Research Article

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

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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.
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.

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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 (LIM-HD) 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

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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 alb-cre 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 μm 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 $p \leqslant 0.05$ was considered to be significant and a $p \leqslant 0.01$ 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.