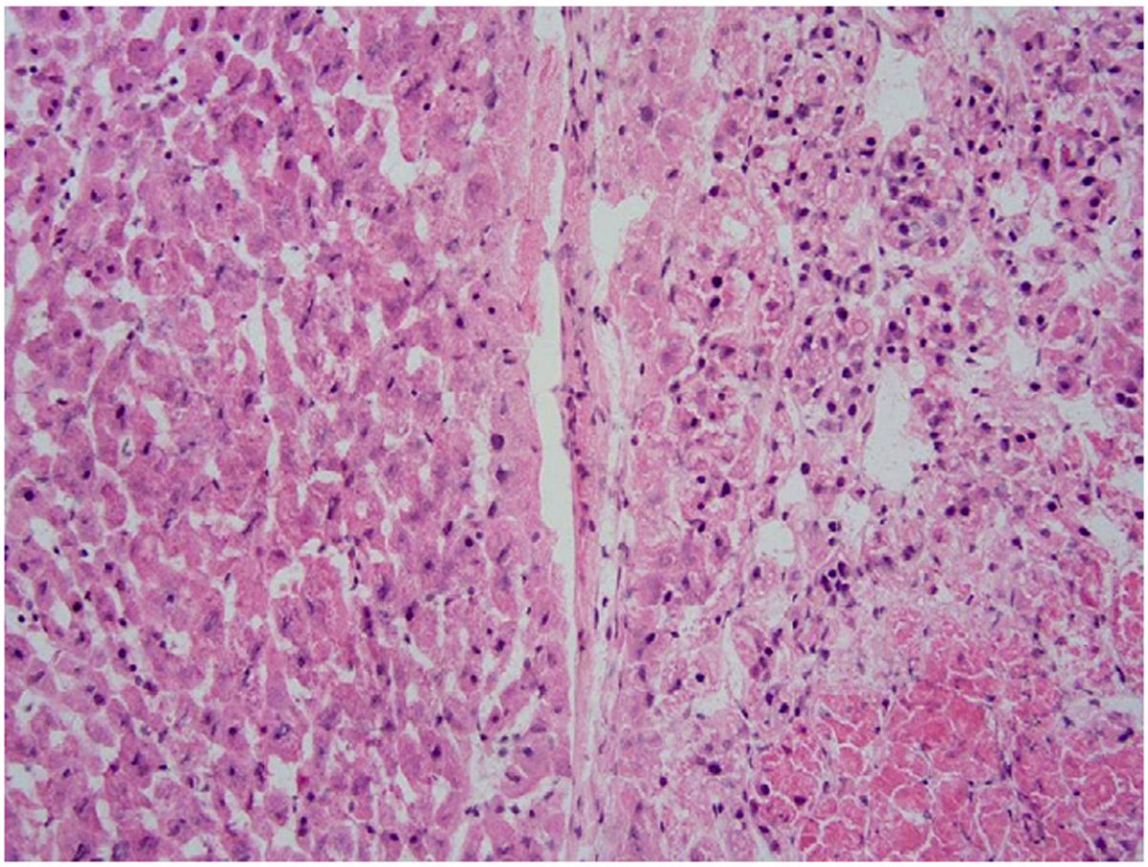

Fig. 3. Border of normal liver parenchyma (left side) and tumor tissue (right side) confirming the tumor nodules to be hepatocellular carcinoma

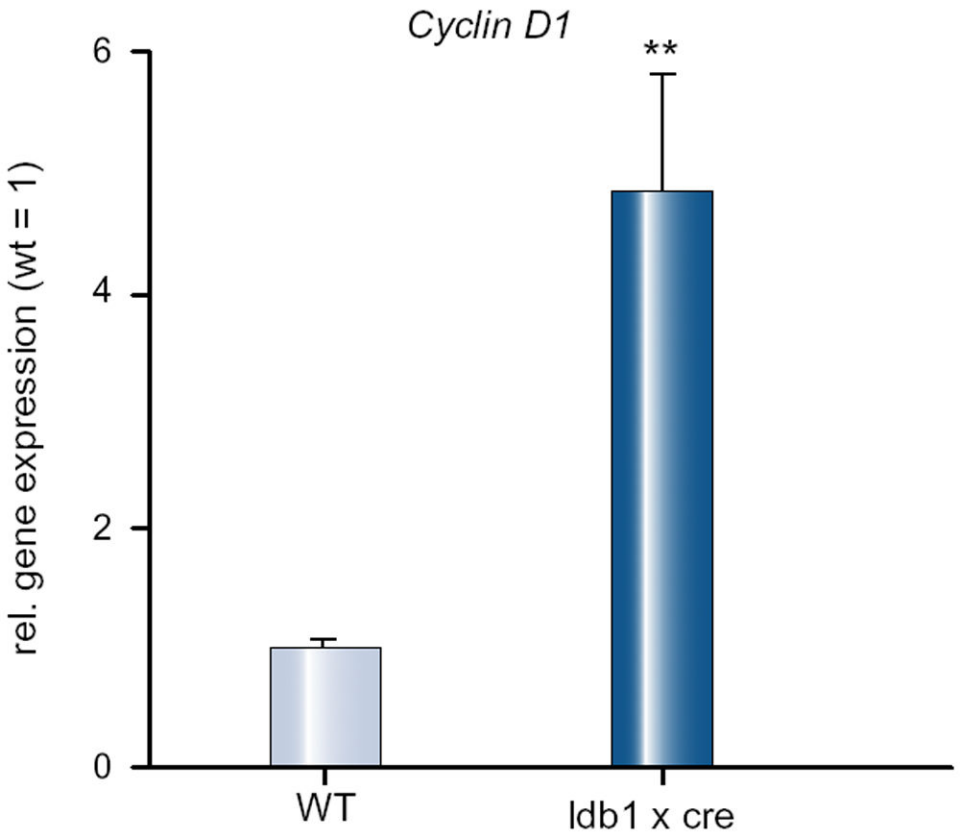

Fig. 4. ***Cyclin D1*** **expression was significantly increased in** ***Ldb1*** $^{-/-}$ **KO mice compared to wild-type animals as measured by RT-PCR (*p* = 0.001)**

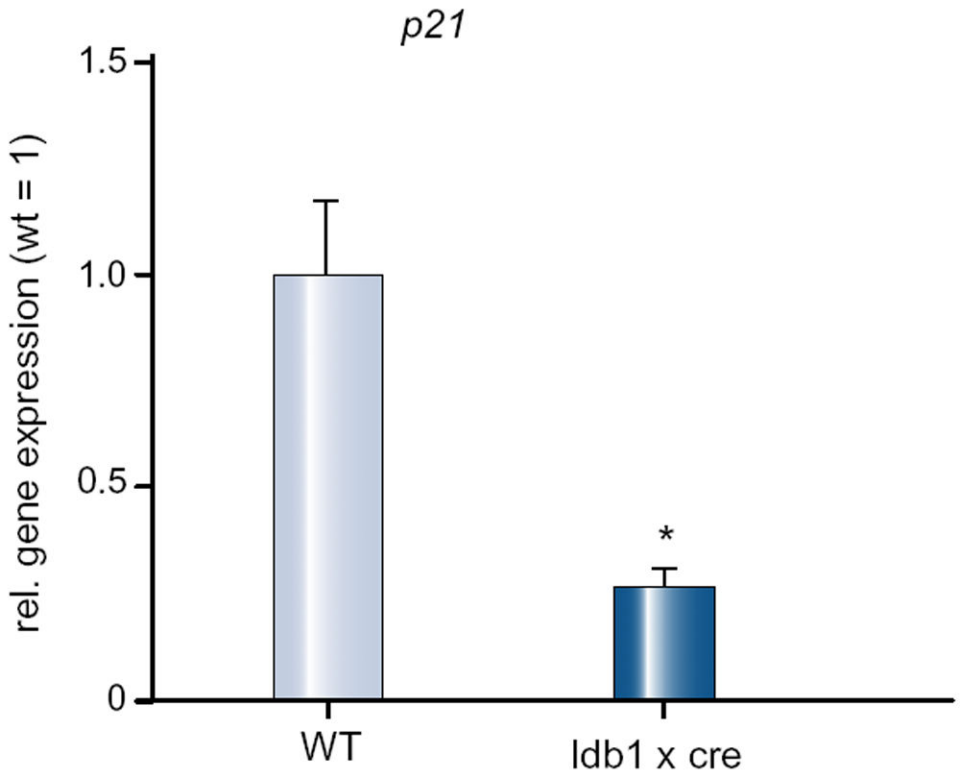

Fig. 5. ***p21*** **expression was significantly decreased in** ***Ldb1***$^{-/-}$ **KO mice compared to wild-type animals as measured by RT-PCR (*p* = 0.034)**

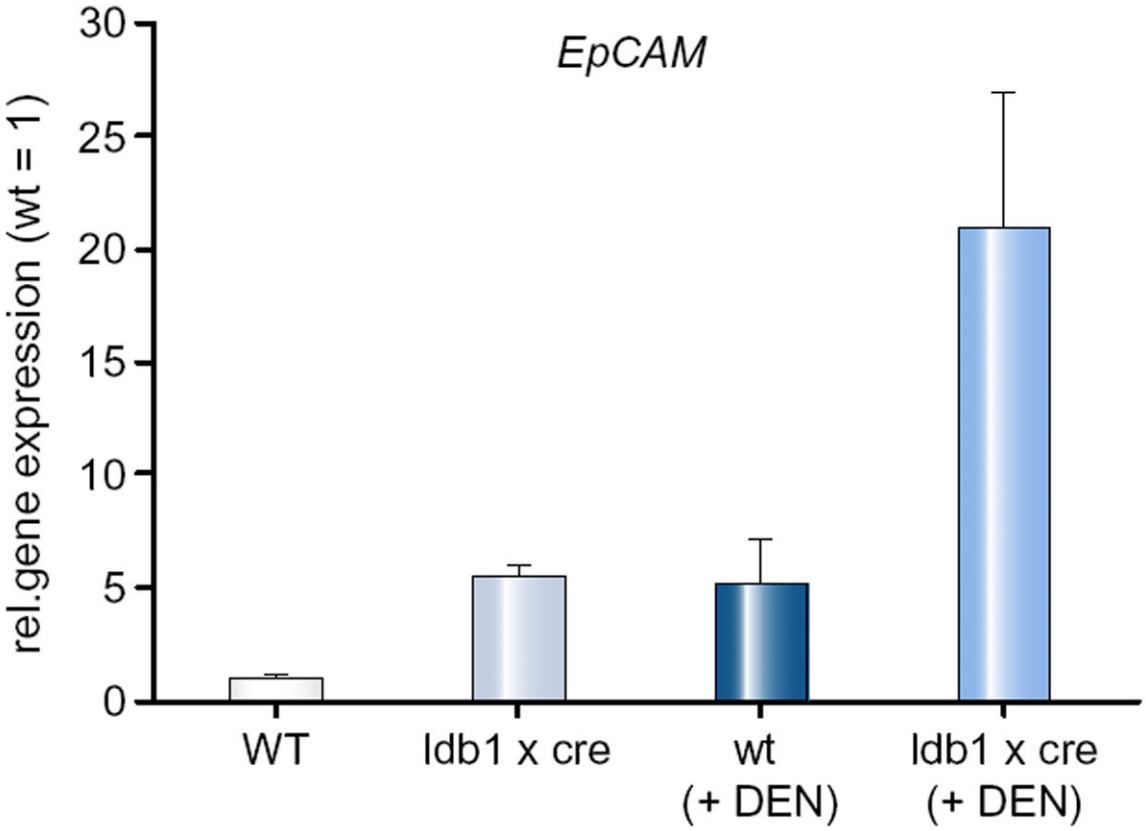

Fig. 6. ***EpCAM*** **expression was significantly increased in** $Ldb1^{-/-}$ **KO mice compared to wild-type animals as measured by RT-PCR (*p* = 0.001)**

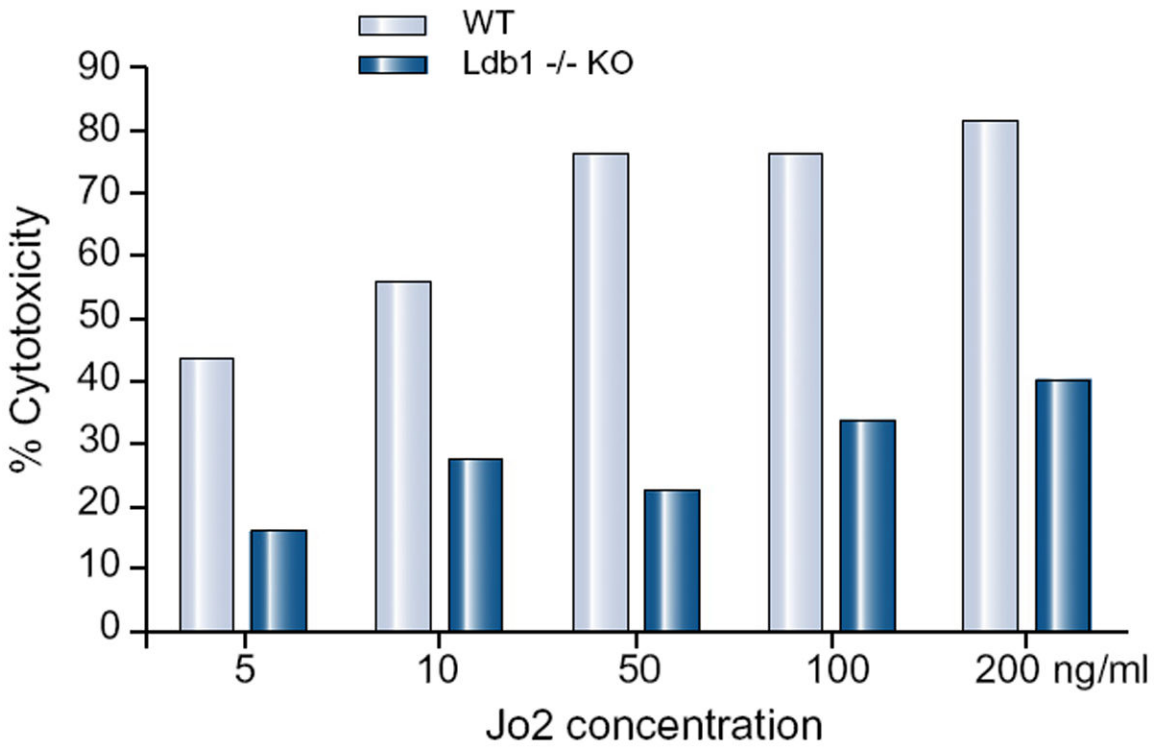

Fig. 7. Ldb1$^{-/-}$ KO mice exhibited a significantly increased resistance towards CD95-mediated apoptosis compared to wild-type animals

Apoptosis was induced by increasing amounts of Jo2 antibody.