Aus dem Veterinärwissenschaftlichen Department

der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Arbeit angefertigt unter der Leitung von

PD Dr. Marlon R. Schneider

# Susceptibility to liver carcinogenesis is increased in a mouse model of conditional E-cadherin deficiency

Inaugural-Dissertation

zur Erlangung der tiermedizinischen Doktorwürde

der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

von Felix Nico Hiltwein

aus Berlin-Zehlendorf

München 2013

Gedruckt mit Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Joachim Braun

Berichterstatter: Priv.-Doz. Dr. Marlon Schneider

Korreferent: Univ.-Prof. Dr. Johannes Hirschberger

Tag der Promotion: 20. Juli 2013

Dedicated to all those closest & dearest to me,

be it my biological, sociological or emotional family.

“The best-laid schemes o' mice an' men / Gang aft agley”

("The best-laid plans of mice and men / Often go awry")

Robert Burns, 1785

# TABLE OF CONTENTS

**I. INTRODUCTION ..... 1**

**II. REVIEW OF THE LITERATURE ..... 3**

**1. Genetically modified mice ..... 3**

1.1. Functional analysis of the genome (in any organism) ..... 3

1.2. Why mice? ..... 4

1.3. Creation of genetically modified mice ..... 5

1.4. Conditional knockout (Cre/loxP) ..... 8

1.5. Analyzing the phenotype of genetically manipulated animals ..... 11

**2. E-Cadherin ..... 12**

2.1. Cadherins in general ..... 12

2.2. E-Cadherin structure, expression and relevance ..... 14

2.3. Connection of E-cadherin with tumors ..... 16

**3. Hepatocellular carcinoma (HCC) ..... 18**

3.1. HCC in humans ..... 19

3.2. HCC in mice (induced by diethylnitrosamine) ..... 20

**III. ANIMALS, MATERIALS AND METHODS ..... 22**

**1. Animals ..... 22**

1.1. *Cdh1* conditional knockout mice ..... 22

1.2. Alb-Cre transgenic mice ..... 22

1.3. Mouse breeding procedure ..... 22

1.4. Animal maintenance ..... 23

1.5. Approval of animal testing and ethical guidelines ..... 23

**2. Mouse genotyping ..... 23**

2.1. Tissue collection ..... 24

2.2. Extraction of DNA from mouse tail tips ..... 25

2.3. Principle of the polymerase chain reaction (PCR) ..... 25

2.4. Assay procedure and PCR protocols ..... 26

**3. Basic overview of mouse sections ..... 27**

**4. Assessment of E-cadherin loss on protein-level ..... 28**

4.1. Collection of liver samples ..... 28

4.2. Preparation of liver samples ..... 28
4.3. Electrophoresis and blotting ..... 29
4.4. Detection of protein ..... 31

**5. Evaluation of gene expression at the RNA level ..... 31**
5.1. Collection of liver samples for RNA analysis ..... 31
5.2. RNA expression analysis using Agilent Microarray ..... 32
5.3. cDNA synthesis for qRT-PCR ..... 32
5.4. Quantitative RT-PCR ..... 33

**6. Analysis of body weight development and organ weight ..... 34**
6.1. Long-term body weight development ..... 34
6.2. Body and organ weight at specific time points ..... 34

**7. Evaluation of serum parameters ..... 34**
7.1. Blood collection ..... 34
7.2. Serum preparation ..... 35
7.3. Clinical chemistry ..... 35

**8. Induction of tumors through use of diethylnitrosamine (DEN) ..... 35**
8.1. Preparation of DEN and dosage ..... 36
8.2. Intraperitoneal (i.p.) application ..... 36
8.3. Safety measures ..... 36

**9. Section of mice with tumors ..... 37**
9.1. Liver and body weight ..... 37
9.2. Other organ weights ..... 37
9.3. Count of tumor lesions ..... 38
9.4. Preparation of tumor samples for RNA analysis, histology and cell culture. ..... 38

**10. Establishment of long term cell culture from tumors ..... 39**
10.1. Preparation of collagenase II ..... 39
10.2. Mincing of tumors and destruction of the organ/tumor structure ..... 39
10.3. Erythrocyte lyses ..... 40
10.4. Re-suspension in cell culture dishes ..... 40
10.5. Maintenance and splitting of cell lines ..... 41
10.6. PCR of cell culture cells ..... 41
10.7. Conservation of cell-lines for future studies ..... 41

**11. Histological analysis ..... 41**
11.1. Hematoxilin and eosin (H&E) staining ..... 42
11.2. Immunohistochemistry (IHC) ..... 42

**12. Histological evaluation of HCC ..... 43**
12.1. Histological quantification of tumors in the liver ..... 43
12.2. Histological evaluation of tumors in the lung ..... 44

**13. Statistical analysis ..... 44**

**14. Primer sequences ..... 45**
14.1. Primers for genomic DNA amplification ..... 45
14.2. Primers for cDNA amplification ..... 45

**15. Materials ..... 46**
15.1. Machines ..... 46
15.2. Consumables ..... 48
15.3. Chemicals ..... 49
15.4. Drugs (used on animals) ..... 53
15.5. Microarray technology ..... 53
15.6. Software ..... 53
15.7. Animals ..... 54

**IV. RESULTS ..... 55**

**1. Spontaneous phenotype of *Cdh1*/Alb-Cre mice ..... 55**
1.1. Generation of *Cdh1* deficient mice ..... 55
1.2. Demonstration of cre-mediated gene deletion ..... 55
1.3. General assessment of the „spontaneous phenotype“ of *L-Cdh1*$^{del/del}$ compared to L-Control mice ..... 56
1.4. Expression analysis on protein level ..... 56
1.5. Expression analysis on RNA level: Agilent Array ..... 57
1.6. Expression analysis on RNA level: qRT-PCR ..... 58
1.7. Body and organ weight ..... 59
1.8. Serum parameters ..... 59
1.9. Long-term body weight monitoring ..... 61
1.10. Histology of L-*Cdh1*$^{del/del}$ vs. L-Control liver ..... 61

**2. Phenotype after tumor induction ..... 63**
2.1. General assessment of the „phenotype after tumor-induction“ of

L-*Cdh*1$^{del/del}$ compared to L-Control mice ..... 63
2.2. Histological nature of DEN-induced tumors in the liver ..... 65
2.3. Body and organ weight ..... 66
2.4. Macroscopical tumor count ..... 69
2.5. Histological tumor count ..... 70
2.6. Survival curve ..... 71
**3. Establishment of primary cells from tumors ..... 71**
3.1. *Cdh1* PCR of cell culture cells ..... 73
**V. DISCUSSION ..... 74**
**1. Spontaneous phenotype ..... 74**
**2. Phenotype after tumor induction ..... 77**
**3. Cell culture permanent lines ..... 78**
**4. Considerations for future projects ..... 79**
**VI. SUMMARY ..... 80**
**VII. ZUSAMMENFASSUNG ..... 82**
**VIII. BIBLIOGRAPHY ..... 85**
**IX. ADDENDUM ..... 94**
**1. Complete names of the genes found to be strongly and significantly regulated on RNA level ..... 94**
**2. List of figures ..... 95**
**X. CURRICULUM VITAE ..... 98**
**XI. ACKNOWLEDGMENT ..... 99**

# ABBREVIATIONS

Country names are abbreviated according to ISO 3166 code.

| Abbreviation | Meaning |
|---|---|
| ABC | avidin-biotin-peroxidase complex |
| ANOVA | analysis of variance |
| AQL | acceptable quality level |
| AT | Austria |
| BSA | bovine serum albumin |
| CAT | chloramphenicol acetyltransferase |
| *Cdh1* | E-cadherin coding gene in mice |
| CDH1 | E-cadherin coding gene in humans |
| cDNA | complementary DNA |
| CH | Switzerland |
| Cre | a tyrosine recombinase enzyme |
| CT | cycle threshold |
| *CV* | central vein |
| DAB | 3,3'-Diaminobenzidine |
| DE | Germany |
| Delta CT | difference between two cycle thresholds (CT) |
| DEN | diethyl nitrosamine |
| DK | Denmark |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMEM/F12 | DMEM + Nutrient Mixture F-12 |
| DNA | deoxyribonucleic acid |
| dNTP | desoxyribonukleosidtriphosphate |
| DTT | Dithiothreitol |
| EC domain | extracellular cadherin domain |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediaminetetraacetic acid |
| EMT | epithelial–mesenchymal transition or transformation |
| ENU | N-ethyl-N-nitrosourea |
| ES cell | embryonic stem cell |
| FCS | fetal calf serum |
| FELASA | Federation of European Laboratory Animal Science Associations |
| fl | "floxed" (floxed sequence) |
| FW | "forward" (primers) |
| G | gauge |
| GAPDH | Glycerinaldehyd-3-phosphat-dehydrogenase |
| GB | Great Britain |

| | |
|---|---|
| GPT | Glutamat-pyruvat-transaminase |
| GV SOLAS | German Society for Laboratory Animal Science |
| H&E | hematoxylin and eosin stain |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| i.p. | intra peritoneal (application) |
| JP | Japan |
| kDa | Kilodalton (atomic mass unit) |
| L-*Cdh1*$^{del/del}$ | Mice with liver-specific ablation of the Cdh1 gene |
| L-Control | Littermates used as controls, considered wildtype-like |
| LBD | ligand-binding-domain |
| LDH | lactate dehydrogenase |
| LMU | Ludwig-Maximilians-Universität |
| loxP (sequence) | binding site for the cre enzyme |
| mRNA | messenger RNA |
| MWW | Mann Whitney Wilcoxon |
| P450 | the cytochrome P450 superfamily of enzymes |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PPF | periportal field |
| qRT-PCR | quantitative real-time polymerase chain reaction |
| RNA | ribonucleic acid |
| RNAse H | a non-specific endonuclease |
| rpm | revolutions per minute |
| RV | "reverse" (primers) |
| SPF | specific pathogen free |
| SSRs | site-specific recombination(s) |
| T-Test | student`s T-Test |
| TAE | mixture of Tris base, acetic acid and EDTA |
| TBS | Tris-buffered saline |
| TBS-T | mixture of Tris-buffered saline and Tween 20 |
| US | United States of America |
| vs. | versus |
| wt | "wild-type" |

# I. INTRODUCTION

The Human Genome Project resulted in the publication of the human genome sequence (covering ~99% of the euchromatic genome) in 2004 (INTERNATIONAL HUMAN GENOME SEQUENCING, 2004). As of today, however, the exact function of many genes is still unknown. If more information could be gained on the functionality of our genes under all kinds of physiological and pathological conditions, a much better concerted effort could be made for the development of new therapies, the detection of predispositions to certain diseases, or novel approaches to medicine in fields like personalized medicine or gene therapy.

Since there are considerable homologies between the genes of humans and other mammals, it is possible to transfer large parts of the functional data gained about the genome of the latter to our effort to understand the human genome more profoundly (and vice versa).

The genomic sequence of several domesticated animals has already been published in its entirety, amongst those the genome of the house mouse (*Mus musculus*). Mice have been used for medical experiments for many decades or even centuries (MORSE et al., 1978). Due to their many advantageous traits as a mammalian model organisms (a large litter size; a fast generation cycle; a small body size and correspondingly small keeping costs; a large number of inbreed strains; an already existing very large pool of information on mouse physiology and on the general traits of mice as experimental subjects (SCHOFIELD et al., 2012); the hardiness and docility of domesticated mice) it seems much more feasible to gain insightful data on the mode of operation of the entirety of the mouse genome, transcriptome and proteome in the near future than it would be with any other mammal, including humans.

Of course, to understand such complex systems, research on them has to be distributed amongst a large number of smaller projects. Each project then deals with a single gene or a group of interacting genes under specific physiological or pathological conditions.

The present project evaluated the relevance of the adhesion molecule E-Cadherin (alternatively: “cadherin-1”, “epithelial cadherin”) and its respective gene *CDH1*

for the homeostasis of the liver in general, as well as its impact on tumors of the liver like hepatocellular carcinoma (HCC).

This specific outlining of the project was chosen with a reason: HCC is one of the leading causes of cancer-related deaths worldwide (JEMAL et al., 2011), and also quite difficult to treat curatively in humans. Any information gained on *CDH1* interaction with HCC could possibly lead to an improvement of treatment options or even better, prevention of the disease in the future.

This project was a collaboration between the “Lehrstuhl für Molekulare Tierzucht und Biotechnologie” (Veterinärwissenschaftliches Department, Tierärztliche Fakultät, LMU München) and the “Molekulare Gastroenterologie” (Medizinische Klinik und Poliklinik II, Campus Großhadern, Klinikum der Universität München, LMU München).

# II. REVIEW OF THE LITERATURE

## 1. Genetically modified mice

### 1.1. Functional analysis of the genome (in any organism)

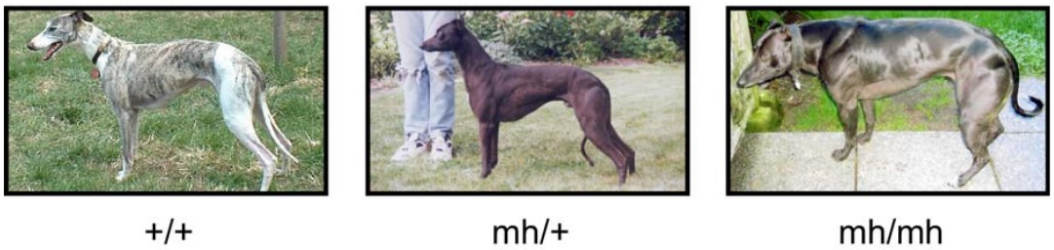


*Fig. 1: "Bully whippet", adapted figure from: (MOSHER et al., 2007).*

To analyze the function(s) of a certain gene in an organism, it is quite useful to compare a population in which the gene is missing (or otherwise altered: overexpressed, for example) to another population in which the gene is not altered. The differences between the populations are likely to be caused by the lack or alteration of the gene in question and give good hints to the functions of the gene. A good example for this would be cattle of the double-muscled Belgian Blue breed (KAMBADUR et al., 1997) or the "bully whippet" (MOSHER et al., 2007), in which the mutation of the myostatin gene causes a very muscular phenotype if the mutation is homozygous (Fig.1).

While it sometimes is possible to find and isolate subpopulations displaying a (preferably single locus) genetic alteration by sheer chance, for systematic research it is considerably more efficient to create such populations artificially (in microorganisms, plants, experimental animals, cell cultures, etc.). There are two major options: forward genetics or reverse genetics.

Forward genetics (phenotype-driven): Use of naturally occurring or artificially induced random mutations in a population. If individuals displaying an alteration of phenotype are detected, they are separated (possibly breed into a new line), the phenotype is characterized thoroughly and their genome is checked for alterations. The techniques used today for this purpose include, but are not limited to: localization of the mutation site using classical animal husbandry in combination with markers like Simple Sequence Repeats (SSRs), genome sequencing (many modern methods being derivatives of the Sanger sequencing method), quantitative

mRNA expression analysis, and many more (SIMON et al., 2012). A nowadays often heard catchphrase and state-of-the-art technique is ‘‘third-generation sequencing’’ i.e. single-molecule sequencing, which helps to decreases errors which might be present in methods (like Sanger sequencing) that rely on amplification of DNA (GUPTA, 2008).

Reverse genetics (genotype-driven): A specific gene is deleted, overexpressed or otherwise altered by gene manipulation techniques. The resulting population is then checked for any changes of phenotype that might have appeared. First established in yeast (STRUHL, 1983), it can also be used in mammals (CAPECCHI, 1989).

Induction of random mutations can be attained by dosing model organisms with chemical substances like the mutagen N-ethyl-N-nitrosourea (ENU), for example (BROWN, 1998). While intrinsically a tool for forward genetics, the large collections of generated mouse strains with identified mutations frequently serve as a tool for reverse genetics studies.

Specific genes can be targeted, for example, by making use of homologous recombination (CAPECCHI, 1989), or additional copies of a specific gene can be brought into the genome at a random location (COSTANTINI & LACY, 1981; GORDON & RUDDLE, 1981).

### 1.2. Why mice?

The biological properties of mice make them a favorable mammalian model organisms: they are easy to handle, replicate fast and generate relatively low initial and maintenance cost even when generating large cohorts to analyze (SCHNEIDER, 2012). Laboratory mice also normally do not endanger research personnel with either severe physical attacks or dangerous zoonoses, since mice in well managed research facilities are kept strictly separated from direct or indirect contact with wild mice and are preferably specific pathogen free (SPF). In contrast, working hazards might be considerable if farm animals or even wild animals are handled for experiments (LANGLEY, 1999).

The nucleic acid sequence of the genome of the house mouse (*Mus musculus*) has been published in its entirety almost simultaneously with the human genome (MOUSE GENOME SEQUENCING et al., 2002; AUSTIN et al., 2004), and the sequence of 17 strains of mice (encompassing most of the mice strains commonly

used in research) had already been reported by 2011 (SCHOFIELD et al., 2012).

It is generally accepted that there is a high degree of homology between the genomes of mice and humans (WINTER, 1988; DELEZOIDE & VEKEMANS, 1994; VENTER et al., 2001) and that the mouse is thus a good model organism to help understand the human genome (ARBOLEDA & VILAIN, 2011).The huge array of technologies available to alter the genotype of mice and to analyze their phenotype additionally contributes to establish mice as one of the best suited experimental models for modern biomedical research (GLASER et al., 2005; CHEON & ORSULIC, 2011).

For example, some of the tools for the genetic manipulation of mammals work best in mice or are even exclusive to mice. Gene targeting by homologous recombination in stem cells and subsequent blastocyst injection has been well established and continually improved in mice (LONGENECKER & KULKARNI, 2009) but is far from being a feasible technology even in other popular lab animals like rats (BRADLEY et al., 1984; LONGENECKER & KULKARNI, 2009; DECHIARA et al., 2010; MEEK et al., 2010). Technologies commonly used to create genetically modified mice are discussed in the next chapter.

The availability of biotechnological tools like embryo transfer and superovulation greatly help not only in the creation but also in the rapid distribution of novel genetically manipulated mouse strains. In mice, embryo transfer is well-established today and enables to bring animals safely into specific pathogen free facilities or to purge a mouse strain of a pathogen (SUZUKI et al., 1996). The fact that many very efficient cryopreservation protocols exist for mouse embryos today (TSANG & CHOW, 2010) does not only aid in transport but also makes inexpensive long-term storage possible. Mouse sperm can also be preserved in liquid nitrogen (MARSCHALL & HRABE DE ANGELIS, 1999; MAZUR et al., 2008), and recently methods have been developed to store mouse sperm for a few months in non frozen media or even at room temperature for short periods (LI et al., 2011). All of this greatly reduces the cost of keeping genetically modified animal model strains for later use and also helps to reestablish mouse facilities, if they become contaminated or after unforeseen disasters like fire or flood.

### 1.3. Creation of genetically modified mice

The report of the first gene-manipulated animal was published in 1974

(JAENISCH & MINTZ, 1974). Mice which passed those alterations to their offspring were first reported in the early eighties (COSTANTINI & LACY, 1981; GORDON & RUDDLE, 1981). Since then it has become possible to insert almost any desired DNA sequences into the genome of laboratory mice, which for example could lead to overexpression of proteins coded by the inserted artificial sequence. It is also possible to target specific, already known genes through the use of homologous recombination (GLASER et al., 2005).

There are many different technologies to create genetically manipulated mice. Two of those technologies are used very commonly today and can be considered crucial for modern biotechnological research with transgenic mice: DNA microinjection in pronuclei and homologous recombination in embryonic stem cells with subsequent blastocyst injection. Both techniques have different advantages and disadvantages. DNA microinjection in pronuclei of fertilized oocytes has been developed in the early 1980s. Compared to other techniques (like transduction, where a virus-vector is used on multicell embryos) it has the advantage that the resulting animals are not chimeras and only very seldom mosaics (HARBERS et al., 1981).

The DNA to be inserted must be designed carefully, and often it is not predictable how efficient the design will be. For example, it often is not known how many regulatory elements of the gene are needed and where they are located. Therefore it is sometimes better to include as much of the gene as possible, including introns and upstream sequences. Sometimes regulatory sequences are contained in adjacent genes and in this case it may be difficult to include all regulatory elements.

Together with the gene of interest, additional DNA can or must be inserted. For example, promoter sequences (this could be an organ-specific or an ubiquitously expressed promoter) and elements that act as reporters (easy to detect sequences or sequences coding a detectable transcript that does not affect the physiology of the test organism) can be included.

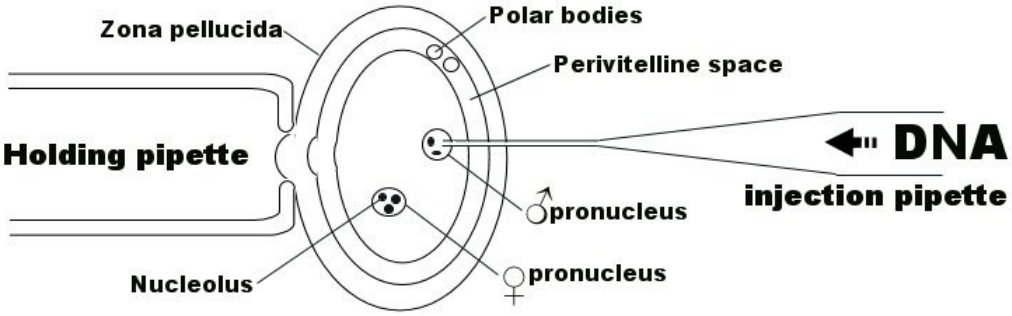


*Fig. 2: Injection of fertilized one-cell eggs. Based on: (SI-HOE et al., 2001)*

After the DNA has been designed, amplified and purified, it can be microinjected into fertilized one-cell mouse eggs (Fig 2). This requires precise equipment (holding pipettes, injection pipettes, microinjection chamber, etc.) and skilled personnel, as well as enough mouse eggs in exactly the right stadium (this can be achieved by checking donor mice for the time of "plugging", which marks the time of insemination by the male mouse).

After injection, the eggs are transferred to the oviduct of pseudopregnant females (females inseminated by non-fertile male mice).

The DNA is normally integrated during the one-cell stage, only in rare cases it is integrated in later stages (which would result in mosaic offspring). How the inserted DNA is expressed depends heavily on the integration site. The integration site as well as the number of copies inserted at that site is random and cannot be dictated by the researcher. It is possible that the integration interrupts the function of another gene, and the resulting offspring has to be carefully checked for this unwanted effect (SI-HOE et al., 2001).

<u>Homologous recombination in embryonic stem cells with subsequent blastocyst injection</u> works only in mice, and has only been established for certain strains of mice. It was first established for the "129" strain. Because the 129 strain genetic background is not desirable for many experimental setups, gene-manipulated 129 mice are generated and then backcrossing is used to give them another strains background while keeping the gene-manipulated site. This is time-consuming, and adequate backcrossing becomes increasingly more difficult the closer the gene-manipulated sequence is linked with (undesirable) 129 traits (SEONG et al., 2004). Strains nowadays deemed suitable as alternatives to 129/SvJ mice include C57BL/6N, C57BL/6JOla, DBA/2N, DBA/1Ola, BALB/c and FVB/N

(CARSTEA et al., 2009). Nonetheless, many popular gene-manipulated mice today are of 129 origin because they were either developed before the existence of alternatives or because 129 ES cells are still considered to be easier to work with.

The ES cells of mice seem to have higher frequencies of homologous recombination than do other cultured mammalian cells. Still, for a successful experiment, the DNA that is used for homologous recombination has to be designed carefully. Most notably, the DNA has to be isogenic, and the homology arms (e.g. the parts that are similar in the artificial DNA and the native DNA of the stem cells) have to be long enough (GLASER et al., 2005).

After stem cells positive for the homologous recombination have been obtained, they can be injected into a blastocyst. This blastocyst preferably hails from a mouse strain with a coat color that is different from the ES cell donor. After the blastocyst has been successfully implanted in a surrogate mother mouse, an embryo derived from cell lines of four different parent mice develops. The tissues of different genetic makeup are not rejected because the embryos immune system is non-existent at first, and later is tolerant to the already-present and "known" proteins of the other genetic strain. The embryo is chimeric in nature (this can be easily detected if the fur is spotted, e.g. made up of different cell lines), but its offspring is not chimeric (and hopefully some of the offspring is derived from the desired cell line, which is the case if the ES cells formed at least some parts of the germline of the chimera) (SEONG et al., 2004).

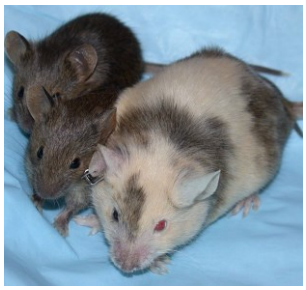

*Fig. 3: A chimeric mouse with typically variegated coat color. Taken from: the public domain.*

### 1.4. Conditional knockout (Cre/loxP)

Limiting the genetic alteration to a single organ is favorable because it prevents the problem of a multitude of effects in multiple organs. If a gene is deleted from all cells of an experimental animal, interesting (but only faint) effects might be missed in one specific tissue because they are obscured by massive effects in another organ. Conditional knockout makes focusing on analyzing the phenotype in the specific tissue easier. Also, the gene to be analyzed, or rather the lack of it, could have a lethal impact on early embryonic stages (LARUE et al., 1994). Conditional knockout can sometimes be a necessity to circumvent this problem.

One common approach to conditional knockout is the Cre-loxP system. This system has been developed in the nineteen eighties (SAUER, 1987) and has been, in parallel with similar systems or even combined with them, in ample use for targeted gene-manipulations since then (ROSSANT & MCMAHON, 1999).

Cre/loxP and analogous systems can be used to delete a sequence from the genome of a cell, even if the cell is part of an already fully developed multi-cell-organism (like a mouse). The deletion happens under defined conditions and can be designed to affect all cells or only a certain type of cells in that organism.

The sequence could be, for example, a complete gene, part of a gene or a sequence regulating a gene. The defined condition can be topical/spatial (e.g. a single organ only) or temporal (e.g. only after timed, extrinsic or intrinsic triggers are present), a combination of both or even more complex conditions (METZGER & CHAMBON, 2001; KOS, 2004).

To create a conditional knockout mouse using cre-loxP, typically (at least) two genetically modified mice strains are used (NAGY, 2000). In the first mouse strain the sequence in question is flanked by two loxP-sequences. The flanked sequence is also often called a “floxed sequence”. The loxP-sequence is not native to eukaryotes, instead originating from the bacteriophage P1 (STERNBERG & HAMILTON, 1981). The sequence is short (ATAACTTCGTATA-GCATACAT-TATACGAAGTTAT) and can be inserted exactly at the desired sites by gene manipulation techniques using homologous recombination. If the sequence is inserted at a site where it does not obstruct the normal reading of the DNA (e.g. in non-functional parts of the noncoding DNA), it alone has no effect. The sequence does not code protein, and it does not cause recombination events by itself. It does enable an enzyme called “Cre” to delete the area between two loxP sites, though. In this process, one loxP site is removed together with the flanked sequence and formed into a cyclic structure, while the other loxP site remains at the point where the DNA has been reconnected. Depending on the orientation of the loxP sites, alternatively, the sequence could be inverted instead, but this variant is normally not used for the purpose of generating knockout mice (KOS, 2004).

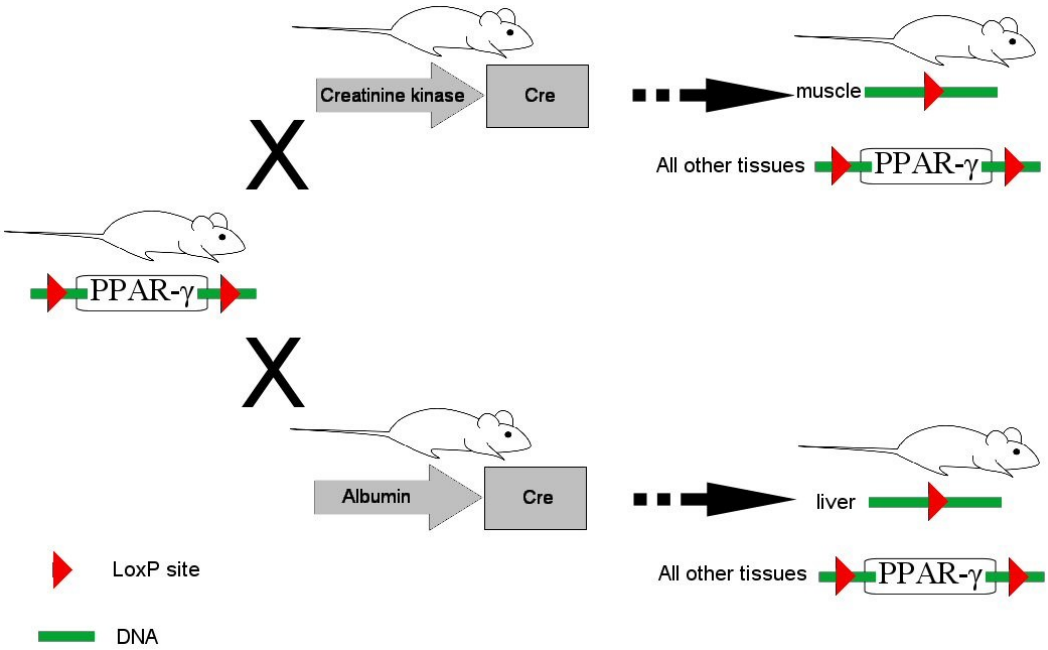


*Fig. 4: In this example of conditional tissue specific knockout, the creatine kinase promoter directs Cre expression only in muscle, or the albumin promoter directs Cre expression only in liver. Accordingly, the gene PPARG (PPAR-γ) is deleted tissue-specific only. Based on: (KOS, 2004)*

In the second mouse strain, the enzyme Cre is expressed. The word “Cre” can be understood as a contraction of the words “creates recombination” or alternatively “cyclization recombination". Like the loxP-sequence, the sequence for Cre is not native to the mammalian genome. If the Cre sequence is inserted at a location where it is under the control of the same promoter as a protein native to the organism (or is inserted together with a specific promoter), Cre can be expressed in a tissue- or cell type-specific manner. A Cre under the same promoter as an organ-specific protein would be only present in this organ (for example: under the same promoter as albumin, which is produced in the liver only). A Cre under a time-specific promoter would be active at certain times (for example: the albumin promoter is only active after the mouse actually begins production of albumin, but not in the early embryonic stages). If the promoter is only active in the organism under certain living conditions, the expression can be triggered intentionally (for example: after feeding the organism a diet containing heavy metals). Alternatively, the *Cre* sequence could be fused with the sequence coding for a ligand-binding-domain (LBD) of a hormone receptor. Cre fused to a ligand-binding-domain can only enter the nucleus (and elicit its effect there) if it has been activated by its ligand (a hormone). If the LBD is mutated and only reacts to

artificial hormone-like substances (and not the native hormone), the effect of Cre can be controlled by application of a substance (e.g. injection of tamoxifen, for example, activates an artificial Cre-ER$^T$) (METZGER et al., 1995).

Once those two mouse strains exist, they can be intercrossed. If the offspring carry both the flanking loxP sequences as well as the ability to produce Cre, the flanked area of their genome is deleted after the aforementioned conditions have been fulfilled (Fig. 4). Since there are a multitude of related systems that can delete genes or trigger them in addition to Cre/loxP, it is possible to create model organisms containing an array of different conditions for the knock-in and/or knockout of multiple DNA sequences (ANASTASSIADIS et al., 2009).

### 1.5. Analyzing the phenotype of genetically manipulated animals

With the creation of mouse mutants, the genotype has been altered. The next question is how this affects the phenotype. In the case of genes that only influence a single locus trait this questions can be answered easily, at least if the trait can be detected with the bare eye or with the technology available to the researcher.

But some traits are influenced by several genes, and sometimes there are redundant genes that take over if the gene of interest is mutated (ZHANG, 2012). In addition, some genes are pleiotropic (influencing two or more distinct phenotypic traits) and this further complicates understanding the relationship between the gene of interest and the phenotype (BECKERS et al., 2009; STEARNS, 2010). Some genes have varying importance during different stages of life. Consequently, analyzing a phenotype can be challenging and time consuming.

Even if genotype and phenotype are thoroughly analyzed, this is often not the whole information needed to transfer the research results to real-life questions of, say, human medicine. This is because the phenotype under challenging conditions (a disease, for example) is still not taken into account. Mice are normally housed under standard conditions and are not at all challenged with the same factors that contribute to human disease. Those conditions have thus to be created artificially. This could, for example, involve confronting mutant mice with pathogens (LECUIT, 2005), narcotics (HALL et al., 2012), lifestyle specific diet (part of the “envirotype”) (BECKERS et al., 2009) or carcinogens (FRESE & TUVESON, 2007; CHEON & ORSULIC, 2011). In addition to giving better insight into the

gene or protein of interest´s function, this approach can possibly also contribute to the establishment of relevant animal models of human diseases.

# 2. E-Cadherin

E-cadherin is a protein that is important for cell-cell adhesion. In humans, it is coded by the gene *CDH1* (obsolete gene symbols are *UVO; CDHE; ECAD; LCAM; Arc-1* or *CD324*). In mice, the equivalent gene is called *Cdh1* (Source: NCBI recources Website 2012).

The word itself can be understood as a contraction of some of the traits of the protein: "e-" stands for "epithelial", while "cadherin" can be understood as "calcium-dependent adherin" (YOSHIDA-NORO et al., 1984). Although the official (mouse) protein name according to the NCBI homepage (as of 2012) is cadherin-1, we have chosen the E-cadherin designation because it is more commonly employed in peer-reviewed publications.

## 2.1. Cadherins in general

The cadherin superfamily is huge, comprising of at least 350 members, and cadherins can be found in an amazingly wide range of organisms (HULPIAU & VAN ROY, 2009). Classical cadherins are typically named according to the organ or tissue in which they are most prominent and/or have been found first (e.g. N-cadherin = "neural cadherin", etc.).

Structurally, all cadherins have the EC domain (extracellular cadherin) containing repeating amino acid sequences of about 110 residues (ß-folded). This definition of the cadherin superfamily includes many proteins whose function has not yet been characterized well. The number and arrangement of the EC domains varies between different cadherins and linkers between successive EC domains are stabilized by $Ca^{2+}$ (some exceptions to this rule exist). Adhesion, even between classical cadherins, can be due to a great variety of different cadherin-cadherin interfaces and many of the mechanisms are still not well understood. The structural basis of cadherin binding to other proteins is often unclear (BRASCH et al., 2012), although some have already been described, like E-cadherin binding to NKLRG1 and to internalin. The natural killer cell receptor KLRG1 binds to a highly conserved site on classical cadherins (LI et al., 2009). Internalin, on the other hand, is a major invasion protein of *Listeria monocytogenes*. It can form a

complex with its human receptor E-cadherin (SCHUBERT et al., 2002).

Cadherins (E-, N-, P-cadherin, etc.) preferentially interact with cadherins of the same type in a homophilic manner when connecting cells and may thus help sorting heterogeneous cell types (TAKEICHI, 1990).

For cell adhesion, cadherins are dependent on $Ca^{2+}$ (VAN ROY & BERX, 2008). A weak form of cell adhesion is achieved by *trans*-interaction between cadherins on opposing cells surfaces. For stronger cell-cell-adhesion, cadherins can be clustered (NELSON, 2008).

Another way to achieve strong cell adhesion is through changes in the actin skeleton. Cadherins can exert a strong influence on actin and vice versa by interacting with it in sophisticated ways (Fig 5). Previously it was assumed that cadherin binds to actin directly (through the cytoplasmic proteins α- and β-catenin). Recently it has been indicated that their influence might be mediated indirectly, using an allosteric switch in α-catenin (NELSON, 2008).

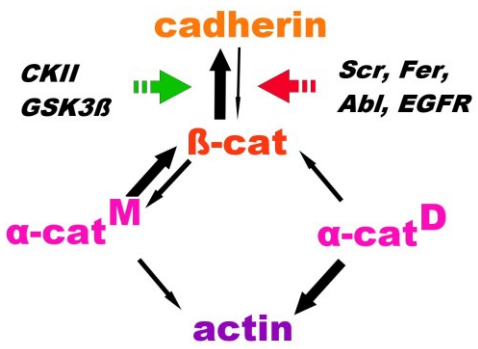


*Fig. 5 Cadherins, catenins and the actin cytoskeleton influence each other, often reciprocally. Based on: (NELSON, 2008).*

The so-called "cadherin-catenin-complex" (and its mode of operation in the cell adhesion and migration) is not understood completely, but models exist that can explain some of its effects (Fig. 6).

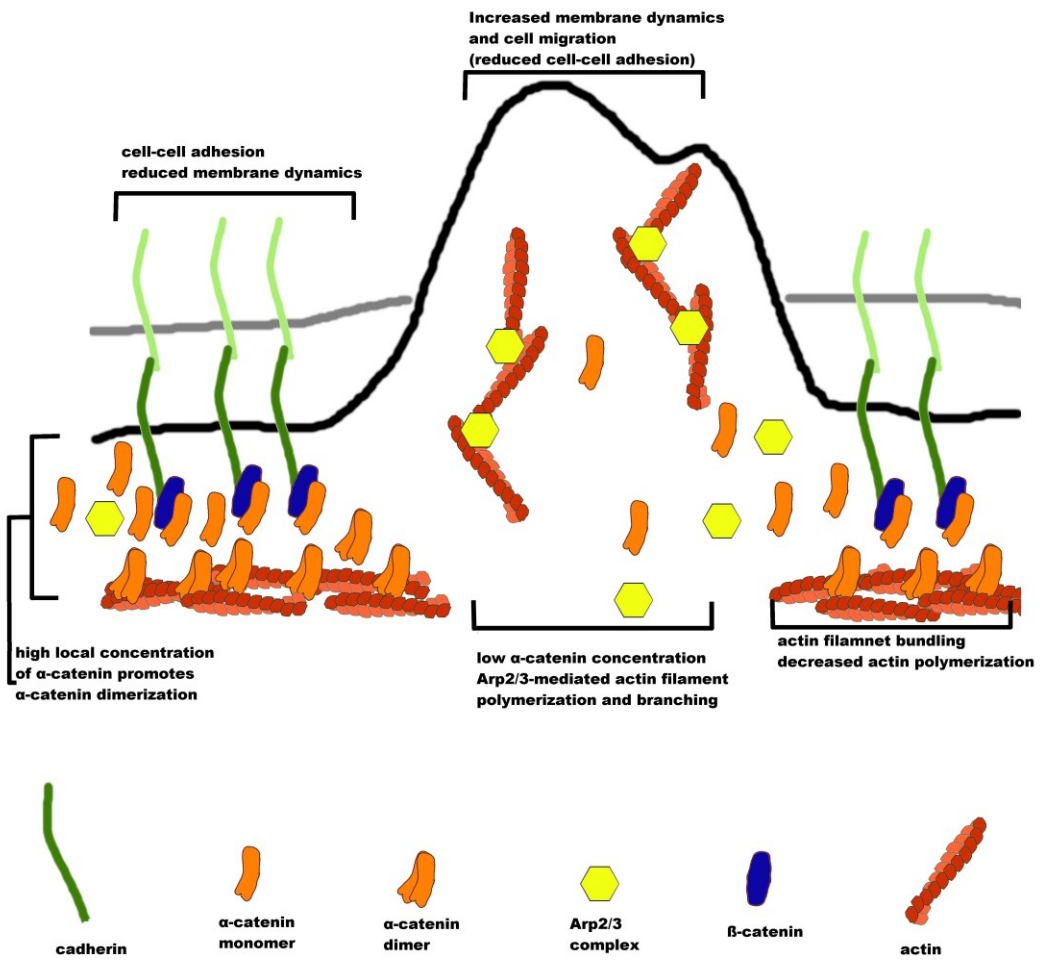


*Fig. 6: This model can explain the regulation of cytoskeleton and membrane dynamics by the cadherin–catenin complex. Cell-cell adhesion is characterized by reduced membrane dynamics. E-cadherin presence can be controlled via catenin, and absence of E-cadherin leads to increased membrane dynamics, which enables cell migration. Based on: (NELSON, 2008).*

## 2.2. E-Cadherin structure, expression and relevance

E-cadherin is considered a "classical cadherin" and a "type I cadherin". Being the founder member of the cadherin family, E-cadherin is often seen as the prototypical cadherin. Its importance for adhesion has been under investigations by scientists since about 1977 (VAN ROY & BERX, 2008; HULPIAU & VAN ROY, 2009).

E-cadherin is a 120 kDa protein in its mature form (OZAWA & KEMLER, 1990). It has five extracellular, immunoglobulin-like domains (EC1 to EC5). It also has a transmembrane α-helix and an extended intracellular domain that binds β-catenin (SCHUBERT et al., 2002). EC5 differs from the other domains (EC1 to 4) and an alternative naming has been proposed (VAN ROY & BERX, 2008).

E-cadherin has a remarkable gene structure because each of the EC domains is coded by two to three exons, with different exons coding for different EC domains, despite the repeating nature of the domains. Also, the boundaries of the exons do not correlate with the EC boundaries in the mature protein (Fig. 7). All of this markedly differs from coding sequences of some other cadherins. The gene *CDH1* contains 16 exons and intron #2 is of special note because it is very probable that it contains regulatory sequences (BERX et al., 1995; VAN ROY & BERX, 2008).

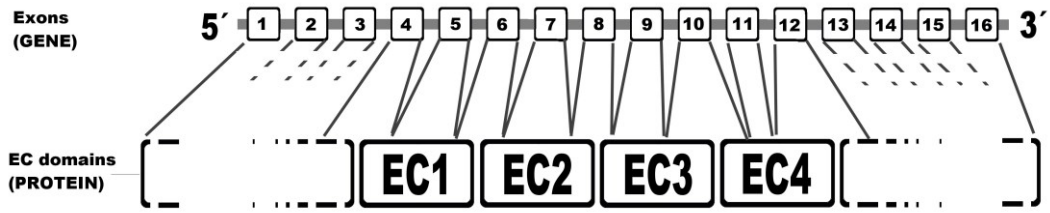


*Fig. 7: The human CDH1 gene structure. Posttranslational modification of the protein yields a mature protein with a sequence markedly different from what could be expected on the basis of the gene sequence. Based on: (VAN ROY & BERX, 2008)*

It is clear that presence of (E-)cadherin is needed for normal cell-cell-contact and cell-sorting, but also down-regulation of E-cadherin is often important if tissues need to be reorganized or are formed *de novo,* such as during embryogenesis. The modulation of E-cadherin is part of a process whereby formerly epithelial tissue temporarily gains mesenchymal qualities (epithelial-mesenchymal-transition = EMT) (KOKKINOS et al., 2010).

Soluble E-cadherin fragments are sometimes increased in serum from patients with skin diseases (FURUKAWA et al., 1994) and loss of E-cadherin expression has been found to correlate with certain skin diseases (FURUKAWA et al., 1997). As the name "epithelial cadherin" already implies, E-cadherin is especially important and pronounced in epithelial tissues and conditional knockout of *Cdh1*

in epithelial tissue often has severe consequences for experimental animal health (SCHNEIDER et al., 2010).

E-cadherin plays a role in the defense against pathogens and its loss or mutation has been associated with decreased barrier function against germs (SCHNEIDER et al., 2010). Interestingly, it can also serve as a receptor for pathogens such as *L. monocytogenes* (VAN ROY & BERX, 2008).

Cadherins are important in the assembly of tight junctions, adherens junctions and desmosomes (LEWIS et al., 1997b; TUNGGAL et al., 2005). Recently it has been hinted that many cell junctions not only are major players in cell-cell adhesion, but also are more important acceptors of cell signaling pathways than it was assumed previously (GREEN & JONES, 1996; MCCREA et al., 2009). Through its importance for cell junctions, E-cadherin (or lack of it) could possibly have impact on the most diverse functions of cells (pathways, regulation of passage of needed or harmful molecules, osmotic balance, etc.).

For experimental set-up of mouse models, it is important to note that E-cadherin has crucial functions during embryogenesis (GUMBINER, 2005). Essentially, using constitutive *Cdh1* knockout models to study the function of E-cadherin in adult mice is pointless because the embryos die around implantation (LARUE et al., 1994).

### 2.3. Connection of E-cadherin with tumors

E-cadherin is the major cell-cell adhesion molecule in epithelial cells, and 80% of human cancers derive from epithelial cells (SEMB & CHRISTOFORI, 1998).

E-cadherin interacts with several proteins important to structure and restructuring processes (ß-catenin, actin) (BEAVON, 2000; GUMBINER, 2005) and thus it is an important player in EMT, which in turn plays an important part in tumorigenesis (TIAN et al., 2011; TIWARI et al., 2012). While EMT normally is a physiological function important to embryogenesis (KOKKINOS et al., 2010), tumor cells can also be more “successful” by using EMT mechanisms.

Analysis of cells from tumors of different origins taken from patients often showed a decrease of E-cadherin expression and/or mutations in the coding sequence. This also proved to be the case for some hepatocellular carcinoma (HCC) samples (MATSUMURA et al., 2001; CHIEN et al., 2011). Also, soluble

E-cadherin fragments are often increased in serum from cancer patients (FURUKAWA et al., 1997).

While a connection between E-cadherin loss and the development or growth of tumors is widely accepted, the relationship could be of quite diverse nature. Is the loss of E-cadherin a consequence of tumors being active (and using mechanisms akin to EMT)? Or is it just a co-causality, where some common mechanism in tumors reduces E-cadherin, but E-cadherin loss does not really contribute to the aggressiveness of the tumor? Or does loss of E-cadherin make the transition of normal cells to “successful” tumor cells easier?

Support for the latter hypothesis comes from both animal models and certain studies from human medicine. Animal models of conditional loss of E-cadherin have shown susceptibility to tumorigenesis in organs like the mammary gland (DERKSEN et al., 2006) and humans carrying a mutation in the *CDH1* gene have a strong predisposition for certain types of cancer, namely Hereditary Diffuse Gastric Cancer and lobular breast cancer (KAURAH & HUNTSMAN, 1993).

Decreased E-cadherin does often increase invasiveness of tumor cells, but by no means is this correlation between E-cadherin level and tumor aggressiveness always the case. Tumor cells with normal E-cadherin levels can be very aggressive (NIEMAN et al., 1999; CHRISTIANSEN & RAJASEKARAN, 2006) and in certain tumors (such as inflammatory breast cancer) E-cadherin is consistently upregulated regardless of histologic type or molecular profile (KLEER et al., 2001; BERX & VAN ROY, 2009).

Upregulation of cadherins other than E-cadherin (N-cadherin) can add to tumor invasiveness and metastasis, even if E-cadherin is not reduced. This leads to the speculation that cadherins act as “homing devices” that can help tumor cells enter into parenchyma if they manage to express enough of the “correct” cadherin for the tissue (e.g. N-cadherin for mesenchymal tissue) (HAZAN et al., 2004).

E-cadherin is generally regarded as a tumor suppressor and loss of the protein seems to be a crucial step for many tumors to be “successful”. Despite this, E-cadherin upregulation might even promote aggressiveness in certain cases. Examples for this have been shown in high-grade gliomas (E-cadherin expression is supposed to be rare in normal adult human nervous system tissue) (LEWIS-TUFFIN et al., 2010) or ovarian carcinoma (ovarian surface epithelium is

normally free of E-cadherin, expressing N-cadherin instead) (SUNDFELDT, 2003).

Metastases from prostate cancer also seem to be more aggressive in certain tissues (like bone) if they express E-cadherin. It has been hypothesized that tumor cells can enter organs far away from the original tumor site if they have low E-cadherin expression, but that they need to "exit EMT" and might upregulate E-cadherin so that they can actually grow at sites of metastasis (PUTZKE et al., 2011).

The cleaved fragments of E-cadherin have been linked with a possible oncogenic potential. Mature E-cadherin has a molecular weight of 120 kDa, but can be cleaved into an extracellular N-terminal 80 kDa fragment (this soluble fragment can enter intracellular space and the bloodstream) and an intracellular C-terminal 38 kDa fragment. Several mechanisms have been proposed to explain how cleavage or the resulting fragments could increase tumor aggressiveness (DAVID & RAJASEKARAN, 2012). Cleavage of E-cadherin could also be an explanation why sometimes tumors with high E-cadherin are equally or even more aggressive than tumors with low expression.

Similar to human medicine, in practical veterinary medicine loss of E-cadherin is often associated with increased aggressiveness of tumors in patients (pets) (SARLI et al., 2004; GAMA & SCHMITT, 2012).

## 3. Hepatocellular carcinoma (HCC)

HCC can develop in humans and many other mammals. Since the main focus of the project underlying this thesis was to gain information relevant to human HCC by using a mouse model, the details of HCC in domesticated animals other than the mouse will not be discussed extensively. It is sufficient to say that HCC is rather uncommon but has been reported in several domesticated animals (GHOLAMI et al., 2006), that it has some qualities similar and some qualities different compared to human HCC (LIPTAK et al., 2004) and that treatment options are available (SEKI et al., 2011). In humans, HCC is one of the most important cancers worldwide (Fig. 8), being diagnosed in more than half a million patients per year worldwide. Of the primary liver cancers, HCC is encountered much more often than other primary liver cancers like cholangiocellular carcinoma (SHIMODA & KUBOTA, 2007). Liver cancer is the fifth most

common cancer in men and the seventh in women (EL-SERAG, 2011) and a leading cause for cancer-related death (JEMAL et al., 2011), ranked amongst the top three in males.

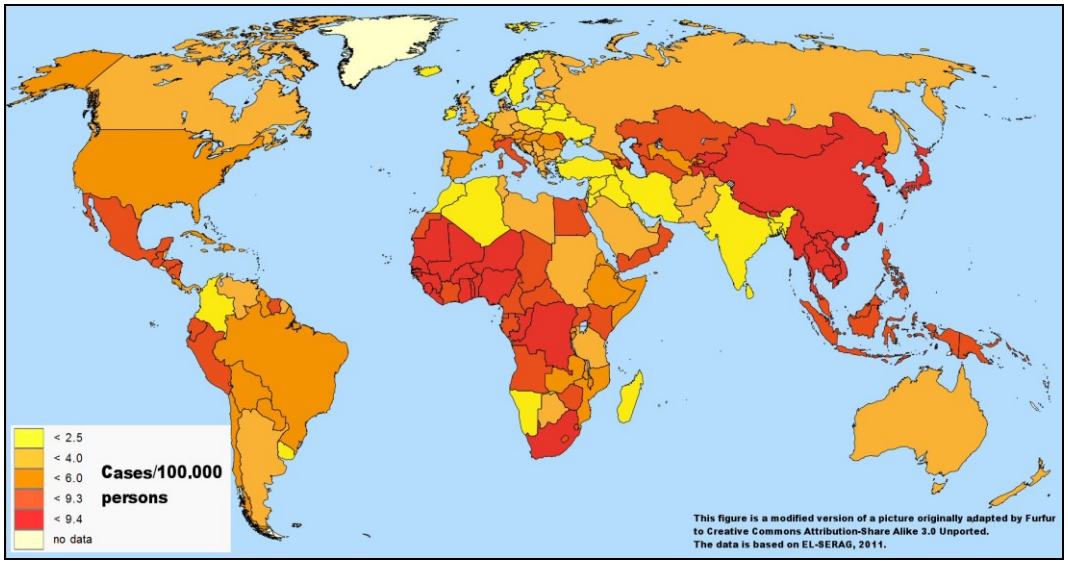


*Fig. 8: World map showing regional variation in the estimated age-standardized incidence rates of liver cancer, based on data from the World Health Organisation. The incidence rates (number of cases per 100,000 persons) pertain to both sexes and all ages. Based on: (EL-SERAG, 2011)*

### 3.1. HCC in humans

The factors causing HCC in humans are diverse, including aflatoxins (GROOPMAN et al., 2008; LIU & WU, 2010), nitrosamines (MITACEK et al., 1999) and other contaminants of food (ADAMSON, 1989), alcohol abuse, genetic predispositions and many more. But possibly the most important factor is hepatitis caused by viral infection (GOMAA et al., 2008). Several different, not closely related viruses causing hepatitis are known today: A, B, C, D and E. Some typically are blood-borne diseases (C, for example) while others typically are smear infections (A, for example). Of the viruses, only B and C are decidedly relevant for HCC (MICHIELSEN et al., 2005). Worldwide 78% of HCC is estimated to be attributable to HBV (53%) or HCV (25%) (PERZ et al., 2006). Prevalence of hepatitis is quite different from region to region and also dependent on the social group. The poor hygienic conditions under which people are forced to live in some areas contribute to this as well as a personal high-risk lifestyle.

Thus hepatitis, and consequently HCC, typically is a disease more prevalent in people who live (or have previously lived) in developing countries. Due to the

close correlation of HCC to virus-induced hepatitis in a distant past, the tumor also seems to afflict predominantly middle-aged to old people rather than young individuals. This reasoning, of course, cannot be applied unrestricted to regions where hepatitis is so prevalent and/or medical standards are so low that infants regularly already get infected perinatally (MICHIELSEN et al., 2005).

Since hepatitis B and C are blood-borne infectious agents (in addition, B is often transmitted sexually), certain sub-groups of the population are much more at risk. Intravenous users of drugs are especially vulnerable to infection. As an interesting remark, this is quite possibly a reason for the very high rate of HCC amongst Japanese that would not be expected for a medically well-covered country like Japan. It is speculated that Japan had a very high rate of intravenous amphetamine-abusers in the turmoil period after the Second World War. Consequently, HCC in Japan mostly afflicts the very old and is predicted to decrease substantially once this generation ceases to exist (MORIYA et al., 1999).

Before the advent of general alertness to the threat of AIDS and other blood-borne diseases, infection with hepatitis did occur easily even without a personal high-risk lifestyle in people of all walks of life (CHIARAMONTE et al., 1996) through poorly sterilized medical equipment or treatments involving serum preparations (SCHREIER & HÖHNE, 2001). The problem persists, since occasionally hygiene in medical facilities in some regions is still far below the necessary level (YERLY et al., 2001). All in all, since neither hepatitis nor alcohol abuse, nor the dangers of food contaminants can be expected to be eradicated in the near future, HCC will continue to be an important disease worldwide.

### 3.2. HCC in mice (induced by diethylnitrosamine)

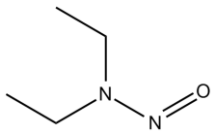


*Fig 9: Diethylnitrosamine structure.*

Mice are a popular model organism for studying different cancer types (FRESE & TUVESON, 2007). While different protocols for the induction of HCC in mice exist, the most common involves the intraperitoneal application of diethylnitrosamine (Fig. 9) (abbreviation DEN; synonym: DANA; DENA; NDEA; N,N-diethylnitrosamine; N,N-diethylnitrous amide). The cancer potential of nitrosamines in general is well known and has been a subject of interest since humans often get exposed to nitrosamines with certain food (JAKSZYN & GONZALEZ, 2006) or if they work in certain

industries (rubber factories, for example) (SPIEGELHALDER & PREUSSMANN, 1983). It should be noted that the cancerogenity of nitrosamines in humans is very strongly suspected but, unlike in rodents, not proven definitely (ABNET, 2007).

DEN is a nitrosamine which has a relatively low toxicity compared to its cancerogenity (DRUCKEY et al., 1967; CHRISTENSEN et al.) and as such it is well suited for the induction of tumors without causing early dropouts in the experimental group. Several variations exist concerning the amount of carcinogen given, the age at which it is given, and possibly which substances (barbiturates for example) are applied in addition to DEN for further promoting tumor development (LEENDERS et al., 2008).

DEN itself is not necessarily the primary tumor causing agent by direct interaction, but it becomes a powerful carcinogen through biotransformation (with a resulting carbonium ion, which can act as an alkylating agent), for example by cytochrome P450 oxigenation (LEWIS et al., 1997a). Since the liver has a very high concentration of cytochrome P450, a lot of the biotransformation of DEN will happen there (RAJEWSKY et al., 1966). In addition, the venous blood from almost all unpaired abdominal organs is directly passed to the liver and a large amount of the DEN injected will reach the liver parenchyma very fast. Due to all these factors, after i.p. injection, DEN causes tumors mostly in the liver and in all other organs the effect will be much smaller.

Like in humans (JEMAL et al., 2011), mouse males are far more often affected by HCC than females and it is suspected that estrogen has a protective effect (NAUGLER et al., 2007).

It should be noted that there has been some research on the exposure of personnel to nitrosamines in animal facilities doing tumor research (ISSENBERG & SORNSON, 1976) and a number of safety measures should be taken when working with such substances (fume hood, gloves, safety goggles, safe disposal of feces of the animals, good ventilation, etc.).

# III. ANIMALS, MATERIALS AND METHODS

## 1. Animals

Since the original creation of the employed mouse lines (POSTIC et al., 1999; BOUSSADIA et al., 2002) was not part of this thesis and only crossing of already genetically manipulated mice strains was done, the gene manipulation procedure will not be described in detail.

### 1.1. *Cdh1* conditional knockout mice

Mice were obtained from The Jackson Laboratory via Charles River. The mouse was originally created by Rolf K Kemler (BOUSSADIA et al., 2002). The mouse has floxed and has the strain name "B6.129-Cdh1tm2Kem/J". The mouse has floxed *Cdh1* (or rather: important exons of *Cdh1* are floxed) and if crossbred with a mouse expressing the enzyme Cre, the floxed exons become lost (Fig. 10). A description of the genotype, phenotype and background of this line can be found (as of February 2013) under the following link:

http://jaxmice.jax.org/strain/005319.html

### 1.2. Alb-Cre transgenic mice

Mice were obtained from The Jackson Laboratory via Charles River. The transgenic mouse was originally created by Mark Magnuson (POSTIC et al., 1999) and has the strain name "B6.Cg-Tg(Alb-cre)21Mgn/J". The mouse expresses Cre in the liver, and can be used to delete floxed sequences specifically in this tissue. A description of the genotype, phenotype and background of this line can (as of February 2013) be found under the following link:

http://jaxmice.jax.org/strain/003574.html

### 1.3. Mouse breeding procedure

To ablate E-cadherin specifically in the liver, mice expressing Cre in the liver only (Alb-Cre) were mated with mice carrying a floxed *Cdh1* allele. Mice were maintained in the C57BL/6 background. Offspring was genotyped and through planned mating, mice homozygous for the floxed allele ($Cdh1^{fl/fl}$) were generated to be used as controls (L-Control) while mice carrying, in addition, the Alb-Cre transgene, formed the experimental group (L-$Cdh1^{del/del}$), which underwent liver-

specific *Cdh1* deletion (Fig. 10). It was taken care that L-*Cdh1*$^{del/del}$ mice were only hemizygous for the Alb-cre transgene.

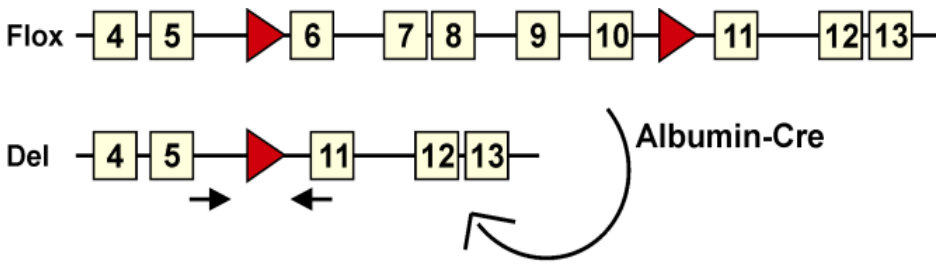


*Fig. 10. Schematic representation of the floxed (Flox) Cdh1 allele and the deleted (Del) allele after Cre-induced recombination. Exon 6 to 10 are lost in the process.*

### 1.4. Animal maintenance

Animals were kept under SPF conditions in macrolon cages type II (type III if group housing was possible). The light cycle was set to 12 hours light and 12 hours darkness. Mice were provided with wood chip bedding, a wooden or plastic shelter, enrichment in the form of paper towels to shred and a spinning wheel, ad libitum access to water and standardized rodent chow (standard rodent diet (V1536, Ssniff, Soest, DE). Care was taken that enrichment was standardized and identical for L-*Cdh1*$^{del/del}$ and L-Control group.

### 1.5. Approval of animal testing and ethical guidelines

The guidelines and laws regarding experiments involving animals in Germany were observed (TierSchG, related laws and regulations; as well as guidelines by the GV-SOLAS, FELASA, responsible veterinary authorities, etc.).

A permit for experimentation on animals was obtained for this thesis project. The respective file numbers at the responsible veterinary authority (Regierung von Oberbayern) are: Az. 55.2-1-45-2531.3-21-09 (training & education purposes) and Az. 55.2-1-54-2532-125-09 (Injection of a cancerogenic substance).

## 2. Mouse genotyping

The designation given to the mice used for internal use in the animal facility and the lab was “fl/fl | +/wt” for knockout and “fl/fl | wt/wt” for animals without knockout, whereas “fl/fl” described the floxed status of the *Cdh1* sequence (in both alleles: homozygote) and “+/wt” or “wt/wt” described the presence or

absence of Cre, respectively.

For generating the mice to be entered into the experiments, “fl/fl | +/wt” mice were crossed with “fl/fl | wt/wt” mice, which resulted in litters in which the genotype of the parents was present again. (Fig. 11).

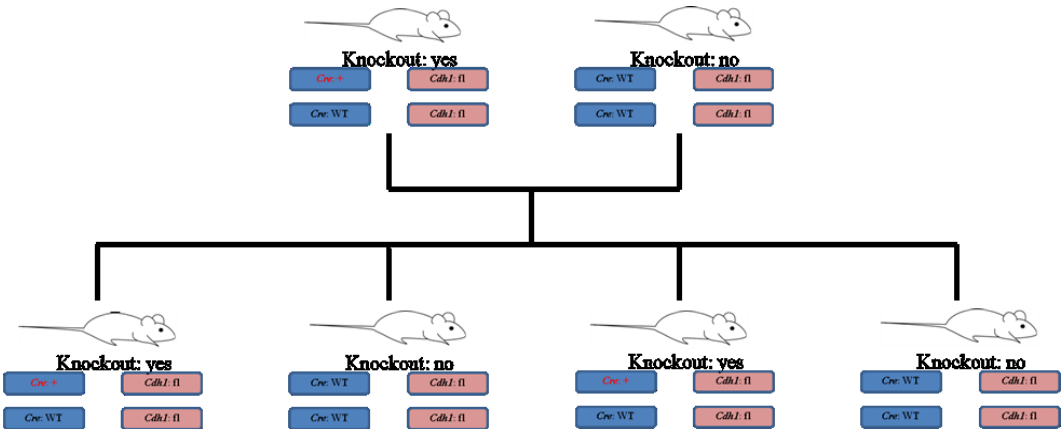


*Fig. 11 Breeding protocol. If random distribution of chromosomes (represented by bluish or rose bars) is assumed, 50% off the offspring are knockout mice.*

Polymerase chain reaction (PCR) was employed to confirm the presence of two floxed Cdh1 alleles and to determine the presence or absence of Cre.

To improve comprehensibility, the above-mentioned designations will generally not be used in this thesis. Instead, in all graphs and in the text, mice will be referred to as “L-*Cdh1*$^{del/del}$” (= liver-specific *Cdh1*-recombination/deletion) and “L-Control” (= littermates with floxed, but not deleted *Cdh1*).

## 2.1. Tissue collection

Two variations of sample collection were used. Mice aged 5 to 6 weeks were marked by the use of ear holes and/or notches using an ear punch device. Then a small part of the end of the tail (ca. 2 to 4 mm) not containing vertebrae was clipped using scissors. The tail wound was sealed by the use of Histoacryl® liquid skin glue (B.Braun, Melsungen, DE). For the mice that were scheduled for tumor-induction at an age of two weeks, it was essential to know the genotype before the induction. Thus, the samples had to be collected earlier (at the age of 3 to 6 days), and tattooing of the pads was used for marking. The ink (Pelikan, Hannover, DE) was injected using a fine (30G) syringe. After collection, the tail clippings were stored at -20° C.

## 2.2. Extraction of DNA from mouse tail tips

For DNA preparation, a modified version of the Promega Corporation protocol (Wizard® Genomic DNA Purification System) was used.

A mixture containing 120 µl EDTA (0.5 M, pH 8.0), 500 µl Nuclei Lysis Solution (Promega, Mannheim, DE) and 17.5 µl proteinase K (concentration, Roche, Mannheim, DE) was added to a 1.5 ml microcentrifuge tube containing a fresh or frozen mouse tail tip and was incubated overnight at 56°C with gentle shaking. On the next day, RNA was eliminated with RNase Solution (Roche, Mannheim, DE). Protein Precipitation Solution (Promega, Mannheim, DE), in combination with centrifugation, removed protein. DNA was precipitated with isopropanol (Merck, Darmstadt, DE). Using centrifugation and 70% ethanol, the DNA was washed and subsequently suspended in 50 µl Rehydration Solution (Promega, Mannheim, DE). If PCR failed due to an overabundance of DNA, further dilution was done with bidistilled water.

## 2.3. Principle of the polymerase chain reaction (PCR)

Polymerase chain reaction is a well-known technique in almost all labs working in the field of life sciences. Thus, only a short overview will be presented here.

The double strand of DNA can be replicated into two double strands of DNA using the enzyme polymerase. A number of steps are required for that:

1) Separation of the double strand through heat
2) Annealing of forward + reverse primers at a lower temperature
3) Activity of the polymerase enzyme

Only the sequence between the primers is amplified (including the binding site). The cycle can be repeated multiple times, producing a very large number of DNA sequence copies. Unless a real-time PCR system is used, the usual method of detection and evaluation of the PCR product is electrophoresis in an agarose gel. DNA copies with the same length will form a “band”, visible after the gel is stained (typically with ethidium bromide).

## 2.4. Assay procedure and PCR protocols

PCR was done in 8-tube-strips (G. Kisker GbR, Steinfurt, DE), using 1 µl of sample at a DNA concentration of about 50 ng/µl. For every sample, 19 µl master mix was added.

Mastermix preparation (per sample):

| | |
|---|---|
| PCR buffer, 10x (Qiagen, Hilden, DE) | 2.00 µl |
| dNTPs, 1 mM (MBI Fermentas, St. Leon-Rot, DE) | 2.00 µl |
| Q-Solution (Qiagen, Hilden, DE) | 4.00 µl |
| $MgCl_2$, 25 mM (Qiagen, Hilden, DE) | 1.25 µl |
| Sense primer, 2 µM | 1.00 µl |
| Antisense primer, 2 µM | 1.00 µl |
| Bidistilled $H_2O$ | 7.65 µl |
| Taq Polymerase, 5 U/µl (Qiagen, Hilden, DE) | 0.10 µl |

The PCR-machine/thermal cycler (Biometra, Göttingen, DE) was programmed for the following temperature cycle:

5 min 94°C (DNA denaturation)
[begin repeatable cycle, x35]
1 min 94°C (DNA denaturation)
1 min annealing temperature (55°C or 58°C)
2 min 72°C (elongation)
[end repeatable cycle]
10 min 72°C (elongation)
Infinite time at 4°C (cooling/storage)

The forward/reverse primers used for genotyping were specific for the *Cre* sequence (58°C annealing temperature) for sites framing a *flox* sequence of *Cdh1* (55°C annealing temperature). The number of cycles was 35 (occasionally increased to 37). The primer sequences are listed in the "Materials" section on page 45.

Electrophoresis was done in a 1.5% agarose gel (containing ethidium bromide) in TAE buffer at 120 V of concurrent flow and detection was done under ultraviolet light.

50x TAE buffer:

| | |
|---|---|
| TRIS | 242 g |
| Glacial acetic acid | 57.1 ml |
| EDTA ph8.0, 0.5 M | 100 ml |

## 3. Basic overview of mouse sections

Before section, mice body weight was documented. In case of drawing blood, mice were anesthetized. Mice were killed by cervical dislocation using the blunt side of a knife blade. Mice younger than 1 or 2 weeks (depending on the pubs size), were killed by cutting the spine close to the head using scissors.

After killing the mice, the body was put belly-up on a tissue-covered polystyrene plate and the paws were affixed using pins. The fur was dampened with isopropyl alcohol or disinfectant to prevent hairs from contaminating samples and the furry outer skin was cut open (one cut along the linea alba and one cut orthogonal to it, roughly 1 to 2 cm cranial of the pelvis) and partly removed from the belly (and, if required, also from the thorax). Then the abdomen was cut open in a similar manner and the flaps of tissue were pinned down on the right and left side to give easy access to the innards of the animal. After inspection and sometimes photography of the opened mouse, the organs of interest were removed and samples gained and/or organ weights and sizes were documented.

Organ samples were either frozen on dry ice, stored in formaldehyde, or kept in RNAlater® (Ambion [part of Invitrogen], Darmstadt, DE). The remaining body was either kept in 4% formaldehyde in PBS for the eventuality of further analysis or was disposed as appropriate (Category 1 material, Regulation (EC) No 1774/2002).

<u>10x PBS pH 7.4:</u>

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $KH_2PO_4$ | 2.4 g |
| $Na_2HPO_4$ | 14.4 g |
| Bidistilled water up to | 1 l |
| Adjusted to pH 7.4 with HCl | |

<u>4% buffered formaldehyde solution:</u>

| | |
|---|---|
| Paraformaldehyde | 40 g |
| 10x PBS pH 7.4 | 100 ml |
| NaOH, 5 M | 250 µl |
| Bidistilled water up to | 1 l |
| Dissolve under heat before correcting pH | |
| Adjusted to pH 7.4 with HCl | |

# 4. Assessment of E-cadherin loss on protein-level

## 4.1. Collection of liver samples

Immediately after killing the animal and opening of the abdomen, the gut was pulled away from the liver, thus making the liver`s stem accessible for fixation with pincers. By carefully applied pulling force (and cutting with scissors where necessary), the liver was removed. On a flat surface the livers lobes were spread out and, using the gall bladder and the outlines of the lobes as orientation, the lobes were identified. The lobes were separated from each other and, using scissors, the lobe chosen for protein analysis was cut into small pieces (ca. 2 x 4 mm). The pieces were immediately placed on dry ice, and after being frozen through they were collected in pre-cooled 1.5 ml plastic microcentrifuge tubes or cell culture plates and stored in a -80°C freezer.

## 4.2. Preparation of liver samples

During the procedure, all samples that were not currently manipulated were stored on ice-cold water. 5 ml round-bottom tubes (Falcon/BD, Heidelberg, DE) were filled with 400 µl to 600 µl Laemmli buffer (1x concentration) and tissue samples (ca. 3 mm diameter).

Laemmli buffer stock (5x concentration):

| | |
|---|---|
| 1M TRIS pH 6.8 | 65.5 ml |
| Glycerol | 100 ml |
| 0.5 M EDTA pH 8.0 | 2.0 ml |
| SDS | 20 g |
| Bromophenol blue | a tiny amount (ca. 0.2 g or less) |
| Ad 200 ml bidistilled $H_2O$ | |

A homogenizer (Homogenizer Miccra, ART Labortechnik, Müllheim) was applied at 23,500 rpm for 30 seconds. After transfer to an eppendorf 1.5 ml cup, the sample was heated to 95°C in a thermomixer (Eppendorf, Hamburg). If required, reducing conditions (cleavage of disulfide bonds) were created by adding 1 µl of 2-Mercaptoethanol per 20 µl of Laemmli buffer before heating to 95°C. After cooling down with ice for 5 min and subsequent centrifugation at 4°C at 13,000 rpm (Table centrifuge 5417R, Eppendorf, Hamburg, DE), the supernatant sample was either used immediately or stored short-term at -20°C.

For measuring protein concentration ("Bicinchoninic acid assay" = "Smith assay") (SMITH et al., 1985), a standard curve ranging from 0 to 8 mg protein/ml

was produced, using bovine serum albumin (= BSA; Roth, Karlsruhe). Measuring of standard and samples was done in 96-well cell culture plates with flat-based wells (Becton Dickinson, Heidelberg).

10 µl sample (or 10 µl standard) was mixed with 40 µl PBS pH 7.4 as well as 4 µl 4% $CuSO_4$ solution (Sigma-Aldrich, Deisenhofen,DE) and 196 µl bicinchoninic acid (Sigma-Aldrich, Deisenhofen,DE). Duplicates were pipetted (e.g. 2 wells with 110 µl each) before incubation at 37°C for 30 min. Absorption at a wavelength of 560 nm was measured using a Microplate Reader (Tecan, Männedorf, CH).

### 4.3. Electrophoresis and blotting

The Mini Protean® 3 cell system (Bio-Rad, Munich, DE) was used to separate proteins. Acrylamide gel was prepared in a glass container under continuous motion (by a magnetic stirrer). Separating gel (5 ml) was poured between the glass plates of the rack and covered with 1 ml bidistilled water. After polymerization for 45 min, the water was removed. Stacking gel (about 1 ml) was poured on top of the separating gel, and the system was fitted with a comb (10 or 15 teeth) to create pouches. After 30 min of polymerization, the gel was ready for use.

Gel acrylamide concentration: depending on the protein molecular weight, either 10% or 12% separating gels were used.

| | | |
|---|---|---|
| Separating gel (10%): | bidistilled water | 4.0 ml |
| | 1.5 M Tris (pH 8.8) | 2.5 ml |
| | 30% acrylamide | 3.33 ml |
| | 10% SDS | 100 µl |
| | 10% ammonium persulfate | 50 µl |
| | Temed | 5 µl |
| Separating gel (12%): | bidistilled water | 3.35 ml |
| | 1.5 M Tris (pH 8.8) | 2.5 ml |
| | 30% acrylamide | 4.0 ml |
| | 10% SDS | 100 µl |
| | 10% ammonium persulfate | 50 µl |
| | Temed | 5 µl |
| Stacking gel (5%): | bidistilled water | 7.0 ml |
| | 1.5 M Tris (pH 8.8) | 1.25 ml |
| | 30% acrylamide | 1.5 ml |
| | 10% SDS | 100 µl |
| | 10% ammonium persulfate | 125 µl |
| | Temed | 5.5 µl |

The gel was placed into the electrophoresis apparatus and the chambers were filled with electrophoresis buffer. The comb was removed and one sample (diluted to contain 40 µg protein) was pipetted per pouch.

Molecular weight standard: PageRuler™ prestained protein ladder (#26616 [former Fermentas # SM0671], Thermo Scientific, St. Leon-Rot, DE).

SDS-PAGE electrophoresis buffer:

| | |
|---|---|
| Tris | 30.3 g |
| Glycine | 144 g |
| SDS | 10 g |
| bidistilled water up to | 1 l |

Protein was drawn into the stacking gel at 100 V for 15 min. Separation of protein was done at 140 V for about 90 min (depending on estimated protein molecular weight).

For semi-dry blotting, a ball-pen labeled PVDF membrane (Millipore, Billerica, MA, US) was activated with methanol (p.a.) for 15 min on a seesaw. On the blotting apparatus (Bio-Rad, Munich, DE) a stack of different layers was arranged (from bottom to top): extra-thick blotting paper (Bio-Rad, Munich, DE), membrane, gel, extra-thick blotting paper. The stack was covered with transfer buffer (20 ml 10x transfer buffer + 40 ml methanol + 140 ml bidistilled water) and fixated with the electrode lid.

10x transfer buffer:

| | |
|---|---|
| Tris | 58.2 g |
| Glycine | 29.2 g |
| SDS | 3.7 g |
| bidistilled water up to | 1 l |

The blotter was set to 15 V for 1 hour. Afterwards, the membrane was flushed with TBS-T. For blocking, 5% instant skimmed milk powder (Roth, Karlsruhe, DE) in TBS-T was applied for 1 hour at room temperature or overnight at 10°C on a seesaw.

10x TBS buffer:

| | |
|---|---|
| Tris | 30 g |
| NaCl | 80 g |
| bidistilled water up to | 1 l |
| pH corrected to 7.4 with HCl | |

TBS-T buffer:

1x TBS with 0.1 % Tween®20 (Sigma-Aldrich, Deisenhofen, DE)

### 4.4. Detection of protein

Antibodies were diluted (see below) in 1% instant skimmed milk powder in TBS-T. Incubation of antibodies was done in 50 ml falcon tubes in an unheated rotating hybridization oven (H.Saur, Reutlingen, DE).

The membrane was flushed with TBS-T and then incubated with the primary antibody for 1 hour at room temperature or overnight at 10°C. After washing with TBS-T, the secondary antibody was applied for 1 hour at room temperature or overnight at 10°C. Following washing with TBS-T, an ECL solution (#34076; #34077, Thermo Scientific, Rockford, IL, US) was applied and incubated for 1 min on a seesaw. CL-XPosure Film (Thermo Scientific, Bonn, DE) was exposed to the membrane (depending on intensity of the fluorescence between 3 seconds and several hours) and developed (Curix60 Tabletop processor, Agfa HealthCare Corporation, Greenville, SC, US).

Primary antibodies diluted:

- goat vs. E-cadherin (#AF748, R&D Systems, Wiesbaden-Nordenstadt, DE) at 1:1000
- mouse vs. actin (#691001, MP Biomedicals, Eschwege, DE) at 1:5000
- rabbit vs. GAPDH (#14C10, Cell Signaling, Frankfurt-Main, DE) at 1:5000

Secondary antibodies (HRP conjugated) diluted:

- donkey vs. rabbit (#NA934V, GE Healthcare, Munich, DE) at 1: 10.000
- sheep vs. mouse (#NA931V, GE Healthcare, Munich, DE) at 1: 10.000
- donkey vs. goat (#sc-2033, Santa Cruz, Dallas, TX, US) at 1: 10.000

## 5. Evaluation of gene expression at the RNA level

### 5.1. Collection of liver samples for RNA analysis

Since we assumed liver RNA expression to be heavily influenced by the circadian rhythm as well as by food intake, care was taken to standardize this influences as good as possible. Mice of all groups scheduled for RNA analysis were put on a grid at 9 o'clock a.m. with no access to chow, where they could not reach other organic material (bedding, feces). Water was given ad libitum. Sections were

started exactly 6 hours later and performed as fast as possible. Preparation of liver for RNA analysis was done by extracting a central piece of the left liver lobe, ca. 4x4x8mm. This work was done on an ice-cooled glass plate (ca. 4°C). The piece was stored in RNAlater® (Ambion [part of Invitrogen], Darmstadt, DE) at 4°C overnight and was transferred to a -20 or -80°C freezer on the next day.

### 5.2. RNA expression analysis using Agilent Microarray

Extraction of RNA from tissue, cDNA synthesis for microarrays and measurement using Agilent Microarray technology (Agilent Technologies, Inc., Santa Clara, CA, US) was kindly done by members of the group of Dr. Helmut Blum (LAFUGA, Gene Center, LMU München), using established protocols. Procession of the raw data was kindly done by junior group leader PD Dr. Stefan Bauersachs.

### 5.3. cDNA synthesis for qRT-PCR

The protocol applied for cDNA synthesis was a modified version of the SuperScript™ First-Strand Synthesis System for RT-PCR protocol (Kit, #11904-018, Invitrogen, Carlsbad, CA, US). The kit also provided the standard reagents.

A sample of 8.0 µl RNA (containing 4 µg total RNA) was pipetted into 250 µl safe-lock tubes, together with 1.0 µl of 10x Buffer and 1.0 µl of DNAseI (#18068-015, Invitrogen, Karlsruhe, DE). After thorough mixing (30 s), incubation at 25°C for 15 min was done. Then 1 µl of EDTA (25 mM) was added and the sample was mixed (30 s) before incubation at 65°C for 15 min. After chilling on ice for 1 min the sample was spun briefly and 1.375 µl of random hexamer primer was added, as well as 1.375 µl of dNTP (10 mM). Incubation at 65°C for 5 min was followed by chilling on ice for 1 min.

A mixture of reagents was added, containing (per sample):

| | |
|---|---|
| 10x RT buffer | 2.75 µl |
| $MgCl_2$ (25mM) | 5.5 µl |
| DTT (0.1 M) | 2.75 µl |
| RNAse-out | 1.375 µl |

Incubation was done at 25°C for 2 min. After adding 1.375 µl Superscript II, three incubation steps followed: 1) 25°C for 10 min; 2) 42°C for 50 min; 3) 70°C for 15 min.

After chilling on ice for 1 min, 1.375 µl RNAse H (Invitrogen, Karlsruhe, DE)

was added, followed by incubation at 37°C for 20 min, and finally chilling on ice for 1 min. The cDNA was stored at -20°C. The housekeeper gene/RNA used to verify presence of cDNA in the samples was *Gapdh*. This housekeeper had been found not to differ much in qRT-PCR of L-*Cdh1*$^{del/del}$ and L-Control (e.g. it was not secondarily affected by the knockout, and thus suitable)

### 5.4. Quantitative RT-PCR

Taq DNA polymerase Kit and HotStar Taq polymerase (Quiagen, Hilden, DE) and 96-well real-time PCR plates (Eppendorf, Hamburg, DE) were used, with every well contained the following: 2.0 µl of sample cDNA (diluted 1:5 with $H_2O$) + 18.0 µl of Master Mix.

Master Mix:

| | |
|---|---|
| 10x buffer | 2.0 µl |
| $MgCl_2$ | 1.0 µl |
| dNTPs | 0.5 µl |
| FW primer | 0.5 µl |
| RV primer | 0.5 µl |
| Q solution | 4.0 µl |
| SYBRGreen (1:2000) | 0.8 µl |
| HotStartTaq | 0.2 µl |
| $H_2O$ | 8.5 µl |

Realtime PCR was performed with a Mastercycler® ep realplex PCR machine (Eppendorf, Hamburg, DE) using the following cycles:

15 min 95°C (DNA denaturation)
[begin repeatable cycle, x56]
30 sec 95°C (DNA denaturation)
30 sec 55°C (primer annealing)
30 sec 72°C (elongation)
20 sec 82°C (DNA quantification)
[end repeatable cycle]
15 sec 95°C (DNA denaturation)
15 sec 60°C
20 min continuously increasing heat to 95°C (melting curve determination)
15 sec 95°C
Infinite time at 4°C (cooling/storage)

As fluorescent reporter, the intercalating dye SYBR® Green (Lonza, Basel, CH)

was used to determine the melting curve. The housekeeper gene *Gapdh* was used to standardize the samples, using the Delta-CT method. Delta-Delta-CT could not be used to compare both groups (L-*Cdh1*$^{del/del}$ and L-Control) because samples (mice) were not paired and there was no reason why any L-*Cdh1*$^{del/del}$ sample should have been associated with a specific L-control sample. Therefore, both groups were averaged before comparing them with each other. Because CT values are logarithmic, adding them up and dividing by n (e.g. the sum of all samples) would result in geometric mean instead of arithmetic mean. To account for this, CT values were linearized (transformed to non-logarithmic numbers) before calculating the mean (e.g. arithmetic mean was calculated) as suggested in literature (SCHMITTGEN & LIVAK, 2008). From the means of both groups, fold-change of cDNA was calculated.

The primer sequences used for qRT-PCR are listed in the "Materials" section (page 45).

## 6. Analysis of body weight development and organ weight

### 6.1. Long-term body weight development

Mice were weighed weekly with a laboratory scale (Laboratory scale, BP4100S, Sartorius, Göttingen, DE). ANOVA of the data was performed with use of the SAS software (SAS Institute Inc., Cary, NC, US).

### 6.2. Body and organ weight at specific time points

Body weight of mice was measured before drawing of blood and section (see the appropriate paragraphs on necropsies). Organs were removed using pincers and fine scissors and placed on a special accuracy weighing machine (Laboratory scale, BP221S, Sartorius, Göttingen, DE). Afterwards organs were further processed for storage (freezing, RNAlater or formaldehyde) or discarded if not needed.

## 7. Evaluation of serum parameters

### 7.1. Blood collection

Before section, mice were anesthetized and blood was collected from the retrobulbar venous plexus. Mice were killed immediately thereafter by cervical dislocation.

### 7.2. Serum preparation

After leaving the blood in an eppendorf cup at room temperature for 1 hour to allow coagulation, spinning at 5000 rpm in a 14 cm diameter rotor centrifuge (Table centrifuge 5417R, Eppendorf, Hamburg, DE) forced the separation of serum from the solid blood components. Serum was pipetted into a new eppendorf cup, centrifuged again to remove any residual erythrocytes and pipetted into the final 1.5 ml eppendorf cup for storage in a -80°C freezer.

### 7.3. Clinical chemistry

Clinical chemistry was kindly performed by staff members of the clinical chemistry lab at the Campus Großhadern (Institut für Laboratoriumsmedizin, Klinikum der Universität München, LMU München).

## 8. Induction of tumors through use of diethylnitrosamine (DEN)

E-cadherin is known to be important to cell-cell-adhesion, and adhesion can be expected to have a great effect on tumor development. Consequently, we submitted our mice not only to normal conditions ("spontaneous phenotype"), but also to the challenge condition of a carcinogen ("phenotype after tumor induction"). Due to the abundancy of publications using DEN for tumor research (LEENDERS et al., 2008), and its ease of application, DEN was chosen as a carcinogen.

A total of 92 animals were injected with DEN (early drop-outs not counted) and necropsy was scheduled to be performed 4, 8 or 12 months later (Table 1). Moribund animals were sacrificed earlier (this applied mostly to the 12-months group).

*Table 1. Number of animals injected with DEN and sacrificed during the experiment coined "phenotype after tumor induction".*

| | 4 months | 8 months | 12 months |
|---|---|---|---|
| L-*Cdh1*$^{del/del}$ | 11 | 15 | 20 |
| L-Control | 12 | 13 | 21 |

### 8.1. Preparation of DEN and dosage

DEN (N-Nitrosodiethylamine; Sigma, St.Louis, MC, US) was diluted in 0.9% NaCl solution (B. Braun Melsungen AG, Melsungen, DE) to a 1:5000 solution. The rubber-stopper capped bottle was protected from direct sunlight and stored at room temperature. While data concerning the degradation of DEN in aqueous solution is lacking, we precautiously refrained from using solutions older than a year.

### 8.2. Intraperitoneal (i.p.) application

We chose a protocol with only one injection of DEN and no additional substances (like barbiturate in drinking water), which are sometimes used in related experiments to further promote tumors (DIWAN et al., 1985; LEENDERS et al., 2008).

A 1-ml syringe fixed with a 30 G needle was used to inject the DEN solution intraperitoneally. Mice pups of 2 weeks of age were weighted and the correct dosis (0.025 ml/g of body weight) of DEN / 0.9% NaCl solution was drawn from the storage bottle (e.g. 5 µg DEN per gram of mouse body weight was applied). The pups were fixated at the neck with thumb and index finger while the body was supported by the palm of the hand, using the little finger to fixate the tail end of the mouse. While injecting, great care was taken to maintain the needle in the abdomen cavity so as not to puncture the liver or another vital organ. To additionally aid in this, mice were held with their head down and their tail up roughly at a 45 degree angle during injection to force the organs and guts away from the site of punctuation by gravity.

### 8.3. Safety measures

DEN is a hazardous substance and can be a danger to personnel working in an animal facility if inhaled (ISSENBERG & SORNSON, 1976) or incorporated via another route. Dangers to the personnel include cancerogenity and, to a much lesser extent, acute toxicity. Preparation and injection of DEN was done under a fume hood. Through the use of rubber-stopper capped bottles, DEN solution always was handled in enclosed containers until entering the animal. Before injection, the bench was covered with sturdy plastic foil which was later discarded safely to remove eventual unnoticed spills. Two pairs of nitrile gloves (AQL 1.5) were donned (double layer) and all materials used in direct contact with the DEN

were discarded with the hazardous lab waste. To prevent DEN excreted by the mice from contaminating the rest of the facility, mice cubs and their mothers were kept in separate filter-enclosed cages and were only handled by experienced animal caretakers (for two weeks after injection). During that time, cleaning of the cages was done separately from standard cleaning and all waste was also immediately packaged separately.

## 9. Section of mice with tumors

After noting mice body weight, mice were anesthesized and blood was gained. Following cervical dislocation, the abdomen was opened without contamination by fur. After inspection of the situs, the liver, lung and spleen were removed. Organ weights were noted and the lesions of the liver were classed according to their size and counted. From some mice of the 12-month-group, tumor tissue was gained for cell culture.

The remaining mouse body with all not-paraffin-embedded organs (e.g. without lung and liver) was kept in 4% formaldehyde in a 50 ml falcon for possible later analysis of alterations (metastases etc.).

### 9.1. Liver and body weight

This was done in the same way as described for all necropsies. The liver weight was intended as a primary variable for some age groups (4 months and 8 months); and body weight was used to calculate the relative liver weight.

### 9.2. Other organ weights

Lung and spleen weight were not conceptualized as primary variables from the very beginning, and thus this measurement was not considered to contribute to the familywise error rate. Instead, documented weight alterations of these organs were used to fortify the observations of the primary variable (relative liver weight).

After weighting, organs were inspected macroscopically from all sides for possible metastases. Alterations were documented with a camera.

### 9.3. Count of tumor lesions

Count of tumor lesions at the livers surface is a method to quickly evaluate the tumor burden, but there are some difficulties:

- a single tumor might create multiple lesions at the liver surface
- the size of irregular formed lesions can often not be measured precisely
- tumors sometimes can be seen, but do not breach the liver surface

As a result measurement is very much dependent on the individual researcher, but can yield quite stable output if always done with the same criteria and by the same person.

The liver was removed from the animal and all lobes were spread open. Lesions were classed (<2 mm | 2-5 mm | >5 mm) and counted. Then the liver was flipped over and the other side´s lesions were counted.

### 9.4. Preparation of tumor samples for RNA analysis, histology and cell culture

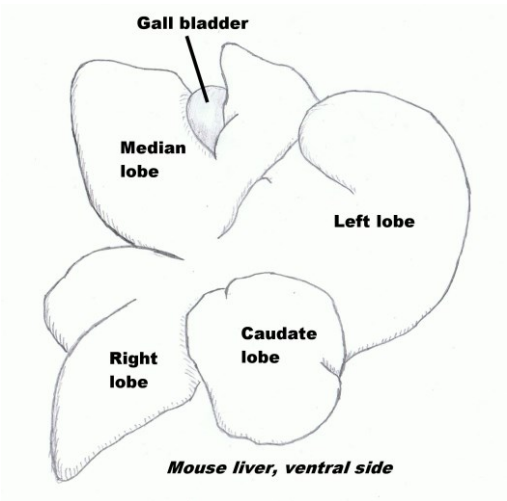


*Fig. 12. Schematic of the mouse liver, based on "The Anatomy of the Laboratory Mouse" by Margaret J. Cook, accessible at Jax informatics.*

The left lobe (Fig. 12) was put into a histological cassette, stored in 4% formaldehyde in PBS and sent to the "Pathologisches Institut" (Universitäts Klinikum Heidelberg) for specialized histological analysis. A part of the median lobe (the part that is located adjacent to the right side of the gall bladder) was also stored in formaldehyde and subsequently embedded in paraffin for histological evaluation as a backup. The rest of the liver was processed as following: liver-tissue that was relatively tumor-free, if available, was cut into small (roughly 2 x 4 mm) pieces and frozen on dry ice for later storage in a -80°C freezer. If available, large tumors (up to 5) were separated and cleaned from healthy tissue and cut into two pieces. Their diameter was documented and one half was

prepared for paraffin embedding while the other half was stored in RNAlater® (Ambion [part of Invitrogen], Darmstadt, DE).

Some tumors of the 12-month DEN group were used for generating cell lines. In this case, the whole necropsy procedure was done under semi-sterile conditions and as fast as possible. For short term storage, sterile dishes with the isolated, tumor-rich liver lobes were kept on ice-cold water. Large single tumors or batches of several smaller tumors were then removed from the liver and put into sterile petri dishes containing a few milliliters of DMEM/F12 cell culture medium (containing no "fetal calf serum" = FCS) and processed quickly, preferably in parallel by a second person.

## 10. Establishment of long term cell culture from tumors

The procedure was established by modifying a protocol kindly provided by members of the workgroup "Endokrinologische Forschung und Endokrinologische Ambulanz" (Medizinische Klinik und Poliklinik IV, Campus Innenstadt, Klinikum der Universität München, LMU München).

### 10.1. Preparation of collagenase II

The enzyme was freshly prepared prior to the necropsy. Collagenase II (Biochrom, Berlin, DE) was dissolved at 1.5 mg per ml of PBS. For the average tumor (ca. 1 cm diameter) around 10 ml of collagenase solution was needed. After sterile filtration (0.22 µm filter), the solution was ready for use.

### 10.2. Mincing of tumors and destruction of the organ/tumor structure

Under a sterile closed-circuit laminar flow cabinet (BDK Luft- und Reinraumtechnik GmbH, Sonnenbühl-Genkingen, DE), the tumors were cleaned of non-tumorous tissue using sterile scissors or a razor blade. Then, the tissue was minced thoroughly (but not too vigorously, which would have destroyed too many cells) in a 10 cm petri dish containing 8 ml PBS/ collagenase II solution. As a rule of thumb, the tissue was minced until most pieces were of roughly 0.5 mm diameter or less. Care was taken to use the blade in a cutting rather than a hitting motion to conserve cells. Cells were then transferred to a Falcon tube (50 ml). To maximize the yield, the petri dish was flushed with the remaining 2 ml of PBS/collagenase II. The falcon was placed for 50 min at 37°C on a shaking incubator (infors AG, Bottmingen, DE) at 110 to 220 rpm.

As an alternative method, the petri dish was sometimes replaced with a 50 ml erlenmeyer flask with wide opening. Small scissors could be inserted through the wide opening to mince the tumor; and the incubation was done in the same flask (covered with sterile aluminum foil).

Incubation was stopped by FCS (PAA Laboratories GmbH, Pasching, AT). A 50 ml Falcon tube filled with enough FCS to later result in a >10% FCS solution was fitted with a 70µm cell strainer/Falcon™ (BD, Heidelberg, DE); and the tumor/collagenaseII/PBS solution was pressed through the strainer with a 10ml glass pipette. The tube was then spun for 5 min at 1200 rpm (17 cm diameter rotor) and the supernatant discarded.

### 10.3. Erythrocyte lyses

The cell pellet was re-suspended in lyses buffer. The volume of buffer added was at least two times of cell pellet volume. Incubation was 7 min at room temperature. The tube was then spun for 5 min at 1200 rpm (17 cm diameter rotor) and the supernatant discarded.

Buffer for erythrocyte lysis:

| | | |
|---|---|---|
| $NH_4Cl$ | 0.15 M | 8.29 g |
| $KHCO_3$ | 1 mM | 1 g |
| $Na_2EDTA$ | 0.1 mM | 37,2 mg |

Add 800 ml bidistilled water
Set pH to 7.2 to 7.4 with HCl
Add bidistilled water up to final volume of 1000 ml

### 10.4. Re-suspension in cell culture dishes

Cells were re-suspended in cell culture medium DMEM/F12+10%FCS+P/S (PAA Laboratories GmbH, Pasching, Austria) and cultured in “BD Primaria™ 60mm Cell Culture Dish, surface-modified polystyrene for enhanced cell culture” (BD, Heidelberg, DE).

After incubating culture dishes overnight, dishes were checked under a microscope (DM IL Mikroskop, Leica, Wetzlar, DE) and the medium was changed. Afterwards the culture was left untouched for around 3 – 4 days. Subsequently, cells were checked regularly and medium was changed twice per week. Splitting was continuously adjusted according to the proliferation rate, since phases of slow or no proliferation and considerable changes of the cell growth were to be expected in the early stages (ROHME, 1981).

### 10.5. Maintenance and splitting of cell lines

Cells were kept in an incubator (Heraeus, Munich, DE) at 37°C and 5% $CO_2$. For splitting, cultures were handled under a closed-circuit laminar flow cabinet. In successfully established cultures, cells had to be split one to two times per week with seeding of ¼ of the cells into a new dish. Cells were kept in 6-well plates, 96-well plates, 6 cm or 10 cm dishes.

For splitting, all reagents, buffers and media were warmed to 37°C. After washing with PBS, trypsin-EDTA (PAA Laboratories GmbH, Pasching, Austria) was added and the cells were incubated at 37°C for up to 5 min. Trypsinated cells were added to DMEM/F12 with FCS and centrifuged. After discarding the supernatant, cells were re-suspended in medium and seeded to new dishes.

### 10.6. PCR of cell culture cells

PCR of cell culture was done as described before for genotyping of mice. Instead of a mouse tail tip, cells of a 6 cm culture dish were scraped off and used.

### 10.7. Conservation of cell-lines for future studies

To conserve cells for later use, they were frozen in liquid nitrogen. Suspended cells were stored in 1.0 ml cryotubes (Nunc A/S, Roskilde, DK) containing as a freeze medium 400 µl DMEM + 320 µl FCS + 80 µl DMSO (PAA Laboratories GmbH, Pasching, AT). Cryotubes were immediately placed in a -80°C freezer for one night and then transferred to a tank containing liquid nitrogen for storage in the gas phase.

To test whether cells were viable, a surplus tube of the batch was removed from the storage after a few days, placed in a 37°C water bath until liquefied and centrifuged for 1 min. The supernatant was discarded and the cell pellet was suspended in cell culture medium and seeded at 37°C and 5% $CO_2$.

## 11. Histological analysis

Formaldehyde-fixated samples were embedded in paraffin wax and Superfrost slides (Themo-Scientific, Braunschweig, DE) were prepared using facilities of the Pathologisches Institut (Campus Innenstadt and Campus Großhadern of the Klinikum der Universität München, LMU München) and the Institut für Tierpathologie (Tierpathologie München, Tierärztliche Fakultät, LMU München).

Some samples were processed entirely (embedding, microtomy, H&E-staining) by cooperation partners (Pathologisches Institut of the Universitäts Klinikum Heidelberg).

### 11.1. Hematoxilin and eosin (H&E) staining

H&E staining performed by cooperation partners (Pathologisches Institut of the Universitäts Klinikum Heidelberg or Pathologisches Institut, Campus Innenstadt, Klinikum der Universität München, LMU München) was done using the respective institute´s procedure. H&E staining performed in our group was done using the following protocol:

Slides were kept at 37°C overnight or alternatively at 50°C for one hour prior to the procedure. After descending ethanol series (Rothishistol → ethanol 50%), slides were rinsed in bidistilled water and stained in Mayer´s hematoxylin for 3-10 min and blued in tap water for 10 min. After ascending ethanol series (water → ethanol 96%), eosine staining (EosinY in ethanol solution) for 5 min followed. After a 25 second step of ethanol and isopropanol each, the ascending ethanol series was continued to Rothihistol. Slides were mounted using Pertex.

Histology reagents were acquired from the following companies: Rothihistol (Roth, Karlsruhe, DE); Hematoxylin (Medite, Burgdorf, DE); Eosin Y (Sigma, St.Louis, MO, US) Ethanol 99% with 2-Butanon 1% (HEMA GmbH & Co. KG, Nurnberg, DE); Isopropanol, technisch (Roth, Karlsruhe, DE); Pertex (Medite, Burgdorf, DE).

### 11.2. Immunohistochemistry (IHC)

After the descending ethanol series, slides were rinsed in PBS. Slides were then boiled in a microwave oven in citric buffer at pH 6.0 for 20 min (or 40 min if samples were resistant enough). Afterwards, 100 ml methanol containing ca. 1% $H_2O_2$ (3 ml of 30% $H_2O_2$ in 100 ml) was used to block endogenous peroxidase activity (30 min incubation). After washing with PBS, slides were incubated with 5% rabbit serum (PromoCell, Heidelberg, DE) in PBS for 30 min. After removal of serum by skidding, the primary antibody (goat vs. E-cadherin diluted 1:100 in PBS, #AF748, R&D Systems, Wiesbaden-Nordenstadt, DE) was incubated 1 hour at room temperature or 17 hours at 4°C. After washing with PBS, secondary antibody (Polyclonal Rabbit Anti-Goat Immunoglobulins/Biotinylated diluted 1:200 in PBS, # E 0466, Dako, Hamburg, DE) was incubated for 1 hour at room

temperature. Following washing with PBS, avidin-biotin-complex (Vectastain Elite ABC Kit, distributed by AXXORA Deutschland GmbH, Lörrach, DE) was incubated for 30 min at room temperature. After washing with PBS, diaminobenzidine (Sigma Fast DAB Tablet Set, Sigma-Aldrich, Taufkirchen, DE) ready-to-use solution was applied. Time for staining varied between 5 seconds and 10 min, depending on the antibody used. Slides were counterstained with a little hematoxylin (only dipping 5 to 20 times; maximum time 1 min), blued, and mounted with Pertex after an ascending ethanol series.

All of the more expensive solutions (antibodies, serum, ABC, DAB) were applied as drops covering just the sample on the slide. To prevent drying out, slides were stored in an enclosed container with high humidity. Positive control was (if available) a slide with a sample that was already known to react. Negative control was the same, but with omission of primary antibody (pure PBS used instead). ABC and DAB were prepared as suggested by the manufacturer(s).

Citric buffer formula: Combine 9 ml stock solution A + 41 ml stock solution B + 450 ml bidistilled water. Stock solution A contains 21.01 g of C6H8O7•H2O in 1000 ml bidistilled water. Stock solution B contains 29.41 g of C6Na3H5O7•H2O in 1000 ml bidistilled water.

## 12. Histological evaluation of HCC

The sample processing, as well as the evaluation and quantification of histologically detectable focal liver lesions was kindly performed by PD Dr. Longerich (Research Group “Molekulare Klassifikation und neue Marker im HCC”, Pathologisches Institut, UniversitätsKlinikum Heidelberg).

### 12.1. Histological quantification of tumors in the liver

For evaluation of tumor areas H&E slides were digitalized using the ScanScope CS system (Aperio Technologies, Vista, CA, US) in combination with Spectrum™ management system (Aperio, version 11.0.0.725). The tumor-occupied areas were measured using the annotation tool of the ImageScope software (Aperio, version 11.0.2.275). See the figure (Fig. 13) for an example.

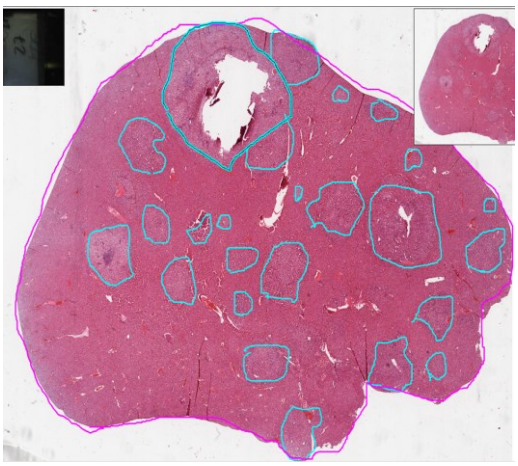

*Fig. 13: Using ImageScope softwares annotation tool, total liver area (pink tool) was measured and set into relation with tumor area (light blue tool). Large tumors sometimes showed necrosis, as can be seen by the destroyed area (histology artifact due to damaged tissue) in the largest tumor.*

### 12.2. Histological evaluation of tumors in the lung

Slides from lungs of animals with HCC were stained H&E and screened for metastases or primary tumors by PD Dr. Longerich.

## 13. Statistical analysis

For comparing body weight gain of L-*Cdh1*$^{del/del}$ vs. L-Control, 2-factorial ANOVA was performed using SAS software (SAS Institute Inc., Cary, NC, US).

For all other statistical tests and for generating graphs, Prism 4.0 (GraphPad Software, La Jolla, CA, US) was used. All graphs show means and SEM. For assessing normal distribution Kolmogorov–Smirnov and/or Shapiro-Wilk normality test was employed if appropriate (appreciable sample size), otherwise normal distribution was ascertained or rejected using information from literature and previous experience. As suitable, *t*-test (two-tailed, unpaired) or Mann-Whitney-U test (two-tailed) was used, and confidence interval was set to 95% ($p<0.05$ denoted with "*", $p<0.01$ with "**" and $p<0.001$ with "***" in graphs with bars).

# 14. Primer sequences

## 14.1. Primers for genomic DNA amplification

| | |
|---|---|
| Cdh1 (oIMR3737) | 5`-CTTATACCGCTCGAGAGCCGGA-3` |
| Cdh1 (oIMR3738) | 5`-GTGTCCCTCCAAATCCGATA-3` |
| Cre1 | 5`-AATCGCCATCTTCCAGCAGG-3` |
| Cre2 | 5`-GATCGCTGCCAGGATATACG-3` |

## 14.2. Primers for cDNA amplification

| | |
|---|---|
| mMMP7_211_FW | 5`-CTGCCCATGACTGGAAAACT-3` |
| mMMP7_291_RV | 5`-TTCTGCAACATCTGGCACTC-3` |
| mScube3_128_FW | 5`-ATGCCATCTGCCAGAATACC-3` |
| mScube3_269_RV | 5`-GGGATGTTGACACAGTCGTG-3` |
| mTff2_154_FW | 5`-TGCTTTGATCTTGGATGCTG-3` |
| mTff2_236_RV | 5`-TCCGATTCTTGGTTTGGAAG-3` |
| mFxyd6_123_FW | 5`-GGTGTTTGCTGTGGTCCTCT-3` |
| mFxyd6_204_RV | 5`-GGGCTTCTGATTGAAACTGC-3` |
| mBicc1_1434_FW | 5`-CATGCAGACAGAAGGCAAAA-3` |
| mBicc1_1514_RV | 5`-GACAGCGGACCGTATTTCAT-3` |
| mAebp1_1747_FW | 5`-AAGAGTTCACGAGGGCTCAA-3` |
| mAebp1_1896_RV | 5`-GTATTGCATGAGCAGGAGCA-3` |
| mArhgap22_1285_F | 5`-ATGCACACTTTGCCTGTCTG-3` |
| mArhgap22_1398_R | 5`-GTTCCCACCAGAGGAGATGA-3` |
| mAdamtsl2_534_FW | 5`-GGTTTGCGTGTCTGGAAAAT-3` |
| mAdamtsl2_680_RV | 5`-AGATGATTGTTGCCCTTTCG-3` |
| mBex1_253_FW | 5`-GTACAGAGGTTTGGGGGTGA-3` |

| | |
|---|---|
| mBex1_282_RV | 5`-GCATGAGGCAAAACTCATCA-3` |
| mGapdhFW | 5'-TCATCAACGGGAAGCCCATCAC-3' |
| mGapdhRV | 5'-AGACTCCACGACATACTCAGCACCG-3' |

# 15. Materials

## 15.1. Machines

| | |
|---|---|
| Accu-jet® pro pipette controller | Brand, Wertheim, DE |
| Agarose gel electrophoresis chamber | MWG-Biotech, Ebersberg, DE |
| Benchtop 96 tube working rack | Stratagene, La Jolla, US |
| Blunt forceps | Aesculap, Tuttlingen, DE |
| Bulldog forceps | Aesculap, Tuttlingen, DE |
| Chyo JL-200 (analytical balance) | Chyo, JP |
| Chyo MJ-3000 (analytical balance) | Chyo, JP |
| Curix60 Tabletop processor | Agfa HealthCare, Greenville, SC, US |
| Digital Camera | Olympus, Hamburg, DE |
| DM IL Flo Mikroskop+digital camera | Leica, Wetzlar, DE |
| DM IL Mikroskop (Cell culture) | Leica, Wetzlar, DE |
| DMC-FZ30 (Digital camera) | Panasonic, Osaka, JP |
| Duran®-Schott glass ware | DURAN Group, Wertheim, DE |
| Electrophoresis chamber | MWG-Biotech, Ebersberg, DE |
| EPS 500/400 Electrophoresis Power Supply | Pharmacia Fine Chemicals, NJ, US |
| Fine scissors | Aesculap, Tuttlingen, DE |
| Gel documentation system | Intas, Göttingen, DE |
| GeneAmp PCR System 9700 | Applied Bisosystems, Foster City, US |
| Glass case for glass rack | Roth, Karlsruhe, DE |
| Heating plate with magnetic stirrer | IKA process equipment, Staufen, DE |

| | |
|---|---|
| Homogenizer Miccra | ART Labortechnik, Müllheim, DE |
| Hybridization oven | H.Saur, Reutlingen, DE |
| Incubator (for cell culture) | Heraeus, Munich, DE |
| Laboratory scale, BP221S | Sartorius, Göttingen, DE |
| Laboratory scale, BP4100S | Sartorius, Göttingen, DE |
| Laminar flow cabinet | BDK, Sonnenbühl-Genkingen, DE |
| Light microscope | Olympus, Hamburg, DE |
| Mastercycler® ep realplex PCR machine | Eppendorf, Hamburg, DE |
| MicroPlate reader | Tecan, Männedorf, CH |
| Microtome | Microm, Walldorf, DE |
| Microwave | Siemens, Munich, DE |
| Mini Protean® 3 Cell | Bio Rad, Munich, DE |
| MS1 Minishaker | IKA process equipment, Staufen, DE |
| Multipette® plus | Eppendorf, Hamburg, DE |
| PCR-machine/thermal cycler | Biometra, Göttingen, DE |
| PIPETMAN® P (1000µl) | Gilson, Limburg, DE |
| PIPETMAN® P (100µl) | Gilson, Limburg, DE |
| PIPETMAN® P (10µl) | Gilson, Limburg, DE |
| PIPETMAN® P (200µl) | Gilson, Limburg, DE |
| PIPETMAN® P (20µl) | Gilson, Limburg, DE |
| PIPETMAN® P (2µl) | Gilson, Limburg, DE |
| Pointed scissors | Aesculap, Tuttlingen, DE |
| Power Pac 300 | Bio Rad, Munich, DE |
| Power Supply PPS200-1D | MWG-Biotech, Ebersberg, DE |
| Rotating shaker | Infors AG, Bottmingen, DE |

| | |
|---|---|
| ScanScope CS system | Aperio Technologies, Vista, CA, US |
| Semidry electroblotting apparatus | Bio-Rad, Munich, DE |
| Spectrophotometer | Beckman, Palo Alto, US |
| Table centrifuge (5417R) | Eppendorf, Hamburg, DE |
| Thermomixer 5436 | Eppendorf, Hamburg, DE |
| Ultraschall Sonorex Super RK 102 H | Bandelin, Berlin, DE |
| UV-Crosslinker | Biometra, Göttingen, DE |
| VakuLab s3000 (Autoclave) | MMM group, München, DE |
| Watch maker forceps | Aesculap, Tuttlingen, DE |
| Water bath SUB14 | Grant Instruments, Royston, GB |

### 15.2. Consumables

| | |
|---|---|
| 70 µm cell strainer/ Falcon™ | BD, Heidelberg, DE |
| 96-well cell culture plates (flat-based) | BD, Heidelberg, DE |
| 96-well real-time PCR plates | Eppendorf, Hamburg, DE |
| BD Primaria™ 60 mm Cell Culture Dish | BD, Heidelberg, DE |
| Blotting paper | Bio-Rad, Munich, DE |
| Centrifugation tube (15 ml, 50 ml) | Becton Dickinson, Heidelberg, DE |
| Centrifuge tube (0.5 ml, 1.5 ml, 2.0 ml) | Eppendorf, Hamburg, DE |
| Chemoilluminiscence film | GE Healthcare, Munich, DE |
| CL-XPosure Film | Thermo Scientific, Rockford, IL, US |
| Cover glass slides | VWR International, Darmstadt, DE |
| Cryotube 1.0 ml | Nunc A/S, Roskilde, DK |
| Doktorand (disposable) | LMU, Munich, DE |
| Filter paper | GE Healthcare, Munich, DE |
| Glass microscope slides | Menzel-Gläser, Braunschweig, DE |

| | |
|---|---|
| Heat sealing foil | Eppendorf, Hamburg, DE |
| Heparinized capillary tubes | Brand, Gießen, DE |
| Histology cassettes | Medite, Burgdorf, DE |
| Multi-well cell culture plates | Becton Dickinson, Heidelberg, DE |
| PCR-reaction-tubes | G. Kisker GbR, Steinfurt, DE |
| Petri dishes (diameter 10 cm) | Becton Dickinson, Heidelberg, DE |
| Plastic tubes (5 ml) | Greiner Bio-One, Frickenhausen, DE |
| PVDF membrane | Millipore, Billerica, US |
| Real-time PCR plates (96 well) | Eppendorf, Hamburg, DE |
| Rundbodenröhrchen, 5 ml | Falcon/BD, Heidelberg, DE |
| Standard rodent chow | Ssniff, Soest, DE |
| Superfrost slides | Menzel, Braunschweig, DE |
| Syringes (2, 5, 10, 20 ml) | Codan Medical ApS, Roedby, DK |

### 15.3. Chemicals

| | |
|---|---|
| 2-mercaptoethanol | Merck, Darmstadt, DE |
| ABC (Avidin-biotin complexes) | Vector Laboratories, Burlingame, US |
| AB: donkey vs. goat AB HRP (#sc-2033) | SantaCruz, Dallas, TX, US |
| AB: donkey vs. rabbit HRP (#NA934V) | GE Healthcare, Munich, DE |
| AB: goat vs. E-cadherin, #AF748 - | R&D Systems, Wiesbaden-Nordenstadt, DE |
| AB: goat vs. MMP-7 - | Santa Cruz Biotechnology, Heidelberg, DE |
| AB: goat vs. mouse antibody HRP | MP Biomedicals, Solon, OH, US |
| AB: monoclonal mouse vs. actin (#691001) | MP Biomedicals, Eschwege, DE |
| AB: monoclonal rabbit vs. GAPDH (#14C10) | CellSignaling, Frankfurt-Main, DE |
| AB: goat vs. E-cadherin (#AF748) - | R&D Systems, Wiesbaden-Nordenstadt, DE |
| AB: rabbit Anti-Goat IG/Biotinylated # E 0466 - | Dako, Hamburg, DE |

| | |
|---|---|
| AB: sheep vs. mouse HRP (#NA931V) | GE Healthcare, Munich, DE) |
| Acrylamide, 30% | Bio-Rad, Munich, DE |
| Agarose | Invitrogen, Karlsruhe, DE |
| Ammonium persulfate, 10% | Bio-Rad, Munich, DE |
| Bichinonic acid | Sigma-Aldrich, Deisenhofen, DE |
| BrdU | Roche, Mannheim, DE |
| Bromphenol blue | Serva, Heidelberg, DE |
| BSA | Roth, Karlsruhe, DE |
| Calcium chloride | Merck, Darmstadt, DE |
| Collagenase II | Biochrom, Berlin, DE |
| $CuSO_4$ | Sigma-Aldrich, Deisenhofen, DE |
| DEPC | Sigma-Aldrich, Deisenhofen, DE |
| DMEM/F12 cell culture medium | PAA Laboratories, Pasching, AT |
| DMEM/F12+10%FCS+P/S | PAA Laboratories, Pasching, AT |
| DNA Rehydration Solution | Promega, Mannheim, DE |
| DNase I Amp Grade, 1U/ µl | Invitrogen, Karlsruhe, DE |
| DNase I reaction buffer, 10x | Invitrogen, Karlsruhe, DE |
| DNase I, Amplification Grade | Invitrogen, Karlsruhe, DE |
| dNTPs (DATP, dTTP, dCTP, dGTP) | MBI Fermentas, St. Leon-Rot, DE |
| DTT, 0.1 M | Invitrogen, Karlsruhe, DE |
| ECL solution (#34076; #34077) | Thermo Scientific, Rockford, IL, US |
| EDTA | VWR International, Darmstadt, DE |
| EDTA solution, 25 mM | Invitrogen, Karlsruhe, DE |
| Enzyme mix | Roche, Mannheim, DE |
| Eosin Y | Sigma, St.Louis, MO, US |

| | |
|---|---|
| Ethanol | Merck, Darmstadt, DE |
| Ethanol 99% with 2-Butanon 1% | HEMA GmbH, Nurnberg, DE |
| Ethidium bromide | Roth, Karlsruhe, DE |
| FCS (fetal calf serum) | PAA Laboratories, Pasching, AT |
| Glacial acetic acid | Roth, Karlsruhe, DE |
| Glycine Merck | Darmstadt, DE |
| HCl | Merck, Darmstadt, DE |
| $H_2O_2$ | Roth, Karlsruhe, DE |
| Hematoxylin | Medite, Burgdorf, DE |
| Hematoxylin solution according to Mayer | Sigma-Aldrich, Deisenhofen, DE |
| Hot-start Taq, 5 U/ µl | Quiagen, Hilden, DE |
| Instant skimmed milk powder | Roth, Karlsruhe, DE |
| Isopropanol p.a. | Merck, Darmstadt, DE |
| Isopropanol, technisch | Roth, Karlsruhe, DE |
| KCl | Roth, Karlsruhe, DE |
| $KH_2PO_4$ | Merck, Darmstadt, DE |
| Loading dye (6x) | MBI Fermentas, St. Leon-Rot, DE |
| Methanol | Merck, Darmstadt, DE |
| $MgCl_2$, 25 mM | Invitrogen, Karlsruhe, DE |
| $MgCl_2$, 25mM | Qiagen, Hilden, DE |
| Molecular weight marker | MBI Fermentas, St. Leon-Rot, DE |
| $Na_2HPO_4$ | Merck, Darmstadt, DE |
| NaCl | Roth, Karlsruhe, DE |
| NaOH | Roth, Karlsruhe, DE |
| Nuclei Lysis Solution | Promega, Mannheim, DE |

| | |
|---|---|
| PageRulerTMPrestained Protein Ladder | MBI Fermentas, St. Leon-Rot, DE |
| Paraformaldehyde | Sigma-Aldrich, Deisenhofen, DE |
| PCR buffer with MgCl2, 10x | Roche, Mannheim, DE |
| PCR buffer, 10x | Qiagen, Hilden, DE |
| Pertex (mounting medium) | Medite, Burgdorf, DE |
| Protein Precipitation Solution | Promega, Mannheim, DE |
| Proteinase K | Roche, Mannheim, DE |
| pUC mix molecular weight marker | MBI Fermentas, St. Leon-Rot, DE |
| Q-Solution | Qiagen, Hilden, DE |
| Rabbit serum | PromoCell, Heidelberg, DE |
| Random hexamer primer | Invitrogen, Karlsruhe, DE |
| Restore Western Blot Stripping Buffer | Thermo Scientific, Rockford, US |
| RNAlater® | Ambion (Invitrogen), Darmstadt, DE |
| Rnase | Roche, Mannheim, DE |
| RNase H | Invitrogen, Karlsruhe, DE |
| RNaseOUT, 40 U/ µl | Invitrogen, Karlsruhe, DE |
| RNeasy Mini kit | Quiagen, Hilden, DE |
| Rothihistol | Roth, Karlsruhe, DE |
| Roti®-Histol | Roth, Karlsruhe, DE |
| RT buffer, 10x | Invitrogen, Karlsruhe, DE |
| SDS | Merck, Darmstadt, DE |
| Sigma Fast DAB Tablet Set | Sigma-Aldrich, Taufkirchen, DE |
| Sodium citrate | Merck, Darmstadt , DE |
| SuperScript First Strand cDNA Synthesis | Invitrogen, Karlsruhe, DE |
| Superscript III RT, 200 U/ µl | Invitrogen, Karlsruhe, DE |

| | |
|---|---|
| SuperScript™ First-Strand Kit | Invitrogen, Karlsruhe, DE |
| SYBR® Green | Lonza, Basel, CH |
| Taq DNA polymeras Kit | Quiagen, Hilden, DE |
| Taq Polymerase, 5U/µl | Quiagen, Hilden, DE |
| Temed | Bio-Rad, Munich, DE |
| Tris | Roth, Karlsruhe, DE |
| Trypsin-EDTA | PAA Laboratories, Pasching, AT |
| Tween®20 | Sigma-Aldrich, Deisenhofen, DE |
| Vectastain Elite ABC Kit - (distributed by) | AXXORA GmbH, Lörrach, DE |
| Washing buffer | Roche, Mannheim, DE |

### 15.4. Drugs (used on animals)

| | |
|---|---|
| 0.9% NaCl solution | B.Braun, Melsungen, DE |
| DEN (N-Nitrosodiethylamine) | Sigma, St.Louis, MC, US |
| Dexamethasone | Sigma-Aldrich, Deisenhofen, DE |
| Diethyl ether | Carl Roth GmbH, Karlsruhe, DE |
| Histoacryl® liquid skin glue | B.Braun, Melsungen, DE |
| Black ink | Pelikan, Hannover, DE |

### 15.5. Microarray technology

Agilent Microarray - Agilent Technologies, Inc., Santa Clara, CA, US

### 15.6. Software

| | |
|---|---|
| GraphPad 4.0 software | GraphPad, LaJolla, CA, US |
| MicrosoftOffice2007 | Microsoft, Redmond, WA, US |
| OpenOffice - | Apache Software Foundation, Forest Hill, MD, US |
| SAS software | SAS Institute Inc., Cary, NC, US |
| Spectrum™ management system | Aperio Technologies, Vista, CA, US |

## 15.7. Animals

B6.129-Cdh1tm2Kem/J (Stock Number: 005319) Charles River, Sulzfeld, DE

B6.Cg-Tg(Alb-cre)21Mgn/J (Stock Number: 003574) CharlesRiver, Sulzfeld, DE

# IV. RESULTS

## 1. Spontaneous phenotype of *Cdh1*/Alb-Cre mice

Mice with liver-specific *Cdh1* deficiency will be referred to as "L-*Cdh1*$^{del/del}$", while littermates with floxed, but not deleted *Cdh1*, will be referred to as "L-Control" (=can be considered wildtype mice for practical purposes).

### 1.1. Generation of *Cdh1* deficient mice

Breeding of mice was successful, and offspring proved to be fertile. It was possible to keep the strain stable at a defined genetic distribution (both L-*Cdh1*$^{del/del}$ and L-Control in every generation). Assuming that presence of Cre does not influence embryonic and early postnatal development, every generation should have had approximately the same number of L-*Cdh1*$^{del/del}$ and L-Control mice. This was the case, as in the three years during the experiments a total of 147 L-*Cdh1*$^{del/del}$ and 122 L-Control was born. By a small margin, this is within the expected range of 118.43 to 150.57 animals of L-*Cdh1*$^{del/del}$ genotype (tested with 95% confidence interval).

### 1.2. Demonstration of cre-mediated gene deletion

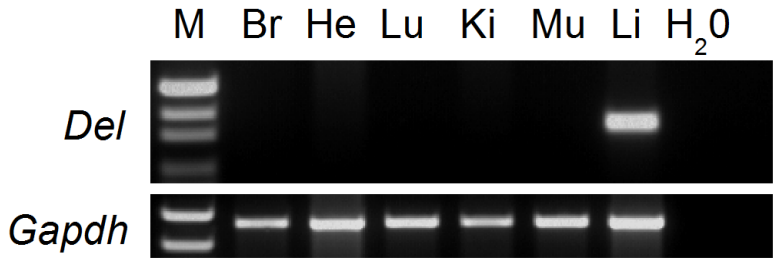


*Fig. 14. Demonstration of cre-mediated gene deletion by PCR ("M"/marker, "Br"/brain, "He"/heart, "Lu"/lung, "Ki"/kidney, "Mu"/muscle, "Li"/liver, "$H_2O$"/negative control). DNA samples isolated from L-Cdh1$^{del/del}$ mouse organs can only be amplified if a gene deletion event has occurred (Del) in the respective organ. Gapdh serves as a positive control.*

Organs (brain, heart, lung, kidney, muscle, liver) were collected from L-*Cdh1*$^{del/del}$ mice and genomic DNA was extracted. Forward- and reverse-primers for PCR were placed far too distant to each other to allow amplification of DNA unless a sequence between them (the floxed part of *Cdh1*) was missing. Marker ("M", to

the left) served as verification of correct band length. As expected, a range of organs did not show amplification of DNA, and only liver ("Li", to the right) did (Fig. 14). $H_2O$ served as negative control. Deletion of *Cdh1* could thus only be detected in the liver, and all other organs remained unaffected.

### 1.3. General assessment of the „spontaneous phenotype" of *L-Cdh1*$^{del/del}$ compared to L-Control mice

Reduction of E-cadherin in the liver had no detectable effect on mouse viability. *L-Cdh1*$^{del/del}$ mice had slightly reduced body weight gain compared to L-Control (see "Long term body weight monitoring", page 61). Neither *L-Cdh1*$^{del/del}$ nor L-Control showed body or organ abnormalities both alive and during necropsies, and generally mice remained healthy until aged well over two years. No significantly increased incidence of tumors could be observed in the genetically altered mice, with only a single spontaneous liver tumor noticed within the whole *L-Cdh1*$^{del/del}$ population in our animal facility over the course of three years.

### 1.4. Expression analysis on protein level

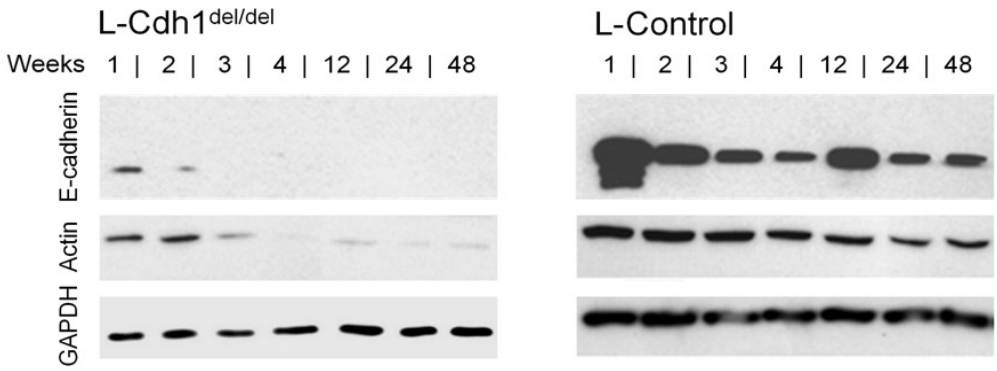


*Fig. 15. Protein expression in L-Cdh1*$^{del/del}$ *and L-control mice liver samples shown by Western blot at different ages (1 week to 48 weeks). Both E-cadherin and actin were reduced in L-Cdh1*$^{del/del}$*. GAPDH serves as loading control.*

To confirm the loss of *Cdh1* in L-*Cdh1*$^{del/del}$ mice, we performed Western blot analysis of the liver from mice at ages 1 to 48 weeks (Fig. 15). E-cadherin in the liver was markedly reduced in L-*Cdh1*$^{del/del}$ at 1 week and was further decreased in older mice. Actin behaved similarly. GAPDH served as a loading control.

### 1.5. Expression analysis on RNA level: Agilent Array

To systematically search for genes differentially regulated by the loss of *Cdh1* in the liver, RNA samples from mice groups aged 1 week, 3 weeks and 6 weeks were analysed using an Agilent array. In 1 week old mice some genes were already regulated, but the effect of the *Cdh1* ablation was still weak (e.g. no statistically significant regulation of any RNA). In 3 week old mice a great number of genes was significantly regulated. At 6 weeks, only a select few genes were still regulated significantly (Fig. 16).

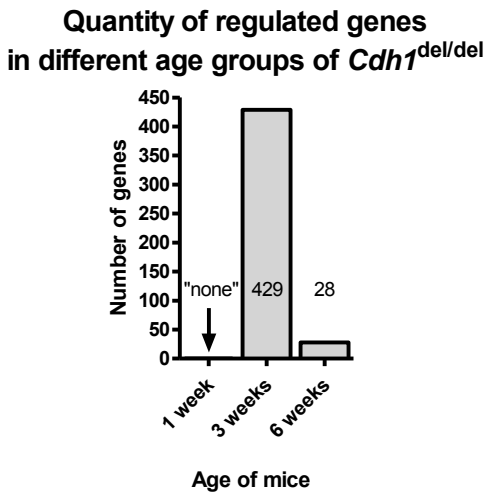


*Fig. 16. Total number of genes with significantly regulated RNA expression in mouse liver according to Agilent arrays in mice aged 1 week (zero genes regulated), 3 weeks (429 genes regulated) and 6 weeks (28 genes regulated).*

Most of the genes remaining regulated at 6 weeks of age showed stronger regulation at 6 weeks than it had been at 3 weeks (Fig. 17).

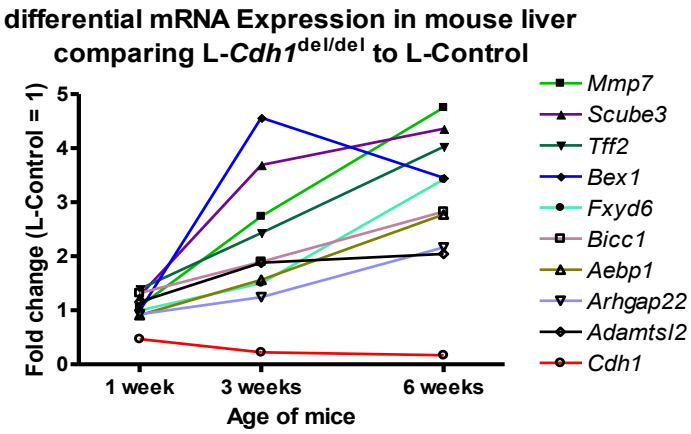


*Fig. 17. RNA expression of 10 of the most noticeable genes (which still remained significantly regulated in the oldest age group and were regulated the strongest) shown at 1 week, 3 weeks and 6 weeks.*

### 1.6. Expression analysis on RNA level: qRT-PCR

To verify the results of the Agilent arrays, qRT-PCR was done. RNA expression of 10 selected genes (the same genes as shown in Fig. 17) was quantified and fold change between L-*Cdh1*$^{del/del}$ and L-Control was determined.

Regulation of the genes shown to be regulated the strongest (and significantly) at 6 weeks of age could be verified by qRT-PCR (Fig. 18), and the results were mostly congruent with the results of the Agilent arrays. The fold change determined in qRT-PCR was consistently stronger than in the arrays for all genes. Only minor alterations occurred in qRT-PCR compared to the arrays concerning the "ranking" of the genes regulated the strongest.

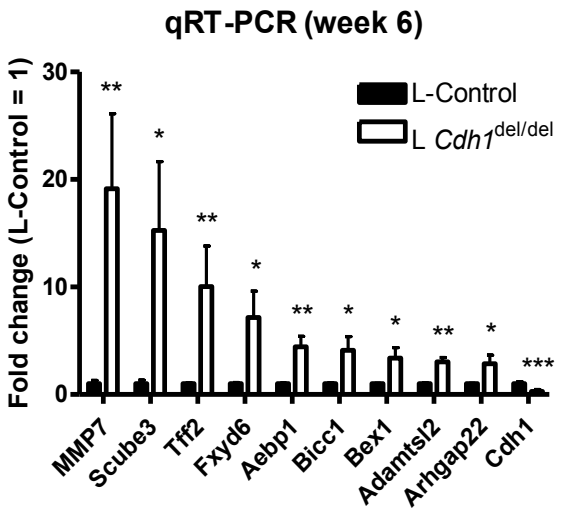


*Fig. 18. RNA expression in mouse liver according to qRT-PCR at an age of 6 weeks.*

## 1.7. Body and organ weight

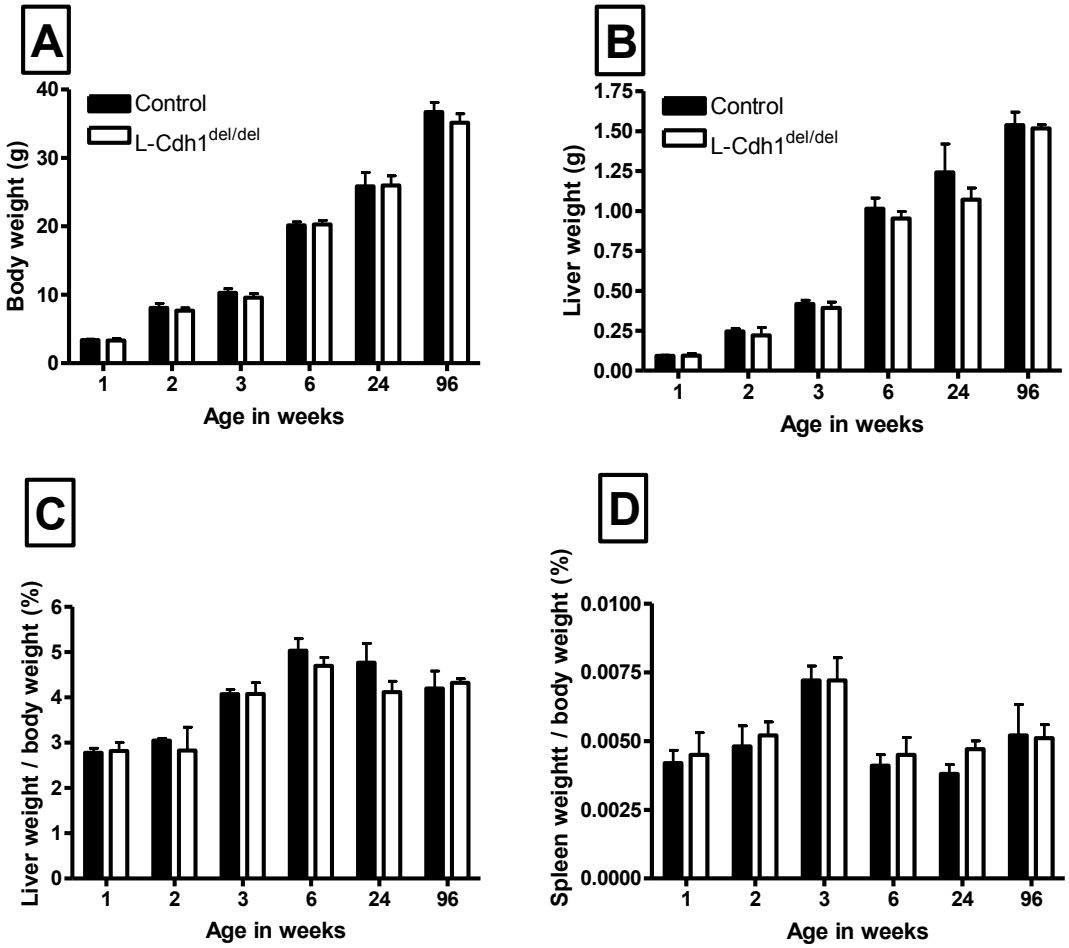


*Fig. 19. Body weight (A), liver weight (B) and calculated relative liver weight (C) of L-Cdh1$^{del/del}$ and L-Control mice at the indicated ages and measured during necropsy. Week 1: n=9+5; week 2: n=3+3; week 3: n=6+7; week 6: n=5+5; week 24: n=3+4; week 96; n=2+2*

*Relative spleen weight (D) was not recorded from all animals at week 1 and 2. Week 1: n=3+3; week 2: n=3+2*

To investigate whether liver specific loss of E-cadherin affected body weight and organ growth, groups of mice at different ages and of both genders were sacrificed and body and organ weights (liver, spleen) were measured (Fig. 19).

## 1.8. Serum parameters

To search for possible effects in blood or serum caused by liver damage due to the

loss of E-cadherin, blood was drawn from the retrobulbar venous plexus and analysed.

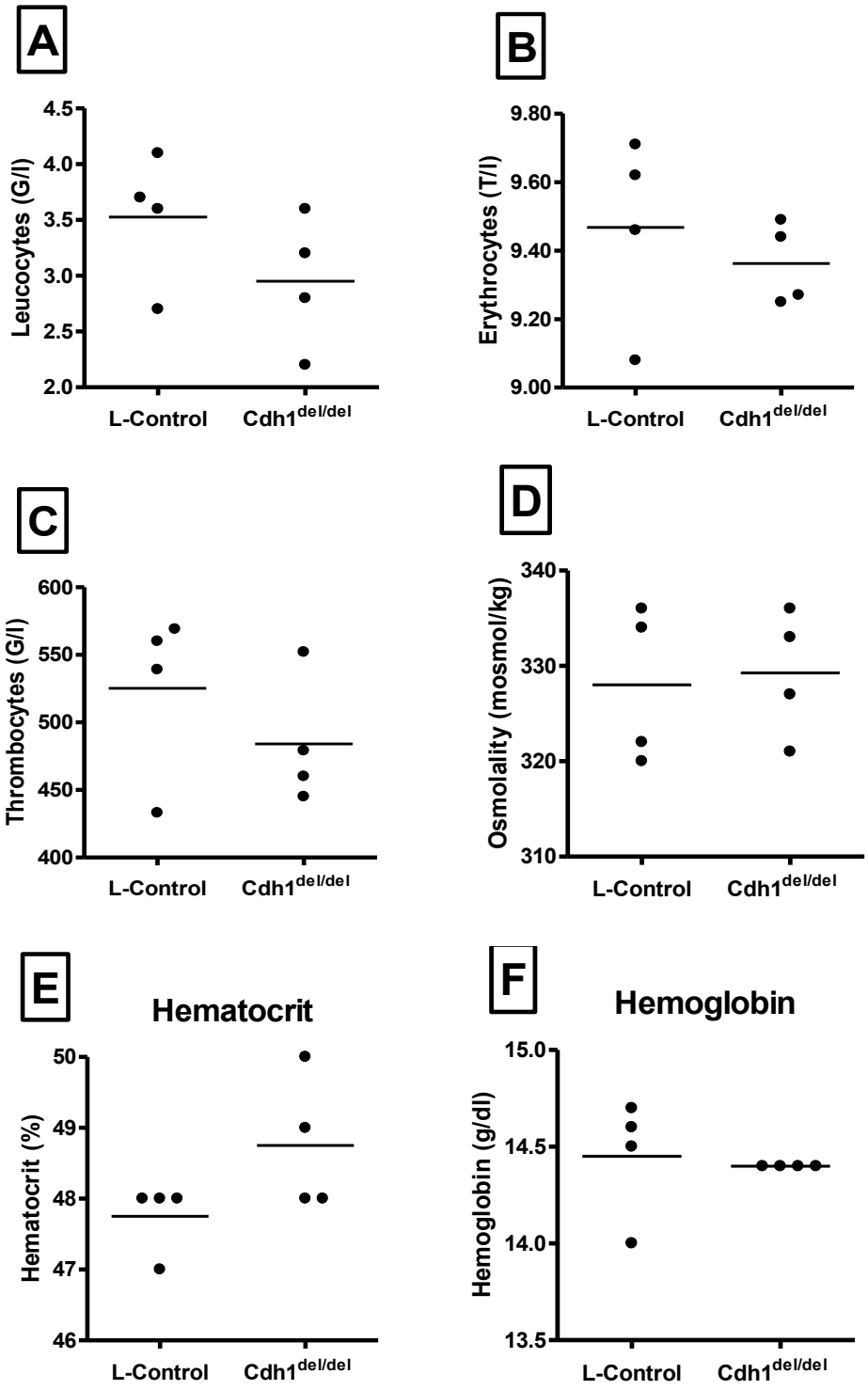


*Fig. 20. Blood and serum parameters of 3 months old male mice, n=4+4 in all graphs (A-F).*

No significant differences between L-Control and L-*Cdh1*$^{del/del}$ could be observed concerning several serum parameters (Fig. 20).

Some of the above serum parameters (plus LDH, Albumin, GPT, Bilirubin) were also analyzed for 6 months old females (n = 3 + 4) and 1 year old females (n = 3 + 4). A trend separating L-Control and L-*Cdh1*$^{del/del}$ was neither observed for single age groups nor for all age groups combined concerning any blood parameter (data not shown).

### 1.9. Long-term body weight monitoring

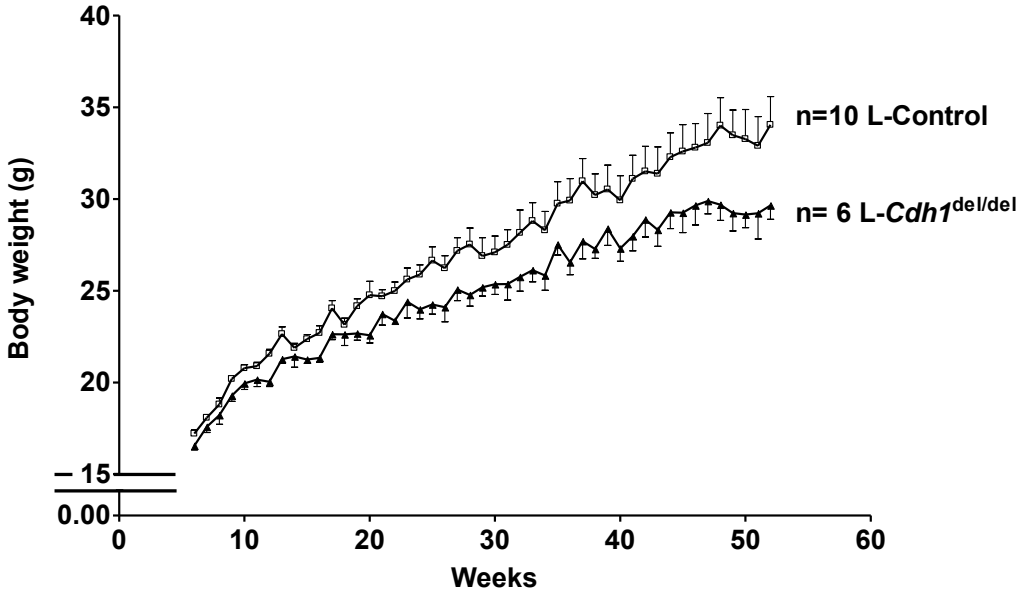


*Fig. 21. Long-term weight development of L-Cdh1$^{del/del}$ and L-Control mice (weekly measurement). Both groups were composed of female mice only.*

Mice were weighted weekly over the course of more than a year. A slight retardation of weight gain could be seen in L-*Cdh1*$^{del/del}$ and 2-factorial ANOVA yielded Pr>F of 0.0175 for comparison of L-*Cdh1*$^{del/del}$ versus L-Control (Fig. 21).

### 1.10. Histology of L-*Cdh1*$^{del/del}$ vs. L-Control liver

Previous mouse models of E-cadherin loss often showed pronounced histological alterations (BOUSSADIA et al., 2002; SCHNEIDER et al., 2010). We therefore analysed formaldehyde fixated livers histologically.

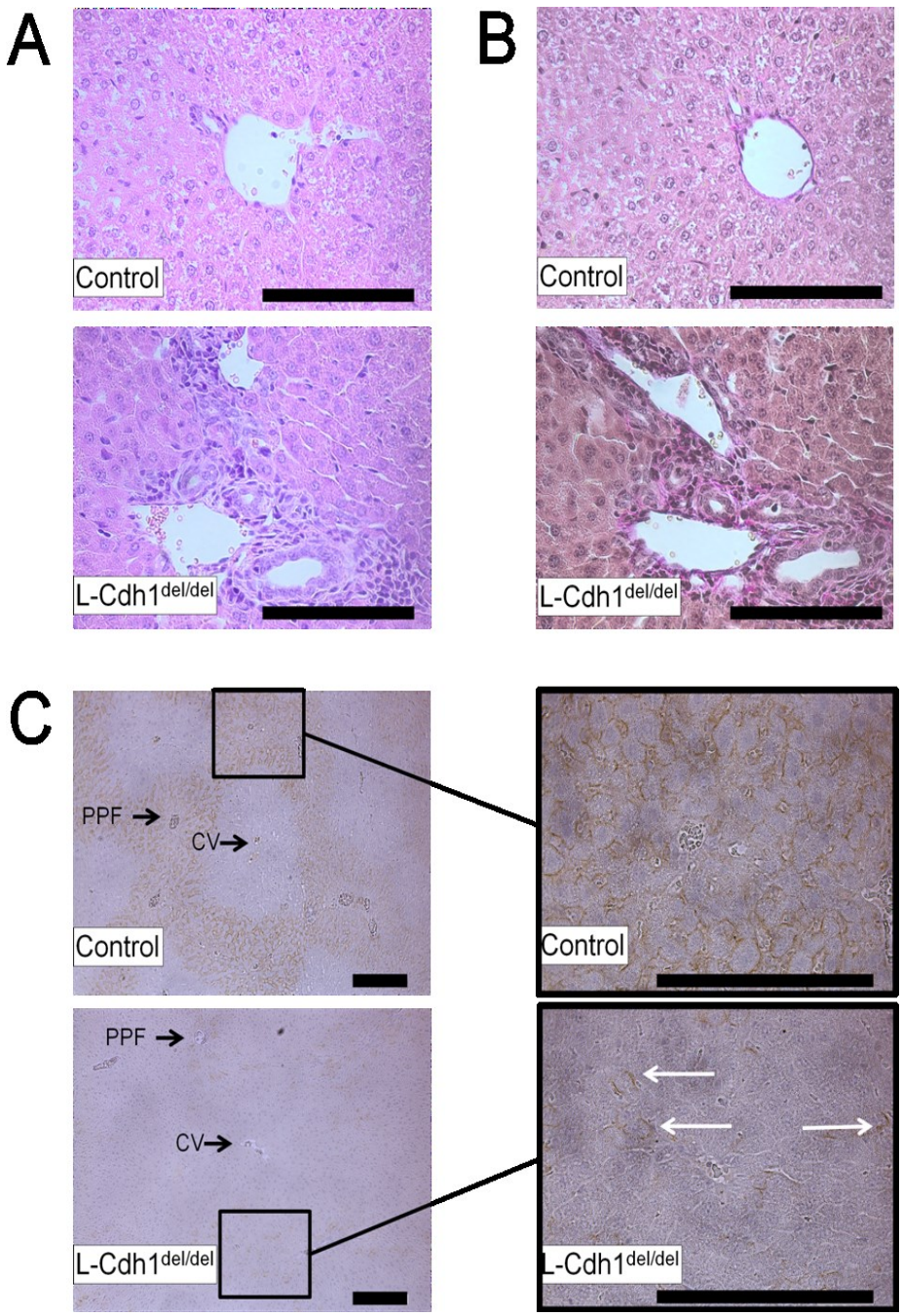


*Fig. 22. Histological changes in the liver of L-Cdh1$^{del/del}$ mice. (A) H&E and (B) Elastica van Giesson staining of livers slides showing a periportal field (age of mice: 5 weeks). L-Cdh1$^{del/del}$ mice are characterized by alterations around the small bile ducts. (C) Immunohistochemistry showing E-Cadherin localization in mouse liver (age: 6 weeks). Control mice display normal distribution of the*

*protein, with small amounts around the central vein (CV) and higher amounts around the periportal fields (PPF). In L-Cdh1$^{del/del}$, E-Cadherin is considerably reduced, but residues remain (white arrows). Black bars on all slides: 200 µm.*

Histologically, L-*Cdh1*$^{del/del}$ livers differed from those of L-Control mice. Alterations of periportal fields consisting of cell proliferation mostly around the small bile ducts (part of the Glisson-Trias) could be seen in H&E-stained liver sections of L-*Cdh1*$^{del/del}$ (Fig. 22 A). The alterations were even moreeasy to notice when EvG staining was used (Fig. 22 B). Collagen (stained red) accumulation seemed to be a component of the lesions. The alterations appeared periodically in mice of all ages with certain fluctuations. While Fig. 22 shows alterations of a periportal field typical for L-*Cdh1*$^{del/del}$, not all periportal fields of L-*Cdh1*$^{del/del}$ mice had alterations of that intensity.

There seemed to be a trend toward more pronounced alterations in younger mice (aged around 5 weeks), but this could not be proven definitively.

Immunohistochemistry confirmed the loss of E-cadherin in L-*Cdh1*$^{del/del}$ livers (Fig. 22 C).

## 2. Phenotype after tumor induction

The carcinogen DEN was injected in groups of male mice at an age of 2 weeks to induce HCC.

### 2.1. General assessment of the „phenotype after tumor-induction“ of L-*Cdh*1$^{del/del}$ compared to L-Control mice

All mice, regardless of their genotype, developed tumors after injection of DEN. Eight months after tumor induction, tumors were always visible on the liver surface to the naked eye. If mice were kept longer than 8 months, they sooner or later fell ill (e.g., 100% of mice developed tumors) and had to be euthanized, if symptoms were so pronounced that they fulfilled the termination criteria. The typical symptoms of progressed stages of liver tumor included:

- an enlarged mass in the abdomen (visible and/or palpable)
- decreased activity of the mouse
- in some cases icterus (visible on ears and pads)

During necropsies, alterations of the liver were frequently obvious (Fig. 23 A). Occasionally, tumorous alterations of the lung were noticed (Fig. 23 B). Histological examination revealed most of those not to be metastases but primary lung tumors (being identified mostly as adenocarcinoma) induced by DEN away from the main site of pharmacological action (Fig. 24). Concerning lung tumors or metastases, no significant difference between L-*Cdh1*$^{del/del}$ and L-Control was detected (data not shown). At necropsy, no visible tumors could be detected in other internal organs or the carcass.

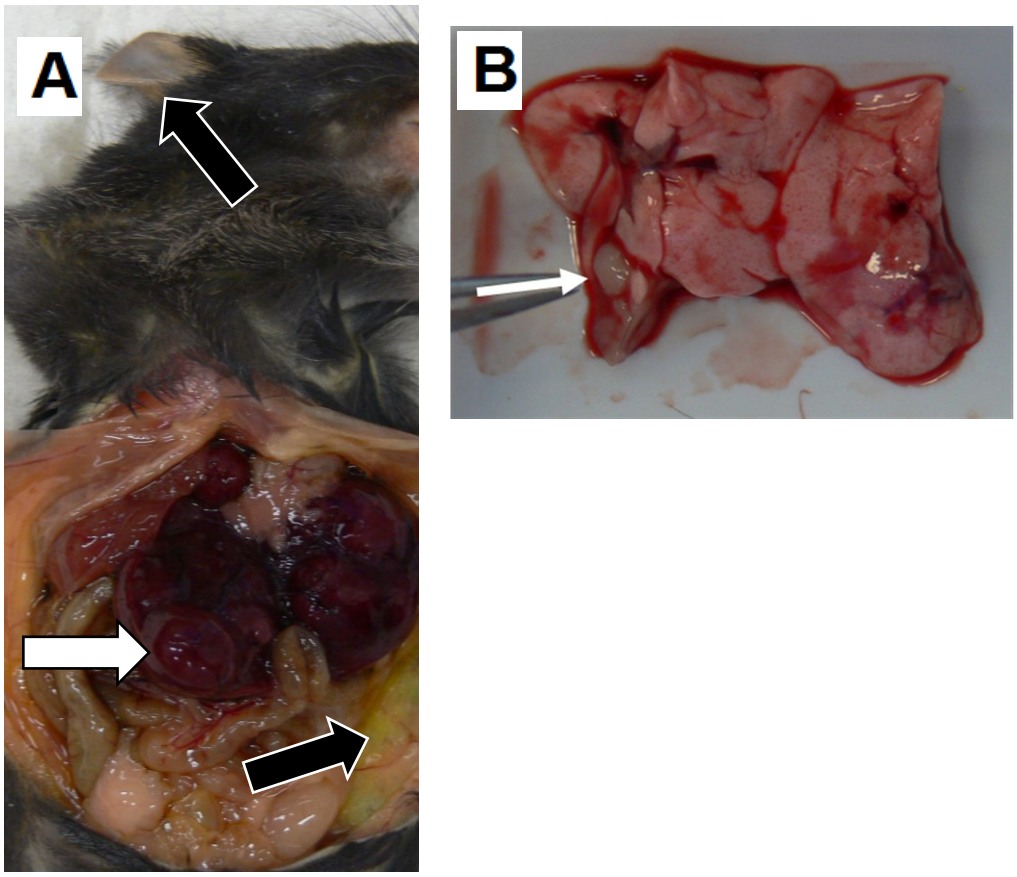


*Fig 23. Necropsy of mouse with tumors.*

*(A) Icteric mouse. Pronounced tumorous alterations of the liver (white arrow) as well as a yellowish touch on the hairless outer skin and in the opened situs (black arrows) can be seen.*

*(B) Conspicuous alteration of the lung (thin white arrow) during necropsy. A subsequent histological examination did not detect liver metastases, the alteration probably being caused by a primary lung tumor instead (adenocarcinoma).*

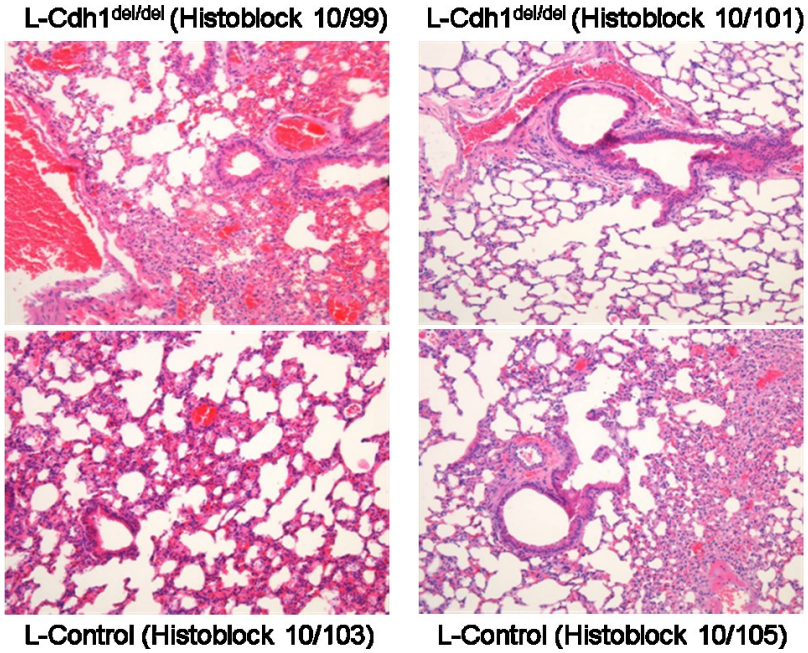


*Fig. 24: Histology of several tumors and/or secondary alterations of the lung. The alterations are most probably primary tumors of the lung and not metastases originating in the liver.*

## 2.2. Histological nature of DEN-induced tumors in the liver

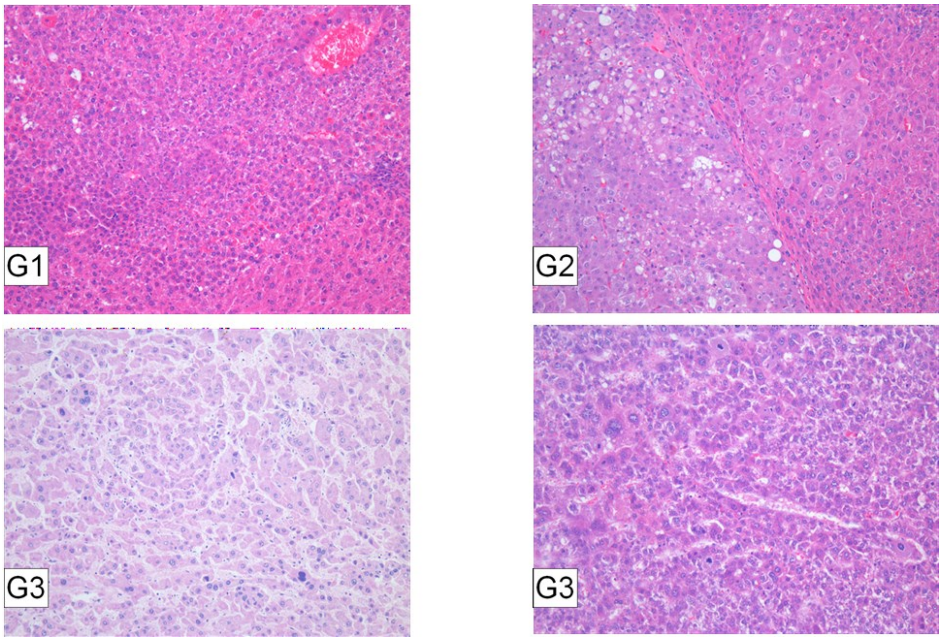


*Fig. 25. A variety of HCC forms could be induced in livers of both L-Cdh1$^{del/del}$ and L-Control mice by DEN. Cancer staging was done, and tumors reaching G3 could be found in both genotypes. G4 was diagnosed only a single time in a Cdh1$^{del/de}$.*

Histological examination confirmed that the tumors in the liver were chiefly HCCs. Often, a multitude of isolated different tumor nodes could be seen and the HCCs had a broad spectrum of (loss of) differentiation. Stages G1 to G3 could be found in mice of the 8 month group and older (Fig. 25), while in mice 4 month after DEN only dysplastic foci were found.

### 2.3. Body and organ weight

Organs of mice were weighted during necropsy four months (Fig. 26), eight months (Fig. 27) and twelve months (Fig. 28) after induction of tumors with DEN.

**Four months after DEN:**

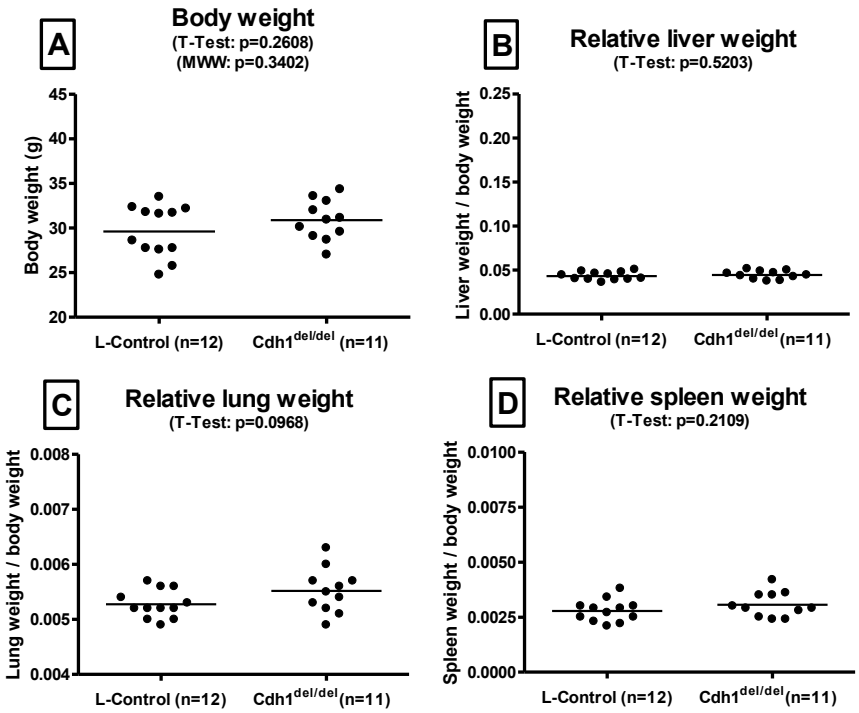


*Fig 26. Body weight (A) and relative weight of liver (B), lung (C) and spleen (D) during necropsy of mice 4 months after tumor induction by DEN.*

**Eight months after DEN:**

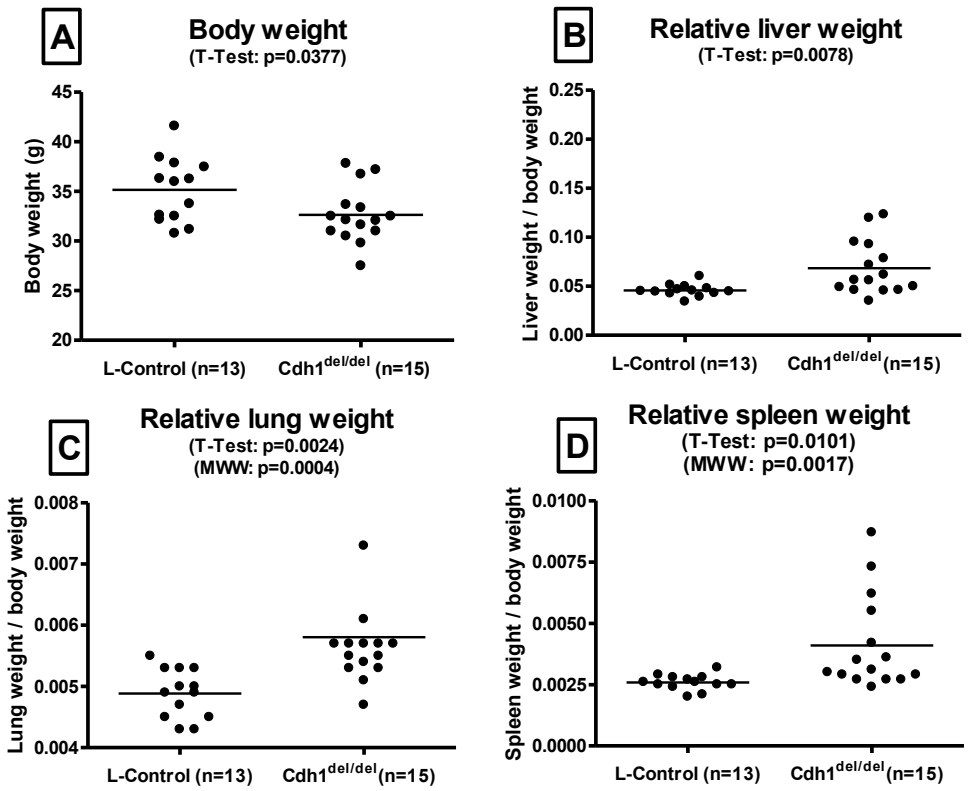


*Fig 27. Body weight (A) and relative weight of liver (B), lung (C) and spleen (D) during necropsy of mice 8 months after tumor induction by DEN.*

**Twelve months after DEN (some mice euthanized prematurely):**

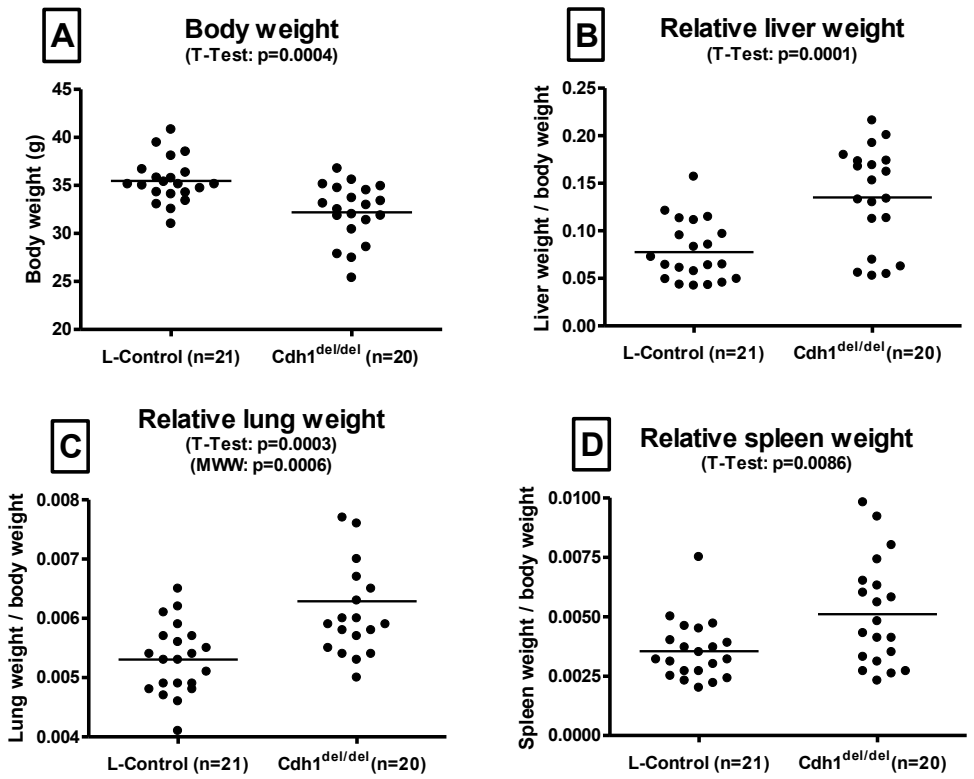


*Fig 28. Body weight (A) and relative weight of liver (B), lung (C) and spleen (D) during necropsy of mice 12 months after tumor induction by DEN. Some mice were euthanized up to 4 months before reaching that age.*

Data was analyzed with unpaired, two-tailed t-test and, if normal distribution could not be assumed, additionally with Mann-Whitney-U test (Mann-Whitney-Wilcoxon = MWW). The differences between L-*Cdh1*$^{del/del}$ vs. L-Control liver, lung and spleen weights were significant (or even highly significant) at 8 and 12 month after DEN injection, while at 4 month there was a trend but not a significant difference yet.

Lung and spleen weight were never intended as primary objectives (primary variables) in this experiment. The main purpose of showing these records is to reinforce the data on the liver alterations (caused by HCC), since increased spleen weight correlates with HCC (OH et al., 2003) and alterations of the lung are also associated with liver failure (MACHICAO & FALLON, 2012). Since the variables are corroborating the single primary objective by being interdependent with it, instead of being primary objectives themselves, adjustment of confidence interval by Bonferroni correction might be excessive. Nonetheless, if Bonferroni

or Bonferroni-Holm correction (VICTOR et al., 2010) is applied to adjust confidence intervals (for 3 hypotheses: relative liver, lung and spleen weight), the data remains significant for all relative organ weights of the 8 and 12 months age groups.

## 2.4. Macroscopical tumor count

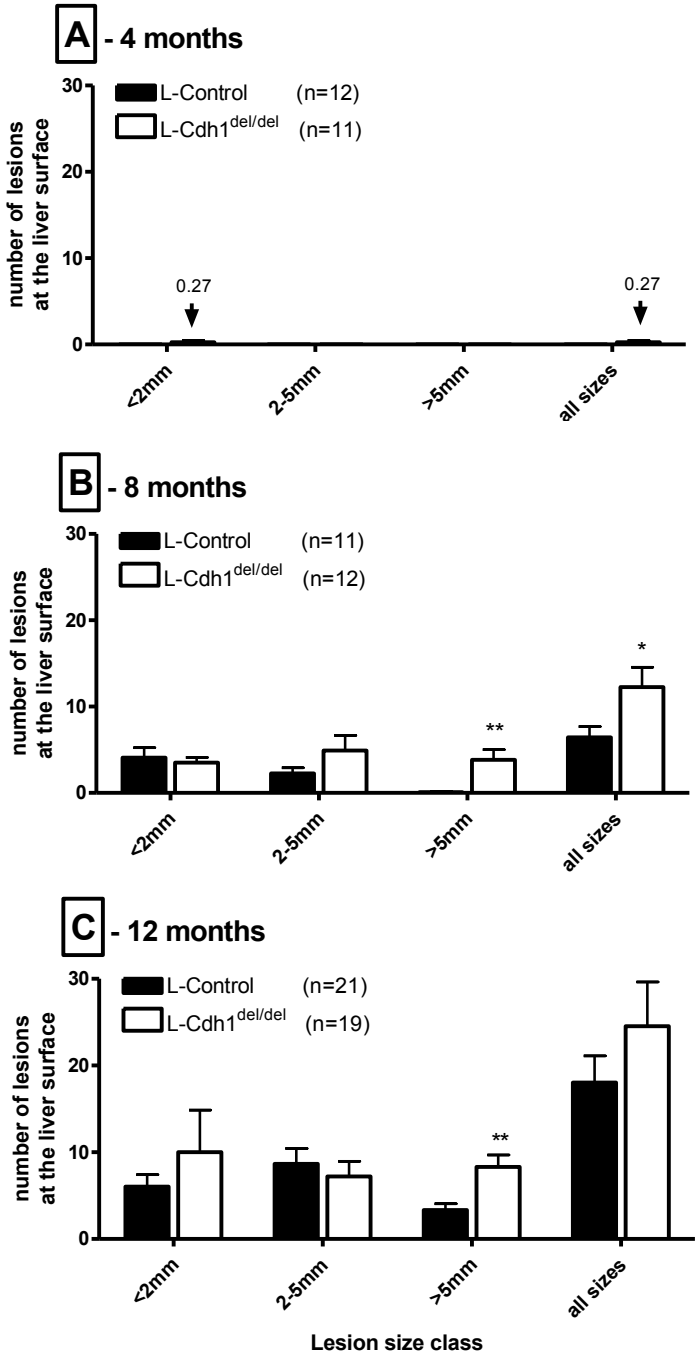


*Fig. 29. Lesions counted on the liver surface at 4 (A), 8 (B) or 12 (C) month after DEN injection. Some mice of the oldest group (C) were euthanized before reaching the planned age.*

Tumor lesions visible on the liver surface were categorized into three size classes and counted (Fig. 29). Groups of 4 and 8 months had normal distribution and t-test was used. The 12 months group did not show normal distribution and Mann-Whitney-U test was applied.

In all age groups, the total lesion number is increased in L-*Cdh1*$^{del/del}$ mice compared to L-Control animals, and especially lesions > 5 mm were eye-catching in the 8 and 12 month groups.

## 2.5. Histological tumor count

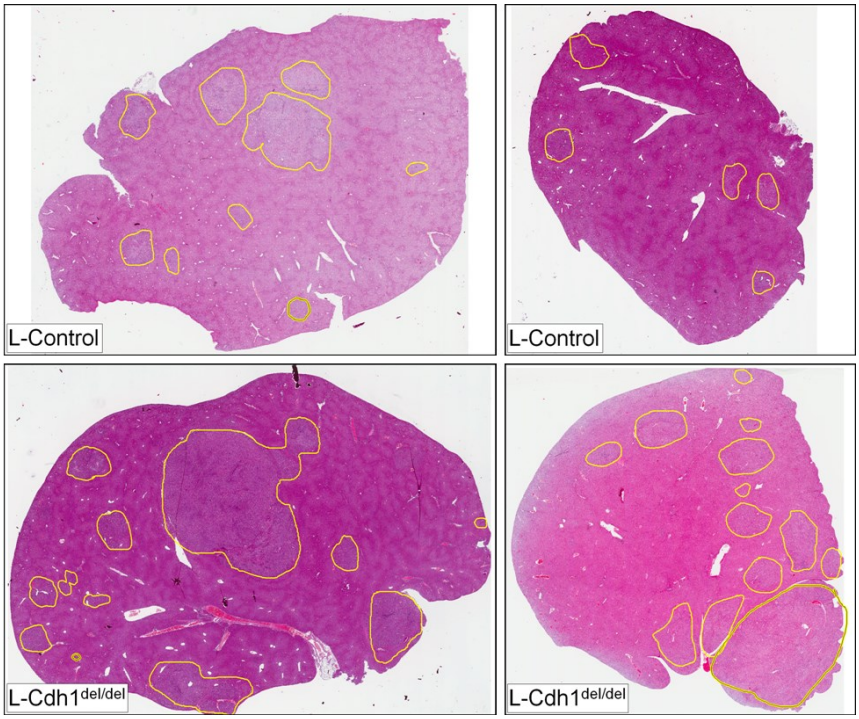


*Fig. 30. Tumorous alterations on liver slides (circled with yellow line).*

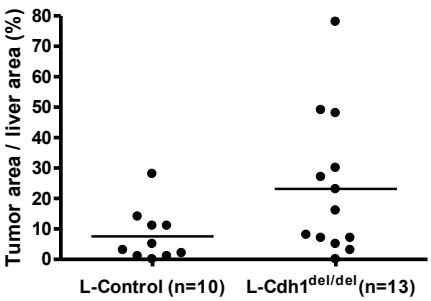


*Fig. 31. Histologic evaluation of liver tumors. Mean tumor area on liver slides of L-Cdh1$^{del/del}$ and L-Control eight months after tumor induction by DEN.*

Histologically, L-*Cdh1*$^{del/del}$ livers showed larger and/or more tumors on slides (Fig. 30). A statistical evaluation showed striking, but not significant

differences (Fig. 31). Mann-Whitney-Wilcoxon test ($p = 0.0628$) was used to evaluate significance (because normal distribution could not be ascertained).

### 2.6. Survival curve

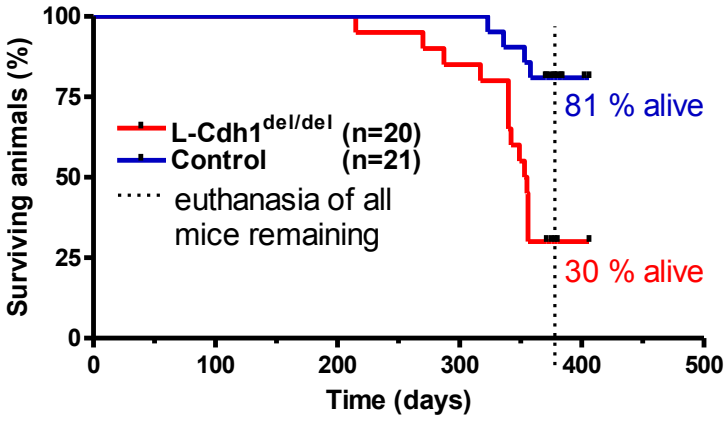


*Fig. 32. Kaplan-Meier plot of mice originally scheduled for necropsy 12 months after DEN injection.*

If kept longer than 8 months after DEN injection, mortality was higher in L-*Cdh1*$^{del/del}$ compared to L-Control (Fig. 32). Mice were euthanized if the termination criteria as specified for this experiment were reached. At 12 months, all remaining mice were euthanized as well. At that point, 81% of L-Control mice were still alive, compared to only 30% L-*Cdh1*$^{del/del}$.

## 3. Establishment of primary cells from tumors

For use in future projects aiming at investigating the molecular mechanisms underlying the function of E-cadherin in liver tumorigenesis, we generated permanent cell lines. Tumors were harvested from L-*Cdh1*$^{del/del}$ and L-control mice 12 months after chemically inducing the tumors with DEN.

Cell cultures isolated from animal tissue can be kept alive for some time and cells can proliferate even if they stem from normal, unaltered tissue. Normal mammalian cells have a limited capability to proliferate, though. At some point they will stop dividing and the cells will stagnate or die. In contrast, we isolated cells from tumors in the hope that permanent cell lines could be created. To prove that permanent cell lines were established, we split the cultures more often than it should have been possible with “normal” mouse cells. We could maintain some

cell cultures for several months with weekly or biweekly splitting, resulting in 20 or more successful passages. The cells therefore had surpassed the hayflick limit (number of possible cell divisions) of about 8 to 11 expected for typical adult mouse cells (ROHME, 1981). After it was clear that the cell morphology remained stable and homogenous, it was assumed that permanent and mostly single-clone cell lines had been achieved.

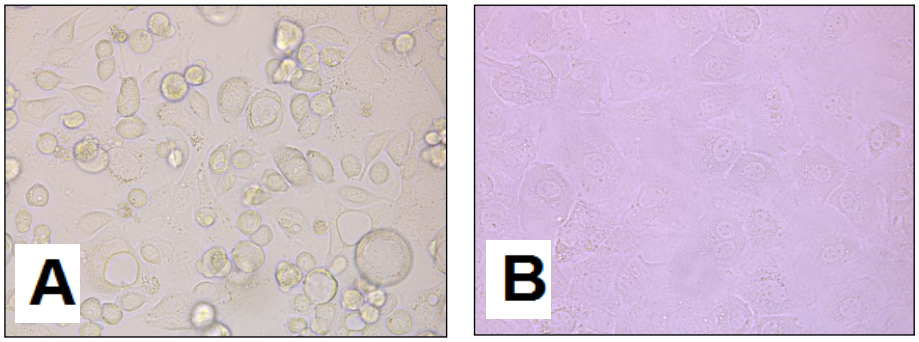


*Fig. 33: cell lines which turned out to have long-term split potential. (A) Cell line "145" was derived from a L-Cdh1$^{del/del}$ mouse (photo 3 months after tumor harvest). (B) Cell line "118" was derived from L-Control mouse (photo: 5 months after tumor harvest), cells at that time still showed a somewhat heterogeneous outlook. The names of the cell lines were derived from the respective donor mouse`s number.*

Around a dozen lines could be kept for an extended period of time (some were subclones of each other). With some lines it was unclear whether they had already surpassed the hayflick limit or not, and some were contaminated with fibroblasts. One line derived from L-*Cdh1*$^{del/del}$ and one line derived from L-Control were chosen for prolonged cultivation and permanent storage: cell lines "118" and "145" (Fig. 33).

"118" was kept for around 7 months (of that, at least 4 month with regular splitting twice per week with 1:4 dilutions) and "145" was kept 5 months (with at least 2 months of regular splitting twice per week with 1:4 dilutions). Afterward, the cells were frozen in liquid nitrogen for storage.

### 3.1. *Cdh1* PCR of cell culture cells

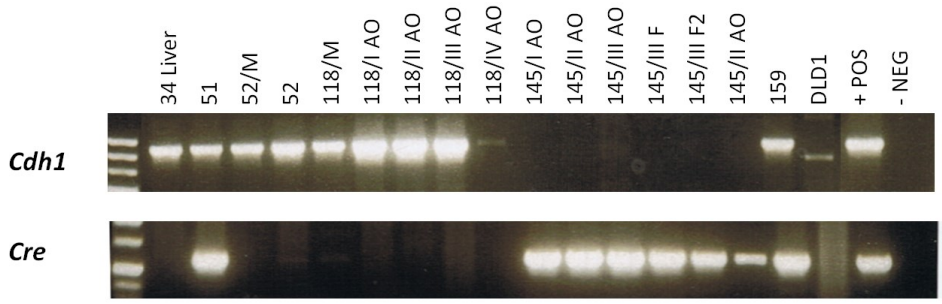


*Fig. 34. PCR of cell culture performed similar to tail clip genotyping. "34 Liver" is normal mouse liver tissue and "DLD1" is a human-derived cell culture (used to test whether non-mouse cells could give a signal in this PCR). "+POS" is a mouse L-Cdh1$^{del/del}$ tail tip and "-NEG" is bidistilled water. All other samples are from cell lines stemming from mouse liver tumors, and the number denotes the animal`s number from which they were derived. "51", "145" and "159" were L-Cdh1$^{del/del}$ mice, while "52" and "118" were L-Control mice. Presence of a Cdh1 signal does not necessarily exclude the possibility of successful knockout, but it proves that at least some of the cells in the population are derived from cells that had been unaffected by Cre in the living animal. Only type "145" cell lines (L-Cdh1$^{del/del}$) show complete loss of Cdh1 positive cells.*

PCR of cell material was done as described previously for routine genotyping of mice. The forward primer utilized had a binding site in exon 10 of *Cdh1*, which should be missing on successful Cre-mediated recombination. Therefore, loss of a signal meant that all cells in the cell population did not possess a functional *Cdh1* gene (or that either reverse or forward primer site had been altered beyond recognition due to direct DEN action or alterations caused by tumor transformation).

While the *Cdh1* PCR product was, as expected, detected in all cells derived from L-Control, it also was present in most L-*Cdh1*$^{del/del}$ cell lines (Fig. 34). Only cells of the "145" type did not show a *Cdh1* signal in the PCR.

# V. DISCUSSION

The aim of the project was to investigate E-cadherin (TAKEICHI, 1990) effect on physiology and pathophysiology of the liver. Special focus was E-cadherin effects on HCC, which is important to human medicine (JEMAL et al., 2011). To this end, we generated liver specific E-cadherin deficient mice using the Cre-Lox system (SAUER, 1987). A group of mice was injected with the chemical carcinogen DEN (LEENDERS et al., 2008).

## 1. Spontaneous phenotype

The protein E-cadherin is known to be essential in many organs. Despite this, liver-specific deletion of *Cdh1* using a cre/loxP system did not significantly affect mouse development or health, and only the impact on postnatal growth was significant (although even here the effects are modest). Single age groups occasionally showed coincidental differences between L-Control and L-*Cdh1*$^{del/del}$ organ weight, but due to the high number of age groups and parameters tested, this can be assumed to be the result of random events (VICTOR et al., 2010). No consistent trend separating control from L-*Cdh1*$^{del/del}$ liver weights was observed. Other organ weights (spleen) did not show a consistent trend separating the two groups, either. In conclusion, L-*Cdh1*$^{del/del}$ were indistinguishable from L-Control mice during necropsies and no conspicuous organ abnormalities were detected macroscopically in any genotype.

This is especially surprising since other models of organ-specific E-cadherin ablation in organs like intestine (SCHNEIDER et al., 2010) or mammary gland (BOUSSADIA et al., 2002) have had severe effects on animal health.

Three hypothesis can be formulated to explain this observation:

1) The loss of *Cdh1* is restricted to a limited number of cells (less than 100% of the tissue) in our model
2) E-cadherin is not essential for the liver under normal conditions
3) The liver has a mechanism to compensate for E-cadherin loss

Possibly a combination of the above is true.

The cre/loxP system is known to affect only a part of the cells in experimental animals. Some analyses have indicated that the percentage of affected target cells in a number of conditional knockout models does not surpass 40% (METZGER & CHAMBON, 2001). The group that originally developed the Alb-Cre mouse, which was used in our experiment, claimed a 90% efficiency of gene recombination after an onset time of around 6 weeks (POSTIC & MAGNUSON, 2000). The absolute evaluation of the recombination efficiency is difficult, because the organ contains target cells (hepatocytes in this case) and a significant amount of non-target cells (connective tissue, blood cells, endothelial cells of the blood vessels, etc.) which might or might not "contaminate" the samples with expressed protein. We could show that L-*Cdh1*$^{del/del}$ lost a considerable amount of E-cadherin in the liver, using qRT-PCR (fold change of 0.1669 for *Cdh1* RNA in 6 week old mice) and Western blot analysis (only minor amounts of E-cadherin detectable in mice older than 3 or 4 weeks). Actin, often used as a loading control in Western blot, turned out to be also decreased. There is a known relationship between the two proteins (NELSON, 2008), and the reduced actin levels demonstrated that E-cadherin loss in our model did have an effect on associated proteins. According to the manufacturer the antibody does not bind a specific type of actin, instead binding an epitope conserved in all actins. In light of this result, we employed GAPDH as a loading control.

While an absolute quantification (in %) is difficult using IHC, we could show that E-cadherin immunostaining in L-*Cdh1*$^{del/del}$ liver was reduced, albeit some E-cadherin positive cells always remained (sometimes concentrated in pockets and sometimes single).

Unlike organs in which E-cadherin has been shown to be crucial (skin, intestine), the liver might not be under a severe mechanical stress. Shear stress and other forms of physical strain are possibly lower in an organ like the liver. The liver architecture is significantly different from the epithelial layers in organs like the skin. The liver parenchyma cells are not packed tight like epidermal skin cells, for example, but are rather optimized for maximum contact with the blood flowing through the liver (BRAET & WISSE, 2012). The liver parenchyma cells are possibly less dependent on a firm connection with their surroundings, in comparison with skin or intestine cells. The fact that the liver is more vulnerable in the case of blunt trauma, compared to other internal organs (POP et al., 2012),

could hint to it having an architecture not optimized for resisting mechanical stress (and thus likely not requiring high shear stress resistance under physiological conditions).

There are several ways how the liver could compensate E-cadherin loss. Another cadherin or pseudo-cadherin could replace E-cadherin (e.g. a related protein takes over the function of E-cadherin). Albeit we could not show significant increase of any member of the cadherin family using RNA arrays, this explanation is still not too far-fetched. The cadherin family is huge and possibly not all members have been identified yet. Consequently, a few of the more exotic cadherin members might be missing on contemporary RNA arrays like the Agilent Array that we employed. Another way to compensate conditional ablation of E-Cadherin could be regeneration and replacement of damaged cells. The liver is known to be capable of impressive feats of regeneration (BOOTH et al., 2012; GENTRIC et al., 2012). Liver stem cells („oval cells“) are located closely to the periportal fields (FAUSTO & CAMPBELL, 2003), and they could replace failing hepatic cells (e.g. cells with induced recombination) quickly. Such stem cells are almost surely not affected by the recombination (the albumin promoter is probably not yet activated) until they have differentiated into functional liver parenchyma cells. The liver therefore could possibly provide a constant stream of (yet not E-cadherin deficient) replacement cells. This peculiar effect is due to the nature of the model we employed, with Cre production being dependent on an activation of regions which are probably dormant in stem cells. Other models, where Cre is produced in all cells regardless of them being stem cells or not, will likely behave different.

Both histology and RNA array results suggested that the L-*Cdh1*$^{del/del}$ knockout mice were affected the most at a young age (around 3-5 weeks). Agilent arrays showed that some genes were strongly and significantly regulated on RNA level in L-*Cdh1*$^{del/del}$ mice liver at 6 weeks, generally increasing the fold change with age. Interestingly, a large amount of genes was statistically significantly regulated at 3 weeks, while at 6 weeks only a few genes were regulated (but those were the genes regulated strongest). A qRT-PCR could confirm the strong regulation of selected genes at 6 weeks of age in L-*Cdh1*$^{del/del}$. This RNA and histology observations could point to remodeling processes, where the animal copes with the loss of E-cadherin. Afterwards, the animal has established a permanent

compensation mechanism by employing alternative proteins to E-cadherin and/or by supplying a constant stream of replacement cells for maintaining the liver parenchyma. Of course this could in some way hamper postnatal development, which would explain the weight differences between L-*Cdh1*$^{del/del}$ and L-Controls. Aside from the slight weight difference, the animals health was virtually unaffected by the *Cdh1* recombination.

Probably all three hypotheses are true to a certain extent: the liver is an organ known for most impressive regeneration of lost cells and thus can probably compensate the incomplete (less than 90%) loss of a protein easily after an adaption and remodelling phase (upregulating certain genes for compensatory mechanisms), especially since it likely is under less physical strain compared to organs like the intestine. Consequently, liver homeostasis is affected much less by organ-specific E-cadherin depletion than originally expected.

## 2. Phenotype after tumor induction

Liver specific deletion of *Cdh1* altered the susceptibility to chemically (DEN) induced liver tumorigenesis. Four months after DEN injection, tumors were already visible on the liver surface of a few L-*Cdh1*$^{del/del}$, while tumors were never seen in the livers of control mice (although small pre-cancer nodes could be seen histologically in both groups). Both control and L-*Cdh1*$^{del/del}$ mice developed liver tumors at the age of 12 months, but the latter reached moribundity faster.

Because mice were still only weakly affected by the carcinogen four months after DEN and mice kept for a longer time often did not even reach an age of 12 month, those age groups (4 and 12 months) were suboptimal for measurement of certain tumor effects. Thus, we can conclude that mice 8 months after DEN injection are the best suited to evaluate quantitative effects of conditional E-cadherin loss on the progress of DEN-induced liver tumorigenesis.

Parameters like body weight, relative liver weight and number of tumor lesions at different ages were evaluated. The total number of macroscopically visible lesions was increased, and lesions also reached larger maximum diameters in L-*Cdh1*$^{del/del}$. Using digitalized slides, the histological tumor area was found to be significantly higher in L-*Cdh1*$^{del/del}$ compared to L-Control mice. This increased tumor burden in L-*Cdh1*$^{del/del}$ was probably the main factor contributing to the

increased liver weight.

The conclusion that can be drawn from the development of tumors in L-*Cdh1*$^{del/del}$ compared to L-Control is that the lack of the functional *Cdh1* gene enables tumors to grow faster. This could have several reasons.

1) Liver cells lacking *Cdh1*, when mutated by DEN, become more aggressive, faster-growing, tumor cells.
2) The genes upregulated to compensate for E-cadherin loss enable tumor cells to be more aggressive. A previous study has, for example, correlated elevated matrilysin (MMP) with more aggressive tumors (GROBLEWSKA et al., 2012).
3) More cells become tumor cells, because less or minor mutations are sufficient to convert a *Cdh1*-defficient cell to a tumor cell (compared to L-Control cells). The increased growth is thus a pseudo-gain in speed: the higher total amount of tumors consequently leads (if Gaussian/normal distribution is assumed) to more outlier super-fast growing tumors. Those outliers overgrow and obscure the presence of the normal-speed tumors.
4) Tissue lacking E-cadherin is not that resistant to tumor invasion and the tumors in the E-cadherin deficient liver can grow more easily.

The first two of the hypotheses stated (or a combination of both) sound the most plausible, because there is an abundance of studies pointing to a connection of tumor aggressiveness with *Cdh1* loss (in the tumor cells) or upregulation of certain proteins like matrilysin (MATSUMURA et al., 2001; CHIEN et al., 2011; GROBLEWSKA et al., 2012; KUMAR et al., 2012). In summary, we could show that in a mouse model of liver specific E-cadherin loss, HCC induced by a carcinogen is more agressive. These findings prove that *Cdh1* acts as a tumor suppressor gene in the liver, matching the results from literature which have shown *Cdh1* relevance for tumors concerning other organs.

## 3. Cell culture permanent lines

Mammalian cells are known to have a limited split potential (hayflick limit), but in contrast to many other mammalian cells, cultured mouse cells (from non-tumorous tissue) are notorious for spontaneous transformation into permanent cell

lines (ROHME, 1981). In addition, primary liver cell cultures are known to face the problem of fibroblasts overgrowing the cells of the intended cell type (WEINSTEIN et al., 1975). Those fibroblast or other non-hepatic cells present might become permanent cell lines through spontaneous transformation, despite their originally non-tumorous nature.

It is therefore not easy to prove that the cells that we cultured are really of the intended cell type (HCC cells), or whether they are liver parenchyma cells at all. The only cells where HCC origin (or at least liver parenchyma origin) can be assumed as very likely are the cells in which no C*dh1* could be detected in PCR (assuming that the destruction of forward or reverse primer site due to random mutations or chromosome aberrations caused by spontaneous transformation is a rare event). Those cells therefore can be presumed to be originally liver cells, because only liver parenchyma cells produce albumin, and consequently in our model also Cre (which in turn leads to partial C*dh1* deletion and loss of exon 10 and thus, the site for the forward primer). The "145" cell line fulfilled the necessary criteria (PCR using *Cdh1*-specific primers did not yield a product) and can be considered to be a HCC permanent cell line without contamination by other cells (like fibroblasts, for example).

The "145" line proved stable, and the cells did survive storage in liquid nitrogen (at least for short periods of time) and can be assumed to be of HCC origin. The cell line therefore has the potential to be used, for example, in subsequent projects investigating the molecular mechanisms underlying the function of E-cadherin in liver tumorigenesis.

## 4. Considerations for future projects

Finally, in light of the good correlation between liver weight and tumor burden 8 months after DEN, for future projects the liver weight may be considered a good primary variable for similar experiments (instead of the often-used tumor lesion count). Weight is much easier to measure than other tumor parameters and the standardization is better in case of several different scientists or technicians doing the measuring. Also, statistics with organ weights are easier to handle than those with classed variables (like tumor lesion size classes). On the downside, some of the more complex information could be missed if only liver weight is gained.

# VI. SUMMARY

This work employed a mouse model of liver specific depletion of the gene *Cdh1* and its respective protein E-cadherin to study the role of this protein in liver homeostasis and pathophysiology. The experiment was done with specific focus on the effects concerning hepatocellular carcinoma (HCC) development.

**Background**: Cadherins are present in all higher organisms, and have been studied rigorously in the past. The cadherin family is huge, encompassing more than 400 (known) members. E-cadherin is the name-giver of that family and is considered to be of great importance to a broad range of physiological and pathophysiological functions. Known functions include cell-cell adhesion and deregulation of E-cadherin (in almost all cases a down-regulation) is associated with increased aggressiveness in both human and animal tumors. Aside from that, E-cadherin is of great importance during embryogenesis.

Worldwide, HCC is an important disease in humans, especially in certain countries (mostly developing countries). While females are only occasionally affected by HCC, it ranks among the top 3 tumor-related death causes in males. The difficulties in treating this tumor curatively make research of genes or proteins relevant to HCC important for human medicine improvement. The existence of a connection between *Cdh1* or E-Cadherin and HCC has been suggested, but more research is still required.

**Methods**: Employing Cre/loxP technology, a mouse model of liver specific E-cadherin depletion was created (L-*Cdh1*$^{del/del}$). The mice were compared to littermates with normal Ecadherin levels (L-Control). Mice body and organ weight was documented at different ages, and liver tissue was analyzed using qRT-PCR (cDNA), Western blot, histochemistry and immunohistochemistry.

To test effects of the reduced E-cadherin on tumor development, a cohort of male mice was injected with a chemical carcinogen (DEN) at two weeks of age to induce HCC, and mice were analyzed 4, 8 or 12 months later.

**Results**: Aside from a slight retardation in weight gain, L-*Cdh1*$^{del/del}$ did not suffer from severe health effects or spontaneous tumor development. Histology showed some alterations around the small bile ducts in the liver (in the periportal fields)

and RNA analysis showed that mice underwent a phase of considerably altered RNA activity (429 significantly regulated genes at 3 weeks of age), but later only a few up/down-regulated genes remained (28 genes at 6 weeks of age). Aside from *Cdh1*, no genes considered cadherin family members were regulated. Western blot analysis, qRT-PCR and IHC confirmed that E-cadherin was down-regulated on RNA level and on protein level in this animal model.

All mice injected with DEN developed tumors, but L-*Cdh1*$^{del/del}$ were affected more heavily, with tumors reaching large diameters faster. If mice were kept longer than 8 months, L-*Cdh1*$^{del/del}$ had to be euthanized significantly earlier than L-Control.

A spin-off of the model was the establishment of a permanent cell line, developed from a liver tumor of a L-*Cdh1*$^{del/del}$ mouse. PCR requiring a functional primer binding site on exon 10 of *Cdh1* could not produce DNA product, indicating that the cell line was a derivative of an E-cadherin negative liver cell.

**Conclusion**: Liver specific E-cadherin reduction had a surprisingly small effect in the present mouse model (compared to the effects of E-cadherin loss in organs like the skin or intestine, as documented in the literature) if mice were not challenged with a chemical carcinogen.

If mice were challenged with experimental HCC induction, lack of E-cadherin had a strong effect on the tumor growth. These findings attest, by an experimental animal model, the importance of E-cadherin for tumor development in the liver. This data reinforces previous observations concerning E-cadherin effects on tumors in studies working with resected human tumors of the liver or with conditional organ specific mouse models studying carcinoma in other organs (like the mammary gland, for example). Therefore, this animal model could help improve the understanding of mechanisms regulating aggressiveness in human tumors.

# VII. ZUSAMMENFASSUNG

Für diese Arbeit wurde ein Mausmodel mit leberspezifischem Ausfall des Gens *Cdh1* und des entsprechenden Proteins E-cadherin genutzt, um dessen Bedeutung in der Homöostase und Pathophysiologie der Leber zu untersuchen. In diesem Experiment wurde dabei besonders viel Wert auf die Effekte gelegt, welche die Entwicklung von Hepatozellulärem Karzinom (HCC) betreffen.

**Hintergrund**: Cadherine gibt es in allen höheren Organismen, und an ihnen wurde bereits intensiv geforscht. Die Cadherin Familie ist zahlreich und umfasst mehr als 400 (bekannte) Proteine. E-cadherin, das ursprünglich namensgebende Protein der Familie, wird mit einer großen Bandbreite an Funktionen in Physiologie und Pathophysiologie assoziiert. Bekannte Funktionen umfassen den Zell-Zell-Zusammenhalt, und eine Deregulation von E-cadherin (in fast allen Fällen eine Herabregulation) wird mit einer vermehrten Aggressivität von Tumoren in Zusammenhang gebracht. Abgesehen davon hat E-cadherin auch noch große Bedeutung in der Embryogenese.

Weltweit ist HCC eine wichtige Erkrankung des Menschen, speziell in bestimmten Ländern (sogenannten "Entwicklungsländern"). Frauen sind zwar verhältnismäßig selten davon betroffen, aber unter Männern nimmt HCC einen der drei Spitzenplätze ein was (Tumor-assoziierte) Todesursachen angeht. Die Schwierigkeiten diesen Tumor kurativ zu behandeln, bedingen dass Forschung an Genen oder Proteinen mit Bedeutung für HCC von Wichtigkeit für eine Verbesserung der Humanmedizin ist. Dass eine Verknüpfung zwischen *Cdh1*/E-Cadherin mit HCC besteht ist bereits bekannt, dennoch ist eine gründlichere Forschungsarbeit notwendig.

**Methodik**: Unter Benutzung der Cre/loxP Technologie wurden Mäuse mit einem leberspezifischem Verlust von E-cadherin generiert (L-*Cdh1*$^{del/del}$). Die Mäuse wurden mit Wurfgeschwistern mit normalen E-cadherin Status (L-Control) verglichen. Körper- und Organgewicht wurde zu mehreren Zeitpunkten gemessen, und Lebergewebe wurde mit qRT-PCR (cDNA), Western blot, Histochemie und Immunhistochemie untersucht.

Um den Effekt von reduziertem E-cadherin auf die Tumorentwicklung zu testen,

wurde eine Gruppe männlicher Mäuse im Alter von zwei Wochen mit einem chemischen Karzinogen (DEN) behandelt um HCC zu erzeugen. Die Mäuse wurden 4, 8, oder 12 Monate später untersucht.

**Ergebnisse**: Abgesehen von einer leicht verringerten Gewichtszunahme waren L-*Cdh1*$^{del/del}$ gesundheitlich weitestgehend unbeeinflusst durch den E-cadherin Verlust. Histologisch waren jedoch einige Veränderungen an/um die feinen Gallengänge (im Periportalfeld) zu erkennen. Eine Messung der RNA ergab, dass die Mäuse eine Phase erheblich veränderter RNA Aktivität durchlaufen (429 signifikant regulierte Gene im Alter von 3 Wochen), aber nicht viele dieser Gene bleiben längerfristig hochreguliert (nur noch 28 im Alter von 6 Wochen). Abgesehen von *Cdh1* war kein Mitglied der Cadherin Familie auf RNA Ebene signifikant reguliert. Mit Hilfe von qRT-PCR, Western Blot und IHC konnte bewiesen werden, dass E-cadherin in diesem Tiermodell auf RNA und auf Protein Ebene herabreguliert ist.

Alle mit DEN injizierte Mäuse entwickelten Tumore, aber L-*Cdh1*$^{del/del}$ Mäuse waren stärker betroffen. Hier erreichten Tumore bereits früher große Durchmesser. Wenn Mäuse länger als 8 Monate nach Karzinogengabe gehalten wurden, mussten L-*Cdh1*$^{del/del}$ signifikant früher euthanasiert werden als L-Control.

Ein Nebenprodukt dieses Models war die Etablierung einer permanenten Zelllinie aus der Leber einer L-*Cdh1*$^{del/del}$ Maus. Eine PCR, welche eine funktionierende Primer Bindungsstelle auf Exon 10 des *Cdh1* Gens benötigt, konnte kein DNA Produkt vermehren. Dadurch konnte bewiesen werden, dass die etablierte Zelllinie ein Abkömmling einer E-cadherin negativen Leber Zelle war.

**Schlussfolgerung**: Leberspezifischer Verlust von E-cadherin hat einen überraschend schwachen Effekt in einem Mausmodel (verglichen mit dem aus der Literatur bekannten Effekt von E-cadherin Verlust in Organen wie z.B. Haut oder Darm) solange die Mäuse nicht mit einem weiteren Faktor (einem Kanzerogen) konfrontiert werden.

Litten die Mäuse an HCC, so hatte der Mangel an E-cadherin einen starken Effekt auf das Tumorwachstum. Dies ist ein tierexperimenteller Beweis für die Bedeutung von E-cadherin für Tumorentwicklung in der Leber. Das Ergebnis

bestätigt frühere Erkenntnisse betreffs des Zusammenhangs zwischen E-cadherin und Tumoren, welche an resezierten Lebertumoren aus der Humanmedizin und anhand von anderen konditionalen E-cadherin defizienten Mausmodellen (z.B. Karzinogene der Milchdrüse) gewonnen wurden. Das verwendete Mausmodell könnte also zu einem besseren Verständnis der Mechanismen dienen, welche die Aggressivität von Tumoren steuern.

# VIII. BIBLIOGRAPHY

Abnet CC. Carcinogenic food contaminants. Cancer Invest 2007; 25: 189-96.

Adamson RH. Induction of hepatocellular carcinoma in nonhuman primates by chemical carcinogens. Cancer Detect Prev 1989; 14: 215-9.

Anastassiadis K, Fu J, Patsch C, Hu S, Weidlich S, Duerschke K, Buchholz F, Edenhofer F, Stewart AF. Dre recombinase, like Cre, is a highly efficient site-specific recombinase in E. coli, mammalian cells and mice. Dis Model Mech 2009; 2: 508-15.

Arboleda VA, Vilain E. The evolution of the search for novel genes in mammalian sex determination: from mice to men. Mol Genet Metab 2011; 104: 67-71.

Austin CP, Battey JF, Bradley A, Bucan M, Capecchi M, Collins FS, Dove WF, Duyk G, Dymecki S, Eppig JT, Grieder FB, Heintz N, Hicks G, Insel TR, Joyner A, Koller BH, Lloyd KC, Magnuson T, Moore MW, Nagy A, Pollock JD, Roses AD, Sands AT, Seed B, Skarnes WC, Snoddy J, Soriano P, Stewart DJ, Stewart F, Stillman B, Varmus H, Varticovski L, Verma IM, Vogt TF, von Melchner H, Witkowski J, Woychik RP, Wurst W, Yancopoulos GD, Young SG, Zambrowicz B. The knockout mouse project. Nat Genet 2004; 36: 921-4.

Beavon IR. The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation. Eur J Cancer 2000; 36: 1607-20.

Beckers J, Wurst W, de Angelis MH. Towards better mouse models: enhanced genotypes, systemic phenotyping and envirotype modelling. Nat Rev Genet 2009; 10: 371-80.

Berx G, Staes K, van Hengel J, Molemans F, Bussemakers MJ, van Bokhoven A, van Roy F. Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1). Genomics 1995; 26: 281-9.

Berx G, van Roy F. Involvement of members of the cadherin superfamily in cancer. Cold Spring Harb Perspect Biol 2009; 1: a003129.

Booth C, Soker T, Baptista P, Ross CL, Soker S, Farooq U, Stratta RJ, Orlando G. Liver bioengineering: current status and future perspectives. World J Gastroenterol 2012; 18: 6926-34.

Boussadia O, Kutsch S, Hierholzer A, Delmas V, Kemler R. E-cadherin is a survival factor for the lactating mouse mammary gland. Mech Dev 2002; 115: 53-62.

Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 1984; 309: 255-6.

Braet F, Wisse E. AFM imaging of fenestrated liver sinusoidal endothelial cells. Micron 2012; 43: 1252-8.

Brasch J, Harrison OJ, Honig B, Shapiro L. Thinking outside the cell: how cadherins drive adhesion. Trends Cell Biol 2012; 22: 299-310.

Brown SD. Mouse models of genetic disease: new approaches, new paradigms. J Inherit Metab Dis 1998; 21: 532-9.

Capecchi MR. The new mouse genetics: altering the genome by gene targeting. Trends Genet 1989; 5: 70-6.

Carstea AC, Pirity MK, Dinnyes A. Germline competence of mouse ES and iPS

cell lines: Chimera technologies and genetic background. World J Stem Cells 2009; 1: 22-9.

Cheon DJ, Orsulic S. Mouse models of cancer. Annu Rev Pathol 2011; 6: 95-119.

Chiaramonte M, Stroffolini T, Lorenzoni U, Minniti F, Conti S, Floreani A, Ntakirutimana E, Vian A, Ngatchu T, Naccarato R. Risk factors in community-acquired chronic hepatitis C virus infection: a case-control study in Italy. J Hepatol 1996; 24: 129-34.

Chien MH, Yeh KT, Li YC, Hsieh YH, Lin CH, Weng MS, Kuo WH, Yang SF. Effects of E-cadherin (CDH1) gene promoter polymorphisms on the risk and clinicopathological development of hepatocellular carcinoma. J Surg Oncol 2011; 104: 299-304.

Christensen HE, Fairchild EJ, Carroll BS, Lewis RJ (1976) Registry of Toxic Effects of Chemical Substances

Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer Res 2006; 66: 8319-26.

Costantini F, Lacy E. Introduction of a rabbit beta-globin gene into the mouse germ line. Nature 1981; 294: 92-4.

David JM, Rajasekaran AK. Dishonorable discharge: the oncogenic roles of cleaved E-cadherin fragments. Cancer Res 2012; 72: 2917-23.

DeChiara TM, Poueymirou WT, Auerbach W, Frendewey D, Yancopoulos GD, Valenzuela DM. Producing fully ES cell-derived mice from eight-cell stage embryo injections. Methods Enzymol 2010; 476: 285-94.

Delezoide AL, Vekemans M. Waardenburg syndrome in man and splotch mutants in the mouse: a paradigm of the usefulness of linkage and synteny homologies in mouse and man for the genetic analysis of human congenital malformations. Biomed Pharmacother 1994; 48: 335-9.

Derksen PW, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, van Beijnum JR, Griffioen AW, Vink J, Krimpenfort P, Peterse JL, Cardiff RD, Berns A, Jonkers J. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell 2006; 10: 437-49.

Diwan BA, Rice JM, Ohshima M, Ward JM, Dove LF. Comparative tumor-promoting activities of phenobarbital, amobarbital, barbital sodium, and barbituric acid on livers and other organs of male F344/NCr rats following initiation with N-nitrosodiethylamine. J Natl Cancer Inst 1985; 74: 509-16.

Druckey D, Preussmann R, Ivankovic S, Schmahl D. Organotrope Carcinogene Wirkung bei 65 Verschiedenen N-Nitroso-verbindungen und BD-Ratten. Z. Krebsforsch 1967;

El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365: 1118-27.

Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 2003; 120: 117-30.

Frese KK, Tuveson DA. Maximizing mouse cancer models. Nat Rev Cancer 2007; 7: 645-58.

Furukawa F, Takigawa M, Matsuyoshi N, Shirahama S, Wakita H, Fujita M, Horiguchi Y, Imamura S. Cadherins in cutaneous biology. J Dermatol 1994; 21: 802-13.

Furukawa F, Fujii K, Horiguchi Y, Matsuyoshi N, Fujita M, Toda K, Imamura S, Wakita H, Shirahama S, Takigawa M. Roles of E- and P-cadherin in the human skin. Microsc Res Tech 1997; 38: 343-52.

Gama A, Schmitt F. Cadherin cell adhesion system in canine mammary cancer: a

review. Vet Med Int 2012; 2012: 357187.
Gentric G, Desdouets C, Celton-Morizur S. Hepatocytes polyploidization and cell cycle control in liver physiopathology. Int J Hepatol 2012; 2012: 282430.
Gholami MR, Hablolvarid MH, Ezzi A. Hepatocellular Carcinoma in Sheep. Archives of Razi Institute, Vol. 61, Nr. 1, Spring (2006) 53-55 2006; Vol. 61: 53-5

Glaser S, Anastassiadis K, Stewart AF. Current issues in mouse genome engineering. Nat Genet 2005; 37: 1187-93.
Gomaa AI, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD. Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. World J Gastroenterol 2008; 14: 4300-8.
Gordon JW, Ruddle FH. Integration and stable germ line transmission of genes injected into mouse pronuclei. Science 1981; 214: 1244-6.
Green KJ, Jones JC. Desmosomes and hemidesmosomes: structure and function of molecular components. FASEB J 1996; 10: 871-81.
Groblewska M, Siewko M, Mroczko B, Szmitkowski M. The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. Folia Histochem Cytobiol 2012; 50: 12-9.
Groopman JD, Kensler TW, Wild CP. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. Annu Rev Public Health 2008; 29: 187-203.
Gumbiner BM. Regulation of cadherin-mediated adhesion in morphogenesis. Nat Rev Mol Cell Biol 2005; 6: 622-34.
Gupta PK. Single-molecule DNA sequencing technologies for future genomics research. Trends Biotechnol 2008; 26: 602-11.
Hall FS, Markou A, Levin ED, Uhl GR. Mouse models for studying genetic influences on factors determining smoking cessation success in humans. Ann N Y Acad Sci 2012; 1248: 39-70.
Harbers K, Jahner D, Jaenisch R. Microinjection of cloned retroviral genomes into mouse zygotes: integration and expression in the animal. Nature 1981; 293: 540-2.
Hazan RB, Qiao R, Keren R, Badano I, Suyama K. Cadherin switch in tumor progression. Ann N Y Acad Sci 2004; 1014: 155-63.
Hulpiau P, van Roy F. Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol 2009; 41: 349-69.
International Human Genome Sequencing C. Finishing the euchromatic sequence of the human genome. Nature 2004; 431: 931-45.
Issenberg P, Sornson H. A monitoring method for volatile nitrosamine levels in laboratory atmospheres. IARC Sci Publ 1976: 97-108.
Jaenisch R, Mintz B. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc Natl Acad Sci U S A 1974; 71: 1250-4.
Jakszyn P, Gonzalez CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. World J Gastroenterol 2006; 12: 4296-303.
Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.
Kambadur R, Sharma M, Smith TP, Bass JJ. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res 1997;

7: 910-6.
Kaurah P, Huntsman DG. Hereditary Diffuse Gastric Cancer. In: GeneReviews. Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, eds. Seattle (WA): 1993:
Kleer CG, van Golen KL, Braun T, Merajver SD. Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol 2001; 14: 458-64.
Kokkinos MI, Murthi P, Wafai R, Thompson EW, Newgreen DF. Cadherins in the human placenta--epithelial-mesenchymal transition (EMT) and placental development. Placenta 2010; 31: 747-55.
Kos CH. Cre/loxP system for generating tissue-specific knockout mouse models. Nutr Rev 2004; 62: 243-6.
Kumar D, Kumar M, Saravanan C, Singh SK. Curcumin: a potential candidate for matrix metalloproteinase inhibitors. Expert Opin Ther Targets 2012; 16: 959-72.
Langley R. Physical hazards of animal handlers. Occup Med 1999; 14: 181-94.
Larue L, Ohsugi M, Hirchenhain J, Kemler R. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc Natl Acad Sci U S A 1994; 91: 8263-7.
Lecuit M. Understanding how Listeria monocytogenes targets and crosses host barriers. Clin Microbiol Infect 2005; 11: 430-6.
Leenders MW, Nijkamp MW, Borel Rinkes IH. Mouse models in liver cancer research: a review of current literature. World J Gastroenterol 2008; 14: 6915-23.
Lewis-Tuffin LJ, Rodriguez F, Giannini C, Scheithauer B, Necela BM, Sarkaria JN, Anastasiadis PZ. Misregulated E-cadherin expression associated with an aggressive brain tumor phenotype. PLoS One 2010; 5: e13665.
Lewis DF, Brantom PG, Ioannides C, Walker R, Parke DV. Nitrosamine carcinogenesis: rodent assays, quantitative structure-activity relationships, and human risk assessment. Drug Metab Rev 1997a; 29: 1055-78.
Lewis JE, Wahl JK, 3rd, Sass KM, Jensen PJ, Johnson KR, Wheelock MJ. Cross-talk between adherens junctions and desmosomes depends on plakoglobin. J Cell Biol 1997b; 136: 919-34.
Li C, Mizutani E, Ono T, Terashita Y, Jia XF, Shi HJ, Wakayama T. Intracytoplasmic sperm injection with mouse spermatozoa preserved without freezing for six months can lead to full-term development. Biol Reprod 2011; 85: 1183-90.
Li Y, Hofmann M, Wang Q, Teng L, Chlewicki LK, Pircher H, Mariuzza RA. Structure of natural killer cell receptor KLRG1 bound to E-cadherin reveals basis for MHC-independent missing self recognition. Immunity 2009; 31: 35-46.
Liptak JM, Dernell WS, Monnet E, Powers BE, Bachand AM, Kenney JG, Withrow SJ. Massive hepatocellular carcinoma in dogs: 48 cases (1992-2002). J Am Vet Med Assoc 2004; 225: 1225-30.
Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environ Health Perspect 2010; 118: 818-24.
Longenecker G, Kulkarni AB. Generation of gene knockout mice by ES cell microinjection. Curr Protoc Cell Biol 2009; Chapter 19: Unit 19 4 4 1-36.
Machicao VI, Fallon MB. Hepatopulmonary syndrome. Semin Respir Crit Care Med 2012; 33: 11-6.
Marschall S, Hrabe de Angelis M. Cryopreservation of mouse spermatozoa: double your mouse space. Trends Genet 1999; 15: 128-31.

Matsumura T, Makino R, Mitamura K. Frequent down-regulation of E-cadherin by genetic and epigenetic changes in the malignant progression of hepatocellular carcinomas. Clin Cancer Res 2001; 7: 594-9.

Mazur P, Leibo SP, Seidel GE, Jr. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol Reprod 2008; 78: 2-12.

McCrea PD, Gu D, Balda MS. Junctional music that the nucleus hears: cell-cell contact signaling and the modulation of gene activity. Cold Spring Harb Perspect Biol 2009; 1: a002923.

Meek S, Buehr M, Sutherland L, Thomson A, Mullins JJ, Smith AJ, Burdon T. Efficient gene targeting by homologous recombination in rat embryonic stem cells. PLoS One 2010; 5: e14225.

Metzger D, Clifford J, Chiba H, Chambon P. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci U S A 1995; 92: 6991-5.

Metzger D, Chambon P. Site- and time-specific gene targeting in the mouse. Methods 2001; 24: 71-80.

Michielsen PP, Francque SM, van Dongen JL. Viral hepatitis and hepatocellular carcinoma. World J Surg Oncol 2005; 3: 27.

Mitacek EJ, Brunnemann KD, Suttajit M, Martin N, Limsila T, Ohshima H, Caplan LS. Exposure to N-nitroso compounds in a population of high liver cancer regions in Thailand: volatile nitrosamine (VNA) levels in Thai food. Food Chem Toxicol 1999; 37: 297-305.

Moriya T, Koyama T, Tanaka J, Mishiro S, Yoshizawa H. Epidemiology of hepatitis C virus in japan. Intervirology 1999; 42: 153-8.

Morse HCI, ALTROCK BW, BAILEY DW, BAKER RW, BENNETT D, BOGGS DR, BOGGS SS, BONHOMME F, CALLAHAN R, CARDIFF RD, CHAPMAN VM, CHERRY M, CHUSED TM, COLCHER D, GREEVE PD, DERINGER MK, DROHAN W, DUNCAN WR, FLAHERTY L, FURTH J, GAUTSCH J, GRAFF RJ, GREEN EL, GREEN MC, HAND P, HARTLEY JW, HELD JR, HERBERMAN RB, HESTON WE, HOFFMAN HA, HOWARD D, HSU E, HUANG H-JS, HUTTON JJ, IVÁNYI P, JOYCE RA, KEELER C, KLEIN J, KRAUSE RM, KUFE D, LERNER R, LIEBELT AG, LILLY F, LYON MF, McCOY JL, MARSHALL JT, MELTZER MS, MILLER DA, MILLER OJ, MOISSET B, III HCM, MOSIER DE, NAUTS HC, OLD LJ, PAIGEN K, PATRENE KD, POTTER M, RODERICK TH, ROSENSTREICH DL, ROWE WP, RUCO L, RUSSELL ES, SAGE RD, SCHIDLOVSKY G, SCHLOM J, SCHWARTZ GM, SELANDER RK, SHIMKIN MB, SNELL GD, STOCKERT E, STRONG LC, TAGLIABUE A, TAYLOR BA, TERAMOTO Y, TODARO GJ, WAKELAND EK, ZALESKA-RUTCZYNSKA Z, ZATZ MM ( 1978) Origins of Inbred Mice. Academic Press

Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG, Ostrander EA. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. PLoS Genet 2007; 3: e79.

Mouse Genome Sequencing C, Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, Attwood J, Baertsch R, Bailey J, Barlow K, Beck S, Berry E, Birren B, Bloom T, Bork P, Botcherby M, Bray N, Brent MR,

Brown DG, Brown SD, Bult C, Burton J, Butler J, Campbell RD, Carninci P, Cawley S, Chiaromonte F, Chinwalla AT, Church DM, Clamp M, Clee C, Collins FS, Cook LL, Copley RR, Coulson A, Couronne O, Cuff J, Curwen V, Cutts T, Daly M, David R, Davies J, Delehaunty KD, Deri J, Dermitzakis ET, Dewey C, Dickens NJ, Diekhans M, Dodge S, Dubchak I, Dunn DM, Eddy SR, Elnitski L, Emes RD, Eswara P, Eyras E, Felsenfeld A, Fewell GA, Flicek P, Foley K, Frankel WN, Fulton LA, Fulton RS, Furey TS, Gage D, Gibbs RA, Glusman G, Gnerre S, Goldman N, Goodstadt L, Grafham D, Graves TA, Green ED, Gregory S, Guigo R, Guyer M, Hardison RC, Haussler D, Hayashizaki Y, Hillier LW, Hinrichs A, Hlavina W, Holzer T, Hsu F, Hua A, Hubbard T, Hunt A, Jackson I, Jaffe DB, Johnson LS, Jones M, Jones TA, Joy A, Kamal M, Karlsson EK, Karolchik D, Kasprzyk A, Kawai J, Keibler E, Kells C, Kent WJ, Kirby A, Kolbe DL, Korf I, Kucherlapati RS, Kulbokas EJ, Kulp D, Landers T, Leger JP, Leonard S, Letunic I, Levine R, Li J, Li M, Lloyd C, Lucas S, Ma B, Maglott DR, Mardis ER, Matthews L, Mauceli E, Mayer JH, McCarthy M, McCombie WR, McLaren S, McLay K, McPherson JD, Meldrim J, Meredith B, Mesirov JP, Miller W, Miner TL, Mongin E, Montgomery KT, Morgan M, Mott R, Mullikin JC, Muzny DM, Nash WE, Nelson JO, Nhan MN, Nicol R, Ning Z, Nusbaum C, O'Connor MJ, Okazaki Y, Oliver K, Overton-Larty E, Pachter L, Parra G, Pepin KH, Peterson J, Pevzner P, Plumb R, Pohl CS, Poliakov A, Ponce TC, Ponting CP, Potter S, Quail M, Reymond A, Roe BA, Roskin KM, Rubin EM, Rust AG, Santos R, Sapojnikov V, Schultz B, Schultz J, Schwartz MS, Schwartz S, Scott C, Seaman S, Searle S, Sharpe T, Sheridan A, Shownkeen R, Sims S, Singer JB, Slater G, Smit A, Smith DR, Spencer B, Stabenau A, Stange-Thomann N, Sugnet C, Suyama M, Tesler G, Thompson J, Torrents D, Trevaskis E, Tromp J, Ucla C, Ureta-Vidal A, Vinson JP, Von Niederhausern AC, Wade CM, Wall M, Weber RJ, Weiss RB, Wendl MC, West AP, Wetterstrand K, Wheeler R, Whelan S, Wierzbowski J, Willey D, Williams S, Wilson RK, Winter E, Worley KC, Wyman D, Yang S, Yang SP, Zdobnov EM, Zody MC, Lander ES. Initial sequencing and comparative analysis of the mouse genome. Nature 2002; 420: 520-62.

Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis 2000; 26: 99-109.

Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007; 317: 121-4.

Nelson WJ. Regulation of cell-cell adhesion by the cadherin-catenin complex. Biochem Soc Trans 2008; 36: 149-55.

Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol 1999; 147: 631-44.

Oh JW, Ahn SM, Kim KS, Choi JS, Lee WJ, Kim BR. The role of splenectomy in patients with hepatocellular carcinoma and secondary hypersplenism. Yonsei Med J 2003; 44: 1053-8.

Ozawa M, Kemler R. Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. J Cell Biol 1990; 111: 1645-50.

Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary

liver cancer worldwide. J Hepatol 2006; 45: 529-38.

Pop PA, Pop M, Iovan C, Boanca C. Hepatic trauma management in polytraumatised patients. Chirurgia (Bucur) 2012; 107: 591-7.

Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem 1999; 274: 305-15.

Postic C, Magnuson MA. DNA excision in liver by an albumin-Cre transgene occurs progressively with age. Genesis 2000; 26: 149-50.

Putzke AP, Ventura AP, Bailey AM, Akture C, Opoku-Ansah J, Celiktas M, Hwang MS, Darling DS, Coleman IM, Nelson PS, Nguyen HM, Corey E, Tewari M, Morrissey C, Vessella RL, Knudsen BS. Metastatic progression of prostate cancer and e-cadherin regulation by zeb1 and SRC family kinases. Am J Pathol 2011; 179: 400-10.

Rajewsky MF, Dauber W, Frankenberg H. Liver carcinogenesis by diethylnitrosamine in the rat. Science 1966; 152: 83-5.

Rohme D. Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. Proc Natl Acad Sci U S A 1981; 78: 5009-13.

Rossant J, McMahon A. "Cre"-ating mouse mutants-a meeting review on conditional mouse genetics. Genes Dev 1999; 13: 142-5.

Sarli G, Preziosi R, De Tolla L, Brunetti B, Benazzi C. E-cadherin immunoreactivity in canine mammary tumors. J Vet Diagn Invest 2004; 16: 542-7.

Sauer B. Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. Mol Cell Biol 1987; 7: 2087-96.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3: 1101-8.

Schneider MR, Dahlhoff M, Horst D, Hirschi B, Trulzsch K, Muller-Hocker J, Vogelmann R, Allgauer M, Gerhard M, Steininger S, Wolf E, Kolligs FT. A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. PLoS One 2010; 5: e14325.

Schneider MR. Genetic mouse models for skin research: Strategies and resources. Genesis 2012; 50: 652-64.

Schofield PN, Hoehndorf R, Gkoutos GV. Mouse genetic and phenotypic resources for human genetics. Hum Mutat 2012; 33: 826-36.

Schreier E, Höhne M. Hepatitis C – Epidemiologie und Prävention. Bundesgesundheitsbl - Gesundheitsforsch - Gesundheitsschutz 2001;

Schubert WD, Urbanke C, Ziehm T, Beier V, Machner MP, Domann E, Wehland J, Chakraborty T, Heinz DW. Structure of internalin, a major invasion protein of Listeria monocytogenes, in complex with its human receptor E-cadherin. Cell 2002; 111: 825-36.

Seki M, Asano K, Ishigaki K, Iida G, Teshima K, Watari T, Tanaka S. En block resection of a large hepatocellular carcinoma involving the caudal vena cava in a dog. J Vet Med Sci 2011; 73: 693-6.

Semb H, Christofori G. The tumor-suppressor function of E-cadherin. Am J Hum Genet 1998; 63: 1588-93.

Seong E, Saunders TL, Stewart CL, Burmeister M. To knockout in 129 or in C57BL/6: that is the question. Trends Genet 2004; 20: 59-62.

Shimoda M, Kubota K. Multi-disciplinary treatment for cholangiocellular

carcinoma. World J Gastroenterol 2007; 13: 1500-4.

Si-Hoe SL, Wells S, Murphy D. Production of transgenic rodents by the microinjection of cloned DNA into fertilized one-cell eggs. Mol Biotechnol 2001; 17: 151-82.

Simon MM, Mallon AM, Howell GR, Reinholdt LG. High throughput sequencing approaches to mutation discovery in the mouse. Mamm Genome 2012; 23: 499-513.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150: 76-85.

Spiegelhalder B, Preussmann R. Occupational nitrosamine exposure. 1. Rubber and tyre industry. Carcinogenesis 1983; 4: 1147-52.

Stearns FW. One hundred years of pleiotropy: a retrospective. Genetics 2010; 186: 767-73.

Sternberg N, Hamilton D. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J Mol Biol 1981; 150: 467-86.

Struhl K. The new yeast genetics. Nature 1983; 305: 391-7.

Sundfeldt K. Cell-cell adhesion in the normal ovary and ovarian tumors of epithelial origin; an exception to the rule. Mol Cell Endocrinol 2003; 202: 89-96.

Suzuki H, Yorozu K, Watanabe T, Nakura M, Adachi J. Rederivation of mice by means of in vitro fertilization and embryo transfer. Exp Anim 1996; 45: 33-8.

Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 1990; 59: 237-52.

Tian X, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, Zhao Y, Harris DC, Zheng G. E-cadherin/beta-catenin complex and the epithelial barrier. J Biomed Biotechnol 2011; 2011: 567305.

Tiwari N, Gheldof A, Tatari M, Christofori G. EMT as the ultimate survival mechanism of cancer cells. Semin Cancer Biol 2012; 22: 194-207.

Tsang WH, Chow KL. Cryopreservation of mammalian embryos: Advancement of putting life on hold. Birth Defects Res C Embryo Today 2010; 90: 163-75.

Tunggal JA, Helfrich I, Schmitz A, Schwarz H, Gunzel D, Fromm M, Kemler R, Krieg T, Niessen CM. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. EMBO J 2005; 24: 1146-56.

van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci 2008; 65: 3756-88.

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV,

Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferriera S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigo R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A, Zhu X. The sequence of the human genome. Science 2001; 291: 1304-51.

Victor A, Elsasser A, Hommel G, Blettner M. Judging a plethora of p-values: how to contend with the problem of multiple testing--part 10 of a series on evaluation of scientific publications. Dtsch Arztebl Int 2010; 107: 50-6.

Weinstein IB, Orenstein JM, Gebert R, Kaighn ME, Stadler UC. Growth and structural properties of epithelial cell cultures established from normal rat liver and chemically induced hepatomas. Cancer Res 1975; 35: 253-63.

Winter RM. Malformation syndromes: a review of mouse/human homology. J Med Genet 1988; 25: 480-7.

Yerly S, Quadri R, Negro F, Barbe KP, Cheseaux JJ, Burgisser P, Siegrist CA, Perrin L. Nosocomial outbreak of multiple bloodborne viral infections. J Infect Dis 2001; 184: 369-72.

Yoshida-Noro C, Suzuki N, Takeichi M. Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. Dev Biol 1984; 101: 19-27.

Zhang J. Genetic redundancies and their evolutionary maintenance. Adv Exp Med Biol 2012; 751: 279-300.

# IX. ADDENDUM

## 1. Complete names of the genes found to be strongly and significantly regulated on RNA level

**Chip: Agilent Array**

| Gen Symbol | Gen Name | Foldchange KO vs. WT | | |
|---|---|---|---|---|
| | | (week 1) | (week 3) | (week 6) |
| Mmp7 | matrix metallopeptidase 7 | 1,1 | 2,7 | 4,8 |
| Scube3 | signal peptide, CUB domain, EGF-like 3 | 1,3 | 3,7 | 4,4 |
| Tff2 | trefoil factor 2 (spasmolytic protein 1) | 1,4 | 2,4 | 4 |
| Bex1 | brain expressed gene 1 | 1 | 4,6 | 3,5 |
| Fxyd6 | FXYD domain-containing ion transport regulator 6 | 1 | 1,5 | 3,4 |
| Bicc1 | bicaudal C homolog 1 (Drosophila) | 1,3 | 1,9 | 2,8 |
| Aebp1 | AE binding protein 1 | 0,9 | 1,6 | 2,8 |
| Arhgap22 | Rho GTPase activating protein 22 | 0,9 | 1,2 | 2,2 |
| Adamtsl2 | ADAMTS-like 2 | 1,2 | 1,9 | 2 |
| Cdh1 | cadherin 1 | 0,47 | 0,22 | 0,17 |
| … | … | … | … | … |
| | | | | |

## 2. List of figures

*Fig. 1: "Bully whippet", adapted figure from: (MOSHER et al., 2007).* **Page 3.**

*Fig. 2: Injection of fertilized one-cell eggs. Based on: (SI-HOE et al., 2001).* **Page 7.**

*Fig. 3: A chimeric mouse with typically variegated coat color. - public domain.* **Page 8.**

*Fig. 4: In this example of conditional tissue specific knockout, the creatine kinase promoter directs Cre expression only in muscle, or the albumin promoter directs Cre expression only in liver. Accordingly, the gene PPARG (PPAR-γ) is deleted only tissue-specific. Based on: (KOS, 2004).* **Page 10.**

*Fig. 5 Cadherins, catenins and the actin cytoskeleton influence each other, often reciprocally. Based on: (NELSON, 2008).* **Page 13.**

*Fig. 6: This model can explain the regulation of cytoskeleton and membrane dynamics by the cadherin–catenin complex […]. Based on: (NELSON, 2008).* **Page 14.**

*Fig. 7: The human CDH1 gene structure. Posttranslational modification of the protein yields a mature protein with a sequence markedly different from what could be expected on the basis of the gene sequence. Based on: (VAN ROY & BERX, 2008).* **Page 15.**

*Fig. 8: World map showing regional variation in the estimated age-standardized incidence rates of liver cancer, […]. Based on: (EL-SERAG, 2011).* **Page 19.**

*Fig 9: Diethylnitrosamine structure - public domain.* **Page 20.**

*Fig. 10. Schematic representation of the floxed (Flox) Cdh1 allele and the deleted (Del) allele after cre-induced recombination. – genuine.* **Page 23.**

*Fig. 11 Breeding protocol. If random distribution of chromosomes (represented by bluish or rose bars) is assumed, 50% off the offspring are knockout mice. – genuine.* **Page 24.**

*Table 1. Number of animals injected with DEN and sacrificed during the*

*experiment coined "phenotype after tumor induction". – genuine.* **Page 35.**

*Fig. 12. Schematic of the mouse liver, based on "The Anatomy of the Laboratory Mouse" by Margaret J. Cook, accessible at Jax informatics.* **Page 38.**

*Fig. 13: ImageScope softwares annotation tool […]- genuine (PD Dr. Longerich)* **Page 44.**

*Fiig. 14. Demonstration of cre-mediated gene deletion by PCR […] – genuine.* **Page 55.**

*Fig. 15. Protein expression in L-Cdh1$^{del/del}$ and L-control mice liver samples […] – genuine.* **Page 56.**

*Fig. 16. Number of genes with significantly regulated RNA expression in mouse liver […] – genuine.* **Page 57.**

*Fig. 17. RNA expression of 10 of the most noticeable genes […] – genuine.* **Page 57.**

*Fig. 18. RNA expression in mouse liver according to qRT-PCR at an age of 6 weeks. – genuine.* **Page 58.**

*Fig. 19. Body weight (A), liver weight (B) and calculated relative liver weight (C) of L-Cdh1$^{del/del}$ and L-Control mice at the indicated ages […] – genuine.* **Page 59.**

*Fig. 20. Blood and serum parameters of 3 months old male mice […] – genuine.* **Page 60.**

*Fig. 21. Long-term weight development of L-Cdh1$^{del/del}$ and L-Control mice (weekly measurement). […]- genuine.* **Page 61.**

*Fig. 22. Histological changes in the liver of L-Cdh1$^{del/del}$ mice. […]- genuine.* **Page 62.**

*Fig 23: Necropsy of mouse with tumors. – genuine.* **Page 64.**

*Fig. 24: Histology of several tumors and/or secondary alterations of the lung. […]- genuine.* **Page 65.**

*Fig. 25. A variety of HCC forms could be induced in livers of both L-Cdh1$^{del/del}$ and L-Control mice by DEN. […]- genuine.* **Page 65.**

*Fig 26. Body weight (A) and relative weight of liver (B), lung (C) and spleen (D)*

*during necropsy of mice 4 months after tumor induction by DEN. – genuine.* **Page 66.**

*Fig 27. Body weight (A) and relative weight of liver (B), lung (C) and spleen (D) during necropsy of mice 8 months after tumor induction by DEN. – genuine.* **Page 67.**

*Fig 28. Body weight (A) and relative weight of liver (B), lung (C) and spleen (D) during necropsy of mice 12 months after tumor induction by DEN […] – genuine.* **Page 68.**

*Fig. 29. Lesions counted on the liver surface at 4 (A), 8 (B) or 12 (C) month after DEN injection. […]- genuine.* **Page 69.**

*Fig. 30. Tumorous alterations on liver slides (circled with yellow line). – genuine.* **Page 70.**

*Fig. 31. Comparison of mean tumor area on liver slides 8 months after tumor induction by DEN. – genuine.* **Page 70.**

*Fig. 32. Kaplan-Meier plot of mice originally scheduled for necropsy 12 months after DEN injection. – genuine.* **Page 71.**

*Fig. 33: cell lines which turned out to have infinite split potential.[…] – genuine.* **Page 72.**

*Fig. 34: PCR (performed similar to tail clip genotyping) showed "118" to be positive for Cdh1 and negative for Cre,* […] *– genuine.* **Page 73.**

# X. CURRICULUM VITAE

[This is a version without curriculum vitae, intended for print and electronic publication]

# XI. ACKNOWLEDGMENT

First off: the mice shall be acknowledged as those who gave the most. Although it can be argued that an apology to them rather than acknowledgment would be appropriate.

Dr. PD Marlon R. Schneider, as my Doctoral advisor, contributed immensely to this work. Amongst his positive qualities most important to doctoral students I would like to point out his practical approach to scientific problems, the speed with which he corrected thesis excerpts that I sent him, the almost round-the-clock availability for (scientific) questions and the considerable autonomy he granted his doctoral students. Aside from that, he also is an amiable guy.

I am indebted to both Prof. Eckhard Wolf and Prof. Frank T. Kolligs, who jointly provided the facilities and resources required for this project. As a side-effect, I was occasionally also provided with impressive medieval choir music (E. W. is an accomplished basso) or a most delicious raclette (Frank is an accomplished host).

I want to thank all the lab colleagues who helped me with practical aid, advice or just plain cheerfulness! This includes, but is not limited to, the following: Dr. Andreas Herbst, Dr. Anna Rettinger, Franziska Stumpf, Sabrina Porada, Mai Le, Freya Jay, Dr. Maik Dahlhoff, Dr. Stefanie Sklenak, Dr. Benjamin Hirschi, Maximilian Marschall, Andrea Ofner, Alexander Philipp, Simon Schön, Dr. Bernadette Ströhle, Isabel Thalhammer, Eva Baehr, Isabelle Flierman, Ding-Dong Zhang, Lixia “Anna” Kong, Florian Flenkenthaler, Kathrin Otte, Daniela Deutsch, Alexander Graf, Tamara Holy, Simona Horn, Josef “daSepp” Millauer and Stefanie Riesemann (most stunning red riding hood I have ever met).

Special thanks goes to Dr. Jessica Grill from whom I “inherited” the mice line and who did a lot of the groundwork on which the project was founded. She also was the one who recruited me to the project, and she showed me quite a few tricks of the trade. We have known each other practically from week one of our veterinary education and have been through many a tough challenge together, be it in academia or in *Aztlán* - always searching for the paydata…

Some cooperation partners and also a couple of plainly altruistic random scientists or technicians from other workgroups aided with this project or the problems

around the project. To mention just a few: Dr. Longerich (it was really pleasant working with him), Dr. Weigand, Dr. Blum, Dr. Krebs, Dr. Bauersachs, Dr. Hantel, Andrea Klanner, Ralph Wimmer, Shuai Lu, Igor Shapiro, Michael Englschall and many more.

For the attentive care that was given to my experimental animals, many thanks to all the mouse facility staff (no, I rather not try naming them all, because if I forget only a single name, that one might be mortally offended) and to their head Dr. Ingrid-Renner Müller (for advice, aid and a motherly attitude).

Timely and accurate lunch planning as well as a positive attitude: the chair for animal husbandry would be bleak (and probably break down anyway) without a secretary like Sylvia Hornig.

Dr. Kathrin Hatzmann kindly cross read this thesis and pointed out typos and other mishaps. She also has been my roommate for eight years. When she recently moved out to go to the next step in her life, as weird as it sounds, it almost felt to me like the end of a marriage (only without the hard feelings).

The project was supported by the Fritz Thyssen Foundation (http://www.fritz-thyssen-stiftung.de), and a sizeable amount of the monetary resources needed for material and personnel (me) were acquired from the foundation. What can I say? Thank you, I like being able to pay my bills! Aside from that, the foundation hosted a refreshing interdisciplinary event that enabled me to speak to some top junior researchers who for once were not in the field of healing and/or cutting mammals open. I really like the humanities.

Last but not least, thanks to the people who have helped me keep sane and unbiased despite the insanity that academic research often is: my family, my friends in general and especially my roommates (past and present). The roomies: Kathrin (vet), Natalie (vet), Lisa (dog), Anna (vet), Maria (teacher), Kelly (teacher/artist), Barbara (vet), Davina (dog) and Stephanie (vet). Yup, my life is filled with vet girls, you pitiable Ph.D. students from the engineering sciences department!