

Article

Establishment and Characterization of the Murine Liver Tumor Cell Line Δ CN60 with Conditional Caspase-8 and IKK γ /NEMO Inactivation and a Hepatocyte-Biliary Progenitor-like Phenotype

Alejandro Cornejo Müller ^{1,2}, Thomas Liehr ³ , Prahlad Balakrishnan ³ , Stefanie Kankel ³, Eva M. Buhl ⁴ , Diandra T. Keller ⁵ , Katharina S. Hardt ⁵ , Sarah K. Schröder-Lange ⁵ , Christian Liedtke ^{1,*}  and Ralf Weiskirchen ^{5,*} 

- ¹ Department of Internal Medicine III, RWTH University Hospital Aachen, D-52074 Aachen, Germany; alejandro.cornejo@gematik.de
 - ² Gematik GmbH, Berlin School of Public Health, D-10178 Berlin, Germany
 - ³ Institute of Human Genetics, Jena University Hospital, Friedrich-Schiller University, D-07747 Jena, Germany; thomas.liehr@med.uni-jena.de (T.L.); bprahlad@gmail.com (P.B.); stefanie.kankel@med.uni-jena.de (S.K.)
 - ⁴ Electron Microscopy Facility, Institute of Pathology, RWTH University Hospital Aachen, D-52074 Aachen, Germany; ebuhl@ukaachen.de
 - ⁵ Institute of Molecular Pathobiochemistry, Experimental Gene Therapy and Clinical Chemistry (IFMPEGKC), RWTH University Hospital Aachen, D-52074 Aachen, Germany; dikeller@ukaachen.de (D.T.K.); khardt@ukaachen.de (K.S.H.); saschroeder@ukaachen.de (S.K.S.-L.)
- * Correspondence: cliedtke@ukaachen.de (C.L.); rweiskirchen@ukaachen.de (R.W.)

Highlights

What are the main findings?

- A new murine hepatoma cell line (Δ CN60) derived from DEN-induced tumors in *Casp8^{f/f}Nemo^{f/f}* mice was established, retaining floxed alleles that allow efficient Cre-mediated simultaneous deletion of Caspase-8 and NEMO.
- Combined Casp8/Nemo deletion reduces proliferation, impairs TNF- α -induced NF- κ B p65 nuclear translocation, and promotes a progenitor-like hepatoma phenotype with increased AFP and CD133 expression and reduced albumin levels.

What are the implications of the main findings?

- Δ CN60 provides a genetically defined and karyotypically stable in vitro system to study Caspase-8/NEMO-dependent TNF- α signaling, NF- κ B activation, and cell death pathways in hepatocytes.
- The model bridges conditional mouse genetics with mechanistic cell culture experiments and may reduce reliance on animal studies while enabling investigation of hepatocyte plasticity and inflammatory signaling in liver disease.



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Abstract

Caspase-8 and the NF- κ B essential modulator (NEMO, also referred to as IKK γ) play critical roles in controlling TNF- α -induced cell death and survival in hepatocytes. The aim of this study was to generate a hepatocyte-derived cell line in which the *Casp8* and *Nemo* genes can be conditionally inactivated simultaneously to investigate the significance of the corresponding signaling pathways. To this end, we induced hepatocellular carcinoma in *Casp8^{f/f}Nemo^{f/f}* mice using diethylnitrosamine and established an immortalized hepatoma cell line from explanted liver tumors, which is subsequently referred to as Δ CN60. Δ CN60 cells still retain floxed *Casp8* and *Nemo* alleles, allowing for efficient Cre-mediated deletion

to generate *Casp8^ΔNemo^Δ* derivatives. Loss of both Caspase-8 and NEMO inhibits cell proliferation, increases the expression of tumor and progenitor markers (AFP, CD133), reduces albumin expression, and blocks TNF- α -induced NF- κ B p65 nuclear translocation. Δ CN60 *Casp8^ΔNemo^Δ* cells display altered sensitivity to prolonged TNF- α exposure, suggesting a potential shift in necroptosis-associated signaling pathways, although necroptotic cell death was not directly demonstrated. Δ CN60 serves as a versatile hepatoma model for investigating Caspase-8/NEMO-dependent TNF- α signaling and hepatocyte plasticity and may help prioritize future in vivo experiments by enabling selected mechanistic questions to be addressed first in vitro.

Keywords: murine hepatoma cell line; caspase-8; NEMO/IKK γ ; conditional gene deletion; TNF- α signaling; NF- κ B activation; necroptosis; hepatocyte plasticity/biliary-progenitor phenotype

1. Introduction

Immortalized cell lines are essential tools in biomedical research, providing standardized, renewable, and experimentally tractable in vitro systems to study cellular and molecular mechanisms under controlled conditions [1]. They allow high-throughput drug and toxicant screening, detailed analysis of signaling pathways, and stable genetic manipulation that is challenging in primary cells or in vivo models. In liver research, robust hepatocytic cell lines are particularly valuable because primary hepatocytes rapidly lose their differentiated phenotype and metabolic competence in culture. Moreover, their availability is limited by donor material and ethical considerations [1]. Immortalized hepatocytic cell lines offer a reproducible platform for long-term experiments, complementing animal models, and align with the 3Rs principles (replacement, reduction, and refinement) in experimental science [2,3].

Several human and murine hepatocytic cell lines are commonly used in hepatology and toxicology research. Human hepatoma-derived lines such as HepG2 [4], Hep3B [4], and Huh7 [5,6] are widely used for studying hepatic metabolism, lipotoxicity, viral hepatitis, and cancer biology. HepaRG cells [7], which are capable of differentiating into hepatocyte-like cells, are often used as substitutes for primary human hepatocytes in xenobiotic metabolism and cholestasis studies. In mouse models, cell lines such as AML12 (derived from non-tumorigenic hepatocytes of a transgenic mouse) [8] and Hepa1-6 (a murine hepatoma line) [9] are employed to explore species-specific liver physiology, oncogenesis, and the effects of genetic manipulation. Together, these models have provided valuable insights into hepatocyte biology, liver regeneration, and the development of liver diseases such as steatosis, fibrosis, and hepatocellular carcinoma (HCC).

However, currently available hepatocytic cell lines have important limitations that restrict their translational relevance. Most widely used lines are tumor-derived and have complex, often incompletely defined genetic and chromosomal abnormalities that can alter signaling pathways, stress responses, and metabolic capacity in ways that do not accurately mimic normal hepatocytes [6,10]. Many lines have reduced expression or activity of drug-metabolizing enzymes, transporters, and nuclear receptors, and show altered bile acid and lipid handling, limiting their suitability for predictive toxicology and pharmacokinetic modeling. Long-term culture can lead to genetic drift and clonal selection, resulting in heterogeneity between laboratories and even between passages within the same laboratory [2]. Furthermore, only a subset of existing lines is directly linked to genetically engineered mouse models, which limits the ability to integrate in vitro findings with in vivo

studies conducted in defined genetic backgrounds. These constraints highlight the need for well-characterized hepatocytic cell lines from mice that are closely linked to specific genetic configurations and can serve as reliable in vitro counterparts to in vivo models.

Caspase-8 and the NF- κ B regulatory subunit NEMO/IKK γ are central nodes in tumor necrosis factor- α (TNF- α) signaling and play a critical role in determining whether hepatocytes undergo apoptosis, necroptosis, or survival and proliferation in response to inflammatory stimuli [11,12]. Disruption of this regulatory network of Caspase-8 and NEMO has previously been associated with activation of receptor-interacting serine/threonine-protein kinase (RIPK1/RIPK3)-dependent inflammatory and cell death pathways in hepatocytes [4,13,14]. The genetic loss of *Casp8* in hepatocytes increases sensitivity to TNF- α , stabilizes RIPK1, and accelerates liver regeneration [15–18]. Conversely, the combined inactivation of *Casp8* and *Nemo* leads to severe liver injury, underscoring the importance of their interaction in controlling cell death and regeneration [18]. To study these pathways in a hepatocyte-specific and genetically manipulable context, free from interference by immune or non-parenchymal cells, a hepatoma cell line carrying conditional alleles of *Casp8* and *Nemo* is particularly appealing.

In this study, we describe the generation of a hepatoma cell line derived from *Casp8^{f/f}Nemo^{f/f}* mice, allowing simultaneous Cre-mediated deletion of both genes in vitro. In particular, we describe the morphology and growth characteristics of the Δ CN60 cell line using light microscopy and we analyzed their ultrastructural features with electron microscopy to evaluate hepatocyte-like organelle architecture and differentiation status. The authenticity of the cell line was confirmed through short tandem repeat (STR) profiling, and its chromosomal landscape was characterized using conventional karyotyping and spectral karyotyping (SKY). Additionally, bulk mRNA next-generation sequencing (RNA-seq) was performed to demonstrate the translational potential of this line. Selected mRNA expression findings were evaluated at the protein level through Western blot analysis. By integrating structural, cytogenetic, and genomic data with targeted protein validation, we provide a comprehensive characterization of this cell line and establish it as a genetically defined, hepatocyte-derived model system. This system will be valuable for studying Caspase-8/NEMO-dependent signaling, TNF- α -induced liver injury, and HCC biology, ultimately providing an in vitro system that may complement in vivo models and help prioritize future animal experiments.

2. Materials and Methods

2.1. Cell Line Origin and Establishment

In our study, we used C57BL/6 mice with LoxP-flanked (i.e., “floxed”) alleles of the *Casp8* and *Nemo* genes [16,17]. *Casp8^{f/f}Nemo^{f/f}* mice were treated with diethylnitrosamine (DEN) according to a standard protocol, leading to the development of HCC approximately 40 weeks after injection. Primary hepatocytes were isolated from HCC-bearing livers using a well-established method and cultured until reaching confluence over a period of approximately two months. This process resulted in the establishment of a stable hepatoma cell line with a *Casp8^{f/f}Nemo^{f/f}* genotype, designated as Δ CN60.

The cell line Δ CN60 was derived from a DEN-induced liver tumor isolated from a male *Casp8^{f/f}Nemo^{f/f}* mouse. The cell line was established by serial culture and passaging of adherent tumor-derived cells and was not generated by single-cell cloning or limiting dilution. Thus, Δ CN60 represents a stable tumor-derived adherent cell population rather than a formally single-cell-derived clone. Unless stated otherwise, experiments were performed between passages 5 and 10 after establishment of this cell line, which was assumed to have occurred after approximately 50 passages.

The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, #D6171) supplemented with 10% fetal bovine serum (FBS, #F7524), 2 mM L-glutamine (#G7513), 1 mM sodium pyruvate (#S8636), and 1× penicillin/streptomycin (#P0781). All reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Mycoplasma contamination in cell culture supernatants was assessed using the Venor[®]GeM OneStep kit (#11-8050; Minerva Biolabs GmbH, Berlin, Germany) essentially as reported previously [19]. Amplified products were separated on a conventional 2% agarose gel prepared in 1× TAE buffer containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (pH 8.6). Electrophoresis was performed for 50 min at 90 V, and DNA bands were visualized by GelRed Nucleic Acid Stain (#41003, Biotium GmbH, Hamburg, Germany). Gel images were acquired using a standard documentation system from Intas Science Imaging GmbH (Göttingen, Germany).

The animal experiments used to generate the tumor material from which ΔCN60 was established were performed under the animal use approval granted on 6 February 2009 by the Review Board for the Care of Animal Subjects of the district government, LANUV, Recklinghausen, North Rhine-Westphalia, Germany, under permit number AZ 8.87-50.10.35.08.349. Specifically, *Casp8^{f/f}Nemo^{f/f}* mice were treated with DEN under this approval, liver tumors were allowed to develop, and tumor-derived hepatocytes were isolated from HCC-bearing livers for subsequent establishment of the ΔCN60 cell line. No additional mice were used for the present study after establishment of the cell line, and no new DEN-induced tumor experiments, tumor isolations, transplantation experiments, or other animal experiments were performed at a later date. All experiments reported in the present manuscript after the initial cell line establishment were conducted exclusively with the established ΔCN60 in vitro cell line, including adenoviral Cre-mediated gene deletion, proliferation and signaling assays, cytogenetic characterization, transcriptomic profiling, microscopy, RT-qPCR, and Western blot analyses. Therefore, the cited 2009 approval applies only to the original animal work required to generate the tumor-derived cell line, whereas the subsequent characterization described here did not require additional animal ethics approval.

2.2. Adenoviral Cre-Mediated Gene Deletion

For Cre-mediated recombination, ΔCN60 cells were infected with adenoviral vectors encoding Cre recombinase. Where indicated, Ad-CMV-Cre/RSV-eGFP (#FVQ005, Kerafast, Newark, CA, USA), hereafter referred to as adv-Cre/GFP, was used to express Cre recombinase and enhanced green fluorescent protein (eGFP) from the same adenoviral construct. In experiments not requiring GFP visualization, an adenoviral Cre vector without eGFP, hereafter referred to as adv-Cre, was used. Control infections were performed with Ad-CMV-eGFP (#FVQ002, Kerafast), hereafter referred to as adv-GFP, or with Ad-CMV-LacZ, hereafter referred to as adv-LacZ. Cre-mediated recombination resulted in deletion of exons 3–4 of *Casp8* and exon 2 of *Nemo*, as determined by PCR using genomic DNA as a template, leading to premature termination and loss or strong reduction in Caspase-8 and NEMO protein expression at the population level. Deletion efficiency was assessed by genomic PCR and Western blotting; however, these bulk assays do not provide a single-cell-based percentage of successfully recombined cells. For *Nemo*, a 460 bp fragment represented the floxed allele, while a 260 bp fragment indicated the recombined knockout allele.

2.3. Functional Assays

Cell growth was monitored for up to 72 h in parental *Casp8^{f/f}Nemo^{f/f}* ΔCN60 cells and adv-Cre-treated *Casp8*/*Nemo*-deficient ΔCN60 cell populations. For growth-curve analyses, three independent biological replicates were analyzed. Stimulation experiments with

TNF- α (#410-MT, Novus Biologicals, Centennial, CO, USA) were performed using a concentration of 10 ng/mL for time-course analyses. Activation of NF- κ B was assessed through Western blotting of nuclear extracts using antibodies against p65 and TATA-box binding protein (TBP), as well as by p65 immunofluorescence with nuclear (DAPI) counterstaining using standard procedures. Expression levels of *Afp*, *Cd133*, and *Alb* were quantified relative to ileum and normal mouse liver. Cell cycle analysis was performed by comparing mRNA expression of cyclin A2 and cyclin D1 in *Casp8^{f/f}Nemo^{f/f}* and *Casp8 Δ Nemo Δ* Δ CN60 cell populations, with the latter referring to the adv-Cre-treated bulk population analyzed in the present study, which does not imply single-cell clonal derivation or complete recombination in every cell. The primers used in these assays are listed in Table S1.

2.4. Short Tandem Repeat (STR) Profiling

The Δ CN60 cell line underwent STR profiling using the CellCheck™ Mouse system, which employs a set of 19 consensus markers specifically designed for mouse species as outlined by the Consortium for Mouse Cell Line Authentication. To analyze the STR profile of Δ CN60, we utilized the Cellosaurus STR Similarity Search Tool CLASTR version 1.4.4 (release 41.0) along with the corresponding mouse STR database from Cellosaurus. The analysis parameters included a scoring algorithm based on Tanabe, a focus on non-empty markers, a score threshold of 40%, and a requirement for at least eight markers to be present.

2.5. Preparation of Δ CN60 Metaphase Chromosomes, Karyotyping, and Molecular Cytogenetics

For karyotype analysis, metaphase chromosomes were isolated from Δ CN60 cells following established protocols involving the induction of mitotic arrest using KaryoMAX colcemid solution (#15212012, Thermo Fisher Scientific, Darmstadt, Germany). After hypotonic treatment and fixation in a methanol:acetic acid mixture, multicolor fluorescence in situ hybridization (mFISH) with different probe sets specific to mouse chromosomes was performed. The probe set 21XMouse (MetaSystems, Altlußheim, Germany) was used according to the manufacturer's instructions; it contains all 21 murine whole chromosome paints as probes, labeled in individual color combinations. To detect intrachromosomal aberrations, 21 chromosome-specific murine multicolor banding (mcb) probe sets were applied as previously described [20]. Twenty metaphases per probe set were thoroughly examined using a fluorescence microscope equipped with a CCD camera, and images were processed through ISIS software (version 5.9.2) to construct the karyotype representation of Δ CN60 cells. The unique features of the blue counterstain 4',6-diamidino-2-phenylindole (DAPI) were utilized by the same software to create an inverted DAPI-banding pattern.

2.6. Next-Generation Sequencing and Data Analysis

Next-generation sequencing (NGS) of Δ CN60 cells was performed following established laboratory protocols designed for RNA sequencing studies [21]. Cells were cultured until they reached about 80% confluence before being lysed with guanidine thiocyanate for total RNA extraction using the cesium chloride cushion method. The quality and quantity of isolated RNA were assessed using UV spectrophotometry and an Agilent TapeStation system before rRNA depletion. This was followed by reverse transcription and indexing with NEBNext Multiplex Oligos designed for Illumina platforms. The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository under accession number GSE325078 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE325078>, last accessed on 26 June 2026).

2.7. Electron Microscopic Cell Analysis

To investigate the ultrastructural characteristics of Δ CN60 cells, samples were fixed in glutaraldehyde and then post-fixed in osmium tetroxide. Dehydration was carried out through an ethanol series before embedding in epoxy resin for further processing. Ultrathin sections approximately 70–90 nm thick were prepared using an ultramicrotome and mounted on copper grids. They were then contrasted with uranyl acetate and lead citrate solutions for examination by transmission electron microscopy (TEM).

2.8. Reverse Transcription Polymerase Chain Reaction

RNA was isolated using the PureLink™ RNA Mini Kit (12183025, Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions, including additional DNase digestion (12185010, Invitrogen, Thermo Fisher Scientific) as described previously [22]. Conventional RT-PCR was performed on cDNA synthesized from Δ CN60 cells using a thermal cycling protocol optimized for various target genes, such as *Hnf4a* and *Rn18s* among others. This process included an initial denaturation followed by multiple cycles at gene-specific annealing temperatures before final extension steps were taken to ensure complete amplification. All primers used in this analysis are listed in Table S1.

RT-qPCR experiments were performed using three independent biological replicates per cell line or condition and three technical qPCR replicates per biological replicate. Statistical testing was performed on biological replicate values after averaging technical replicates. Transcripts with undetermined C_t values or signals below the detection limit in all replicates of one group were reported as not detected and were not subjected to conventional parametric significance testing based on substituted C_t values.

2.9. Western Blot Analysis

Protein extracts from Δ CN60 cells and primary mouse hepatocytes were analyzed using standardized methodologies to ensure uniform loading across all gel electrophoresis lanes. The proteins were then transferred onto a 0.45 μ m nitrocellulose membrane (GE10600001, Amersham, Cytiva, Marlborough, MA, USA) and probed with specific primary antibodies. Chemiluminescent signals were visualized using SuperSignal West Dura Extended Duration Substrate (#34076, Thermo Fisher Scientific) and the iBright™ FL500 imaging system (Invitrogen, Thermo Fisher Scientific). All antibodies used for Western blot analysis are listed in Table S2.

2.10. Phalloidin Stain

For F-actin visualization, Δ CN60 cells were seeded at 4×10^4 cells per well in four-chamber glass slides suitable for tissue culture (#354114, Corning Inc., Falcon, Corning, NY, USA). After allowing time for attachment, cells were fixed with 3.7% paraformaldehyde and permeabilized for F-actin staining using phalloidin-Alexa Fluor 488 (#A12379, Invitrogen, Thermo Fisher Scientific) and 4',6-diamidino-2-phenylindole (DAPI, #D1306, Thermo Fisher Scientific) as a nuclear counterstain before imaging by fluorescence microscopy using a Nikon Eclipse E80i fluorescence microscope (Nikon, Düsseldorf, Germany) equipped with the NIS-elements Vis software (Software version 3.22.01), following procedures described previously [23].

2.11. Statistical Analysis

Data are presented as the mean $\pm$ SD unless indicated otherwise. Statistical analyses were performed using GraphPad Prism (version 8.4.2). Normally distributed data were analyzed using Shapiro–Wilk test and comparisons between two groups were done using

an unpaired two-tailed Student's *t*-test. Statistical significance was defined as $p < 0.05$. The number of biological and technical replicates is indicated in the respective figure legends.

3. Results

3.1. Establishment of Δ CN60 and Conditional *Casp8*/*Nemo* Deletion

The Δ CN60 cell line was established from DEN-induced HCC arising in *Casp8*^{f/f} *Nemo*^{f/f} mice and maintained in vitro until stable proliferation was achieved (Figure 1A). We confirmed that Δ CN60 cultures are mycoplasma-free, as routine PCR-based screening using the Venor® GeM OneStep kit consistently showed no detectable mycoplasma contamination (Figure S1). Genetically, Δ CN60 retains floxed *Casp8* and *Nemo* alleles, enabling conditional gene deletion by Cre recombinase. Adenoviral delivery of Cre (adv-Cre) efficiently generated detectable *Casp8*^Δ and *Nemo*^Δ alleles at the bulk population level, as demonstrated by the appearance of the 260 bp *Nemo* knockout fragment alongside the 460 bp floxed fragment (Figure 1B). Western blot analysis confirmed a reduction in NEMO protein levels after infection (Figure 1C), demonstrating that this system provides a suitable cellular platform to analyze the biological functions of the Caspase-8/NEMO network and its downstream signaling. Efficient adenoviral transduction of Δ CN60 cells was further verified by fluorescence microscopy following infection with adv-GFP or adv-Cre/GFP at different multiplicities of infection, demonstrating robust GFP expression after 24 and 48 h (Figure 1D). In addition, Western blot analysis confirmed a marked reduction in Caspase-8 protein levels in adv-Cre/GFP-infected cells compared with adv-GFP controls, validating efficient Cre-mediated deletion of the floxed *Casp8* alleles at the protein level (Figure 1E).

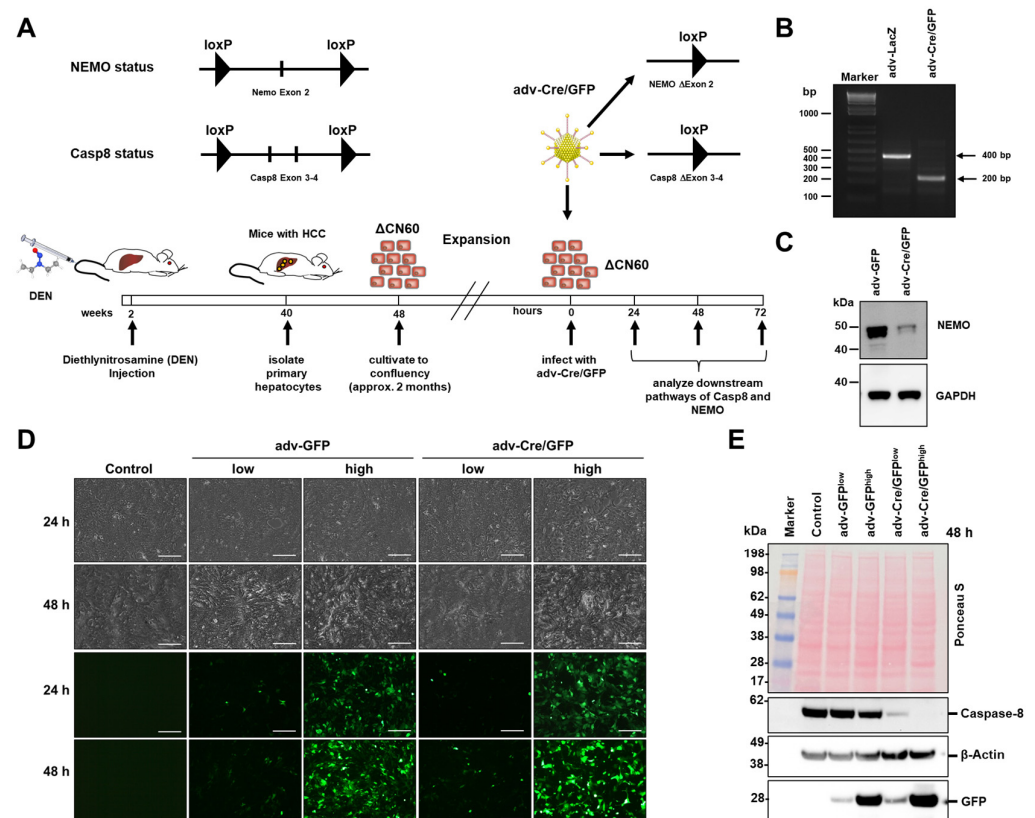


Figure 1. Establishment of the Δ CN60 hepatoma cell line and conditional deletion of *Casp8* and *Nemo*. (A) Scheme describing the generation of the Δ CN60 cell line from DEN-induced hepatocellular carcinoma (HCC) in *Casp8*^{f/f} *Nemo*^{f/f} mice, followed by the use of adenoviral Cre recombinase to delete floxed *Casp8* and *Nemo* alleles. (B) PCR analysis of genomic DNA from Δ CN60 cells infected

with Ad-CMV-Cre/RSV-eGFP (adv-Cre/GFP) or control adenovirus Ad-CMV-LacZ (adv-LacZ), showing the 460 bp floxed *Nemo* band and the 260 bp recombined *Nemo*^Δ allele, indicating efficient Cre-mediated recombination. (C) Western blot analysis confirming reduced NEMO protein levels in adv-Cre-infected ΔCN60 cells compared with floxed controls infected with adv-Cre/GFP. (D) ΔCN60 cells were infected for 3 h with the control virus adv-GFP or vector adv-Cre/GFP at a multiplicity of infection of 2×10^8 viruses/mL (low) or 2×10^9 viruses/mL (high). Representative light microscopy and fluorescence microscopy images were taken after 24 h and 48 h, demonstrating effective transduction. (E) Western blot analysis of Caspase-8 expression in ΔCN60 cells 48 h after infection with increasing amounts of adv-GFP or adv-Cre/GFP. While control virus infection (adv-GFP) does not affect Caspase-8 protein levels, adv-Cre/GFP infection leads to a marked reduction in Caspase-8 expression, demonstrating efficient Cre-mediated reduction in *Casp8* expression at the protein level. β-Actin was used as a loading control, and GFP detection confirmed successful adenoviral infection. Corresponding Ponceau S staining of the membrane is shown to demonstrate equal protein loading and transfer efficiency across the analyzed samples.

At the transcriptional level, parental ΔCN60 cells expressed *Casp8* and *Nemo/Ikbkγ* transcripts at the TPM values shown in Table 1. These values indicate detectable expression of both genes in the ΔCN60 bulk transcriptome, without implying a universal threshold-based classification as low, moderate, or high expression (Table 1).

Table 1. Expression of cellular regulators in ΔCN60 cells.

Gene	Gene Description	Gene Id	Transcript Id	TPM
<i>Afp</i>	alpha fetoprotein	ENSMUSG00000054932	ENSMUST00000042755.7	29.657458
<i>Alb</i>	albumin	ENSMUSG00000029368	ENSMUST00000031314.10	13.777514
<i>Casp8</i>	caspase 8	ENSMUST00000190213.2	ENSMUSG00000026029	57.648647
			ENSMUST00000165549.8	39.945768
			ENSMUST00000027189.15	22.521594
<i>Ccna2</i>	cyclin A2	ENSMUSG00000027715	ENSMUST00000029270.10	87.682636
<i>Ccnd1</i>	cyclin D1	ENSMUSG00000070348	ENSMUST00000093962.5	629.633846
<i>Cd133/Prom1</i>	prominin 1	ENSMUSG00000029086	ENSMUST00000087441.11	24.50765
			ENSMUST00000074113.13	1.860301
<i>Ikbkg</i>	inhibitor of kappaB kinase gamma	ENSMUSG00000004221	ENSMUST00000064407.10	23.286547
			ENSMUST00000114127.8	16.919647
			ENSMUST00000114128.8	5.881418
			ENSMUST00000114133.9	4.684198
<i>GAPDH</i> ¹	glyceraldehyde-3-phosphate dehydrogenase	ENSMUSG00000057666	ENSMUST00000118875.8	4128.128844
			ENSMUST00000117757.9	2200.218561
			ENSMUST00000073605.15	961.568394

¹ For the comparison of transcript levels of the listed genes, the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) is displayed. The complete mRNA expression profile of ΔCN60 cells, as observed by NGS, can be found in Table S3. TPM stands for transcripts per million. Descriptive terms in the text, such as low, moderate, or high expression, refer only to a relative qualitative interpretation within the ΔCN60 transcriptome and are not intended to represent universal TPM thresholds.

3.2. Proliferation, Cell Cycle Control, and Tumor Differentiation Marker Expression

Adv-Cre-treated *Casp8/Nemo*-deficient ΔCN60 cell populations displayed significantly reduced proliferation compared with their *Casp8*^{f/f}/*Nemo*^{f/f} counterparts when monitored over 72 h (Figure 2A). Cell-cycle analysis after adv-Cre infection showed impaired progression in *Casp8/Nemo*-deleted cells, indicating a decreased fraction of cells entering the S-phase. At the transcriptional level, the induction of cyclin D1 (*Ccnd1*) and cyclin A2 (*Ccna2*) was lower in *Casp8*^Δ/*Nemo*^Δ hepatoma cells relative to floxed controls (Figure 2B). Additionally, parental ΔCN60 cells expressed the tumor-associated markers alpha-fetoprotein (AFP)

and CD133, while showing lower albumin levels compared to normal liver, indicating a hepatoma phenotype. Upon deletion of *Casp8* and *Nemo*, *Afp* and *Cd133* mRNA levels increased further, while *Alb* expression decreased, suggesting additional dedifferentiation and acquisition of progenitor-like features (Figure 2C). Therefore, Δ CN60 not only models HCC biology, but also indicates that *Casp8/Nemo* loss may promote dedifferentiation in hepatoma cells. In addition, the altered signaling environment created by combined *Casp8/Nemo* deletion may influence TNF- α -dependent cell death pathways that have previously been linked to RIPK1/RIPK3 signaling [3,13,14,24]. However, the present study did not directly investigate necroptotic signaling or necroptotic cell death mechanisms in Δ CN60 cells.

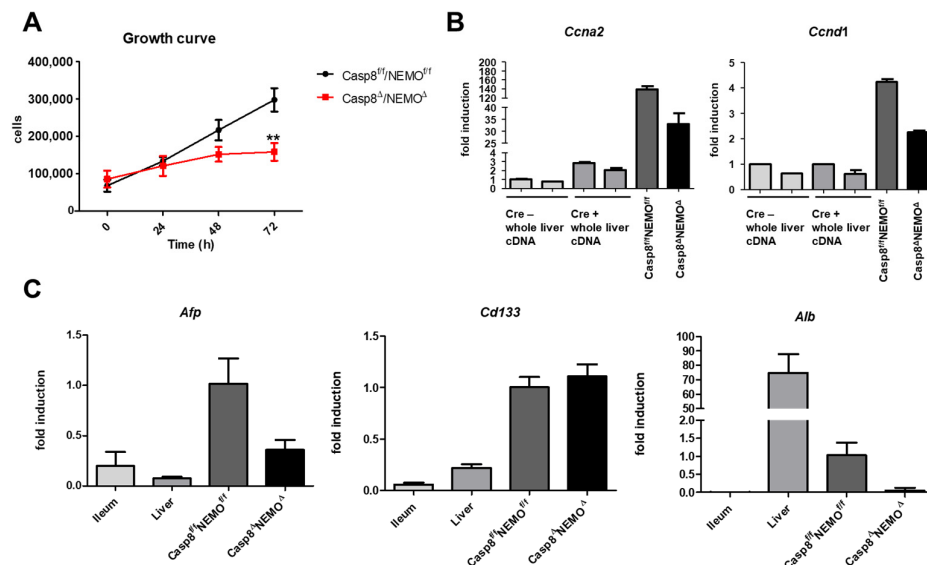


Figure 2. Simultaneous deletion of *Casp8/Nemo* reduces proliferation, alters cell-cycle gene expression, and promotes dedifferentiation in Δ CN60 cells. (A) Proliferation of parental *Casp8^{fl/fl}/Nemo^{fl/fl}* and adv-Cre-treated *Casp8/Nemo*-deficient Δ CN60 cell populations was monitored over 72 h. Data are shown as the mean $\pm$ SD from three independent biological replicates per condition and time point. $p = 0.003$ for the overall effect of *Casp8/Nemo* deletion at 72 h. (B) Relative mRNA expression of cyclin A2 (*Ccn2*) and cyclin D1 (*Cnd1*) in *Casp8^{fl/fl}/Nemo^{fl/fl}* versus *Casp8^Δ/Nemo^Δ* Δ CN60 cells, and in whole-liver cDNA from Cre-negative versus Cre-positive *Casp8^{fl/fl}/Nemo^{fl/fl}* mice, showing diminished induction of these cell-cycle regulators upon *Casp8/Nemo* deletion. (C) Expression of the tumor/progenitor markers α -fetoprotein (*Afp*) and CD133/Prominin-1 and the hepatocyte differentiation marker albumin (*Alb*) in parental and *Casp8/Nemo*-deficient Δ CN60 cells, quantified relative to ileum and normal liver, demonstrating increased *Afp* and *Cd133* and decreased *Alb* expression after *Casp8/Nemo* loss, indicative of further dedifferentiation and acquisition of progenitor-like features. ** $p < 0.001$.

3.3. NF- κ B p65 Nuclear Translocation

Stimulation with TNF- α (10 ng/mL) in *Casp8^{fl/fl}/Nemo^{fl/fl}* Δ CN60 cells led to a rapid nuclear accumulation of NF- κ B p65, whereas *Casp8^Δ/Nemo^Δ* cells exhibited significantly reduced TNF- α -induced nuclear p65, both biochemically (Figure 3A) and by microscopy (Figure 3B). Quantification of p65-positive nuclei confirmed a notable decrease in NF- κ B activation in adv-Cre-infected cells compared with adv-LacZ-infected cells (Figure 3C). While *Casp8/Nemo* deletion has previously been linked to RIPK1/RIPK3-dependent cell death signaling in hepatocytes, the present study primarily demonstrates altered TNF- α responsiveness in Δ CN60 cells rather than directly characterizing RIPK1/RIPK3 activation.

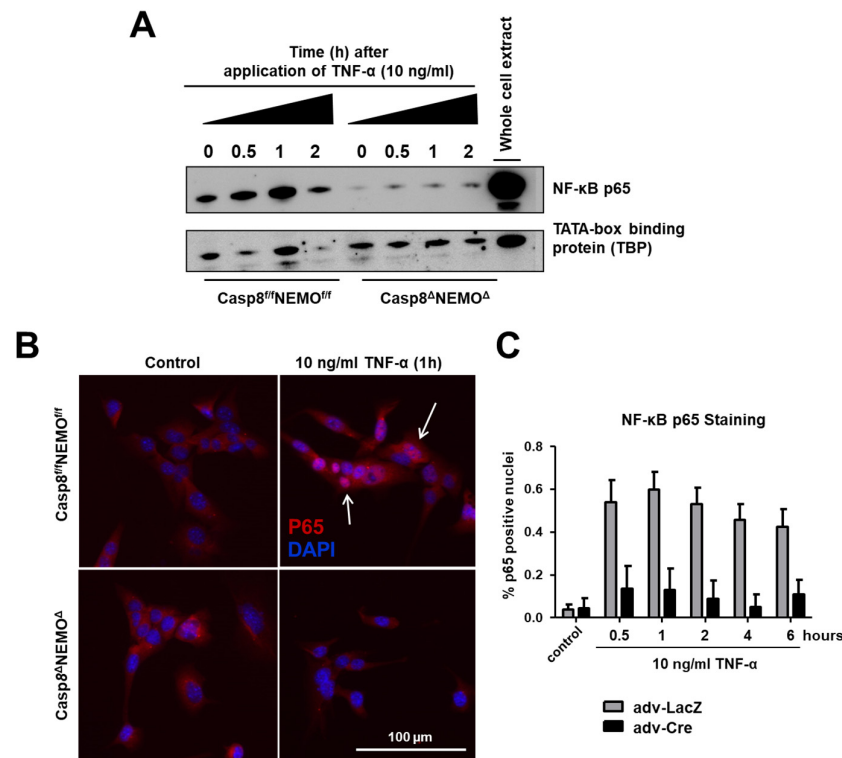


Figure 3. TNF- α -induced NF- κ B p65 nuclear translocation is impaired in *Casp8 Δ Nemo Δ* Δ CN60 cells. (A) Western blot analysis of nuclear extracts from *Casp8^{f/f}Nemo^{f/f}* and *Casp8 Δ Nemo Δ* Δ CN60 cells stimulated with TNF- α (10 ng/mL) for the indicated times, probed for NF- κ B p65 and the nuclear marker TATA-box binding protein (TBP). The results show robust TNF- α -induced nuclear accumulation of p65 in floxed cells but strongly reduced nuclear p65 in *Casp8/Nemo*-deficient cells. (B) Immunofluorescence staining for p65 (red) with DAPI nuclear counterstaining (blue) in *Casp8^{f/f}Nemo^{f/f}* and *Casp8 Δ Nemo Δ* Δ CN60 cells treated with TNF- α (10 ng/mL), illustrating prominent nuclear translocation of p65 in floxed cells (white arrows) and its attenuation in *Casp8/Nemo*-deleted cells. (C) Quantification of p65-positive nuclei in adv-LacZ- versus adv-Cre-infected Δ CN60 cells after TNF- α stimulation confirms significantly decreased NF- κ B activation upon combined loss of *Casp8* and *Nemo*, validating Δ CN60 as a robust model for TNF- α -dependent NF- κ B signaling.

3.4. Morphological, Ultrastructural, and Cytoskeletal Characterization of Δ CN60 Cells

Under phase-contrast light microscopy, Δ CN60 cells formed a tightly adherent monolayer of predominantly polygonal cells with an epithelial-like morphology and moderate size heterogeneity, consistent with their hepatocytic origin from DEN-induced HCC (Figure 4A). Individual cells exhibited abundant cytoplasm and centrally to eccentrically located round nuclei with one or more prominent nucleoli, features typical of hepatocyte-derived tumor cells. At higher confluence, cells established close cell–cell contacts and organized into compact clusters and sheet-like arrangements, while occasionally less differentiated, spindle-shaped cells were observed at the periphery of colonies, reflecting the tumor-derived and partially progenitor-like nature of the line. Fluorescence staining with Alexa Fluor-conjugated phalloidin further revealed a well-developed F-actin cytoskeleton with intense cortical actin along cell borders and a fine submembranous network, underscoring the epithelial organization of Δ CN60 and the integrity of intercellular contacts (Figure 4B). Nuclear counterstaining with DAPI confirmed a largely uniform nuclear size and chromatin distribution, without evidence of contaminating non-parenchymal cell populations, in agreement with the hepatocyte-derived and genetically defined origin of the cell line.

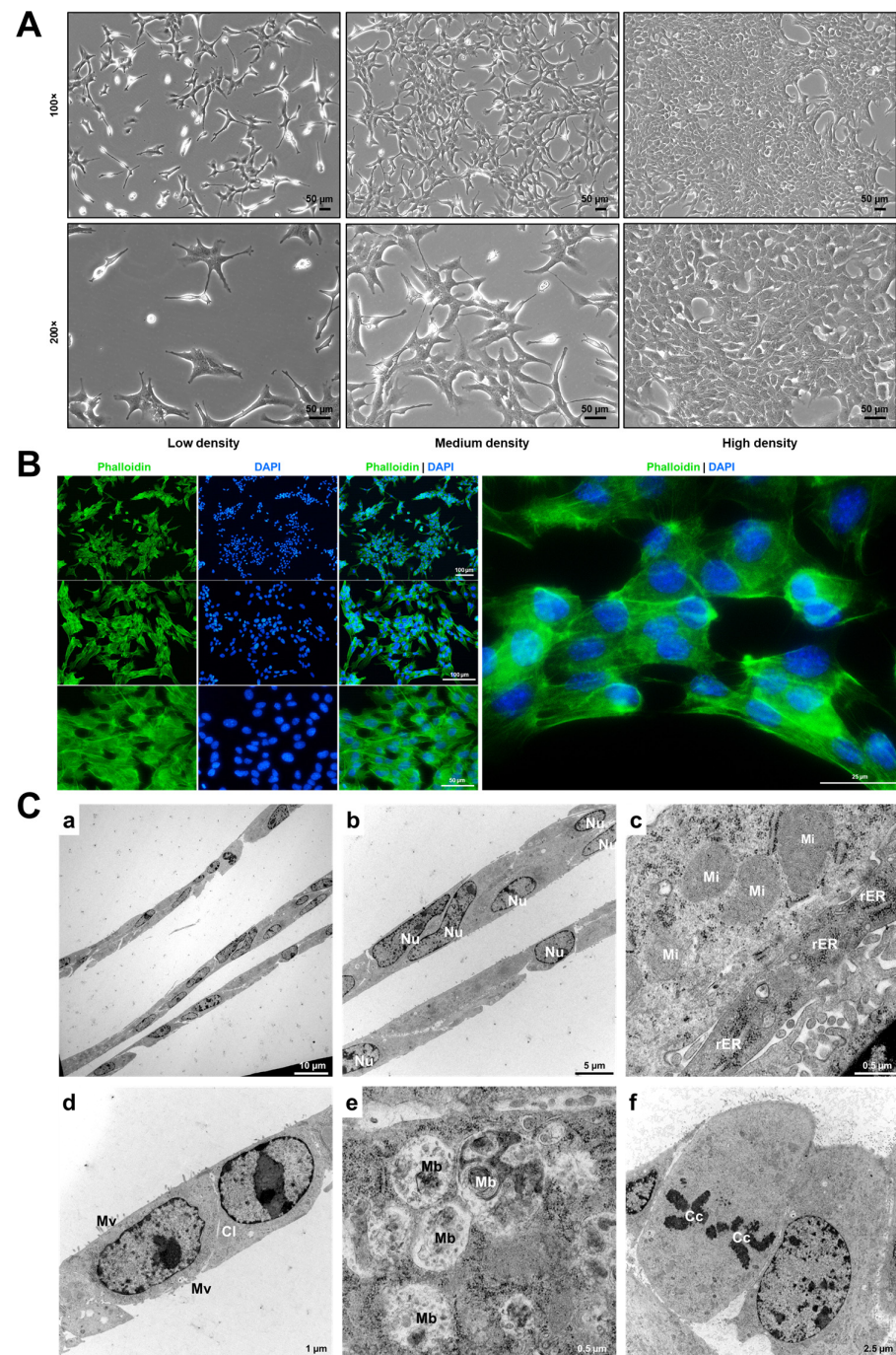


Figure 4. Morphological, cytoskeletal, and ultrastructural characterization of Δ CN60 cells. **(A)** Phase-contrast microscopy of Δ CN60 cultures at different cell densities showing a predominantly polygonal, epithelial-like morphology with abundant cytoplasm and round nuclei containing one or more prominent nucleoli. At low density, individual cells are clearly separated, whereas increasing confluence leads to the formation of compact clusters and sheet-like monolayers with pronounced cell–cell contacts. Occasionally, less differentiated spindle-shaped cells are observed at the colony periphery, reflecting the tumor-derived and partially progenitor-like nature of the line. Representative images are shown at 100 $\times$ and 200 $\times$ magnification. Scale bars: 50 μ m. **(B)** Visualization of the F-actin cytoskeleton in Δ CN60 cells. Cultured cells were stained with Alexa Fluor 488TM phalloidin to detect filamentous actin (green), and nuclei were counterstained with DAPI (blue). Fluorescence microscopy images acquired at different magnifications reveal a prominent cortical F-actin belt along cell borders and a fine submembranous actin meshwork, supporting the epithelial organization and stability of intercellular contacts in the Δ CN60 monolayer. Scale bars correspond to 100 μ m, 50 μ m, or 25 μ m as

indicated. (C) Transmission electron microscopy of Δ CN60 cells illustrating hepatocyte-derived ultrastructure. Low- and intermediate-magnification images show cells with large, oval, predominantly euchromatic nuclei (Nu) and a thin rim of cytoplasm. High-magnification views reveal elongated mitochondria (Mi) and extended cisternae of rough endoplasmic reticulum (rER), indicating preserved metabolic and secretory capacity. Canaliculus-like lumina (Cl) between adjacent cells are highlighted, and microvilli-like membrane protrusions (Mv), some of which are partially lost during fixation, are indicated. An additional figure depicting these microvilli-like structures is provided in Figure S2. Cytoplasmic regions containing multivesicular and multilamellar bodies (Mb), corresponding to late endosomal/lysosomal or autophagic compartments, are also detectable. In addition, mitotic cells with condensed chromosomes (Cc) demonstrate the proliferative capacity of the cell population. Images were captured at magnifications of (a) 1000 $\times$, (b) 2784 $\times$, (c) 27,800 $\times$, (d) 6000 $\times$, (e) 27,800 $\times$, and (f) 3597 $\times$, respectively. Scale bars: 10 μ m (a), 5 μ m (b), 0.5 μ m (c), 1 μ m (d), 0.5 μ m (e), and 2.5 μ m (f).

TEM provided further insight into the ultrastructural organization of Δ CN60 cells and confirmed their hepatocytic identity. Ultrastructural examination revealed a high nuclear-to-cytoplasmic ratio with round to oval nuclei containing dispersed euchromatin and distinct nucleoli, indicative of active transcriptional activity typical of tumor cells (Figure 4C). The cytoplasm contained numerous mitochondria and well-developed rough endoplasmic reticulum cisternae, consistent with the preserved protein-synthetic and metabolic functions of hepatocyte-derived cells. The overall organelle organization and abundance suggested a partially dedifferentiated hepatoma phenotype rather than fully mature hepatocytes. In higher-magnification TEM images, intercellular clefts resembling canaliculus-like lumina were observed between adjacent cell membranes, and short microvilli-like membrane protrusions were visible within these structures (Figure S2). Electron-dense membrane appositions at lateral cell borders were consistent with junction-like cell–cell contacts and supported the epithelial organization observed by light microscopy, although definitive assignment to specific tight or adherens junction subtypes would require additional junction-marker analyses. Together, the light microscopic, ultrastructural, and cytoskeletal analyses confirm that Δ CN60 represents a hepatocyte-derived hepatoma line with epithelial organization, preserved hepatocyte-like organelle architecture, and a well-developed cortical actin network, reflecting the partial dedifferentiation and plasticity inferred from its transcriptional and functional characteristics.

3.5. Genetic Characterization of Δ CN60 Cells

STR profiling was conducted to assess the genetic identity of Δ CN60. The STR analysis revealed distinct profiles that differentiate this cell line from other murine hepatocytic lines, such as AML12 and Hepa1-6, used in our laboratory (Table 2).

Table 2. Short tandem repeat (STR) profiling of Δ CN60 cells.

STR Marker ¹	Known Allele Range ¹	Sample Results (Hepa1-6) ¹	Sample Results (AML12) ²	Sample Results (Δ CN60)
STR 1-1 (MCA-1-1)	10–19	14, 15, 16	11	10,16
STR 1-2 (MCA-1-2)	12–30.1	13	13	19
STR 2-1 (MCA-2-1)	8–17.1	14	9	15
STR 3-2 (MCA-3-2)	9–25	10	12	14
STR 4-2 (MCA-4-2)	13–23.3	18.3, 19.3	20.3	20.3
STR 5-5 (MCA-5-5)	11–22	17	14,15	14, 17
STR 6-4 (MCA-6-4)	12.2–21	18, 19	15.3	18
STR 6-7 (MCA-6-7)	11–26	15	12	15

Table 2. Cont.

STR Marker ¹	Known Allele Range ¹	Sample Results (Hepa1-6) ¹	Sample Results (AML12) ²	Sample Results (Δ CN60)
STR 7-1 (MCA-7-1)	19.2–33.2	25.2	29	27.2
STR 8-1 (MCA-8-1)	6–19	15	14, 15	16
STR 9-2 (MCA-9-2) ³	7.1–19	NN	15	16
STR 11-2 (MCA-11-2)	12–26.2	16	18	16
STR 12-1 (MCA-12-1)	15–24.1	17	19	17
STR 13-1 (MCA-13-1)	11–20.1	17.1	15	17
STR 15-3 (MCA-15-3)	12–31.3	17, 18, 19	21.3	22.3
STR 17-2 (MCA-17-2)	11–22	14	13, 15	15, 16
STR 18-3 (MCA-18-3)	13–28	16, 17	21	17
STR 19-2 (MCA-19-2)	10–16	10, 11	13	12, 13
STR X-1 (MCA-X-1)	15.2–20.1	25	26	26

¹ Data were taken from [25]; ² data were taken from [21]; ³ data were taken from [26]. The chromatograms of the individual variant sites are depicted in Figure S2. “NN” indicates that no reliable allele call was available for the respective marker in the referenced dataset.

The karyotypic analysis of the Δ CN60 cell line based on inverted DAPI-banding revealed an unaltered chromosomal structure, indicating stability in its genetic makeup (Figure 5A). Representative metaphase spreads demonstrated a normal diploid complement of 40 murine chromosomes without obvious structural abnormalities or aneuploidy. To further investigate chromosomal integrity, we utilized multicolor fluorescence in situ hybridization (mFISH) analysis, which allowed visualization of the distinct spectral color signature of each chromosome (Figure 5B). This analysis confirmed the findings obtained by inverted DAPI-banding and demonstrated the absence of numerical or interchromosomal rearrangements in Δ CN60 cells. The stability of the karyotype suggests that the cellular behavior observed in experimental settings is likely to remain consistent and reproducible over time. Finally, high-resolution mcb analysis was performed to further refine the cytogenetic characterization (Figure 5C). This approach allows the detection of intra- and interchromosomal rearrangements larger than approximately 5 Mb. Consequently, no molecular-cytogenetically detectable chromosomal aberrations, including inversions, insertions, translocations, or large copy-number alterations, were identified in the Δ CN60 cell line.

3.6. Transcriptomic Profiling Reveals a Core Hepatocytic Program with Partial Loss of Mature Metabolic Functions

To further confirm the hepatocytic origin of Δ CN60 cells, we conducted bulk mRNA NGS (Table S3) and examined curated marker panels for hepatocytes (Table S4), hepatic stellate cells (Table S5), cholangiocytes (Table S6), and Kupffer cells (Table S7). Δ CN60 cells showed strong expression of numerous hepatocyte-enriched transcription factors, such as *Hnf4a*, *Foxa1-3*, *Hhex*, and *Prox1*, along with classic hepatocyte products like *Alb*, *Apob*, *Cp*, *Ambp*, *Gc*, and genes related to the urea cycle (*Asl*, *Ass1*, *Glul*). Additionally, essential enzymes involved in mitochondrial β -oxidation and cholesterol/lipid biosynthesis, including *Acadm*, *Acat2*, *Acly*, *Hmgcs1*, *Msmo1*, and *Scd*, were expressed at high TPM values, indicating that Δ CN60 maintains a significant hepatocyte-like metabolic program. However, several characteristics of fully differentiated adult hepatocytes exhibited either low or absent expression, such as the gluconeogenic genes *G6pc* and *Pck1*, the glycolytic regulator *Gck*, the bile acid synthesis enzyme *Cyp7a1*, and the bile acid transporter *Slc10a1*.

There was also minimal expression of the glucose transporter *Slc2a2* (Glut2), suggesting a partial metabolic dedifferentiation commonly seen in hepatoma cell lines. Δ CN60 also expressed fetal/tumor-associated hepatocyte markers like *Afp* and *Gpc3*, consistent with previous findings of AFP and CD133 expression at the mRNA and protein levels, further confirming its classification as a hepatoma line with progenitor-like characteristics.

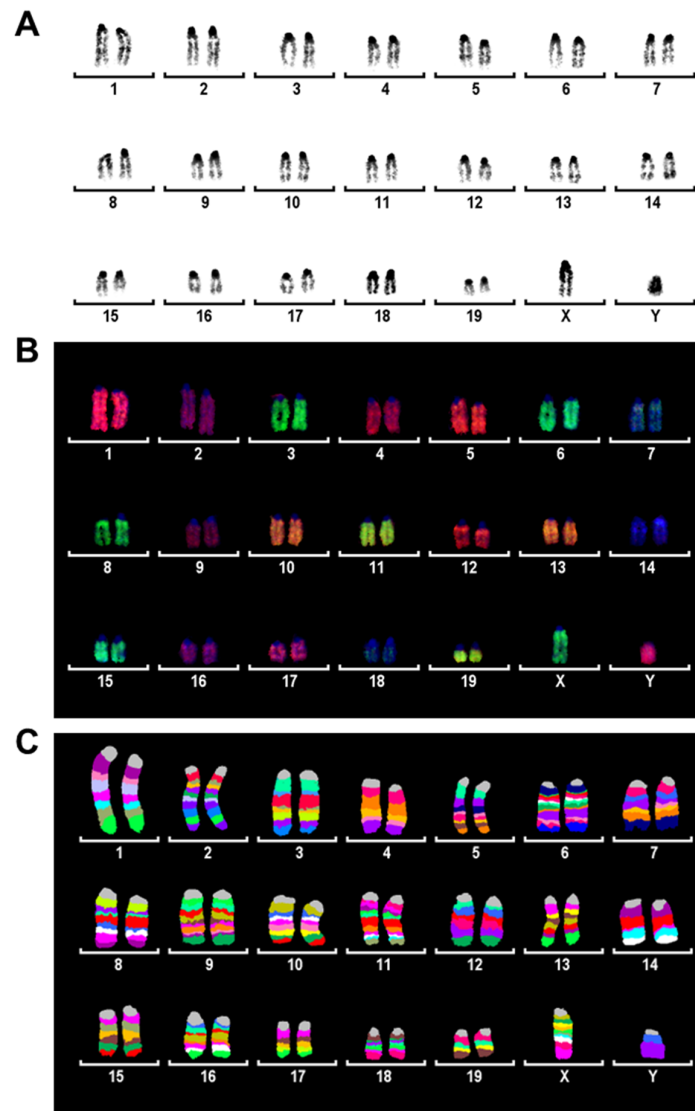


Figure 5. Cytogenetic characterization of Δ CN60 cells by multicolor fluorescence in situ hybridization (mFISH) and multicolor banding (mcb). (A) Representative metaphase spread of Δ CN60 cells after colcemid arrest and hypotonic treatment, visualized by inverted DAPI-banding, showing a normal diploid complement of 40 murine chromosomes. (B) mFISH analysis of the same metaphase spread, in which each chromosome is labeled with a specific spectral color combination, confirming the absence of numerical or interchromosomal rearrangements. (C) Analysis summarizing 24 chromosome-specific mcb experiments performed in the Δ CN60 cell line, allowing high-resolution detection of intra- and interchromosomal rearrangements. No chromosomal alterations were identified, supporting the cytogenetic stability of Δ CN60 cells.

3.7. Minimal Hepatic Stellate and Kupffer Cell Signatures but Pronounced Biliary/Progenitor Marker Expression

Analysis of hepatic stellate cell markers showed that canonical myofibroblastic genes indicative of hepatic stellate cells, such as *Acta2*, *Des*, *Gfap*, *Tagln*, *Pnpla3*, *Fap*, and *Hgf* were not detected in Δ CN60 cells. Fibrillar collagens (*Col1a1*, *Col1a2*, *Col3a1*) and proteoglycans

(*Bgn*, *Dcn*) were present but at low TPM values compared with *Gapdh*, indicating some extracellular matrix production. In contrast, several matricellular and secreted factors typically associated with stellate cells and tumor stroma, including *Cyr61/Ccn1*, *Sparc*, *Ctgf/Ccn2*, *Igfbp3*, and particularly *Igfbp7*, showed moderate to very high transcript levels, suggesting that Δ CN60 hepatoma cells themselves produce components that, in vivo, would partly derive from non-parenchymal cells.

The cholangiocyte marker panel revealed a surprisingly strong biliary/progenitor gene signature in Δ CN60 cells. Δ CN60 showed very high expression of the ductular keratin *Krt19* and the secreted phosphoprotein *Spp1* (osteopontin), together with substantial expression of the transcription factors *Sox9*, *Hnf1b*, and *Onecut2* that are characteristic of biliary epithelial cells and liver progenitors. Additional cholangiocyte-associated genes were also clearly expressed, including *Aqp1*, *Itgb4*, *Slc4a2*, *Lcn2*, *Elf3*, *Muc5b*, and the trefoil factors *Tff1* and *Tff2*, further supporting a biliary/progenitor-like transcriptional component. By contrast, some markers of mature large bile ducts such as *Agr2* and *Kcnn2* were not detected, and *Krt7*, *Cftr*, and *Cldn4* were present only at low levels, indicating a partial rather than fully cholangiocytic differentiation state. Taken together with the hepatocyte marker profile, these data support a hybrid hepatocyte-cholangiocyte or liver-progenitor-like phenotype of Δ CN60 cells.

Similarly, Kupffer cell-specific genes were largely absent from the Δ CN60 transcriptome. Key macrophage markers, including *Adgre1* (F4/80), *Adgre4*, *C1qa*, *C1qb*, *C1qc*, *Cd163*, *Clec4f*, *Marco*, *Msr1*, *Tnf*, and *Il1b*, were not expressed, which argues against contamination with liver-resident macrophages or a macrophage-like identity. Instead, Δ CN60 expressed a subset of innate immune and iron-handling genes that are known to be shared between hepatocytes and macrophages, such as *Cd14*, *Tlr4*, *Slc40a1*, and *Ftl*, consistent with the established role of hepatocytes in sensing inflammatory stimuli and regulating iron metabolism rather than indicating a Kupffer cell origin. Additional stress- and signaling-related genes including *Cd38*, *Mmp13*, *Ppard*, *Stard5*, and *Hfe* were also expressed, highlighting that Δ CN60 is an inflammation-responsive cell line. Overall, the NGS data confirm the hepatocytic origin of Δ CN60, reveal a loss of several functions typical of fully differentiated hepatocytes, and uncover pronounced cholangiocytic/progenitor and inflammatory signatures that likely contribute to the specific behavior of this *Casp8/Nemo*-conditional hepatoma model.

To validate our NGS data, we next performed quantitative real-time RT-qPCR and Western blot analysis for representative markers. In accordance with the RNA-seq data, RT-qPCR analysis demonstrated that Δ CN60 cells maintain robust expression of core hepatocytic markers such as *Alb*, *Hnf4a*, *Foxa2*, *Krt18*, *Apob*, and *Apoe*, while also expressing high levels of fetal/progenitor and iron-handling genes, including *Afp*, *Sox9*, *Fth1*, and *Ftl1*. Several transcripts differed significantly from AML12 hepatocytes, a non-tumorigenic murine hepatocyte cell line commonly used as a reference model for differentiated hepatocytes, further supporting the idea of a hepatocyte-derived yet progenitor-biased tumor phenotype (Figure 6A). Western blot analysis confirmed the presence of HNF4 α and β -catenin in Δ CN60 cells. It also showed expression of the cell cycle regulator cyclin D1, indicating a proliferative hepatoma state. Detection of vimentin and ferritin light chain (FTL1) further supported partial epithelial plasticity and altered iron metabolism characteristic of transformed, dedifferentiated liver cells (Figure 6B). The consistent mRNA and protein expression profiles validate the transcriptomic characterization of Δ CN60, confirming its mixed mature-fetal hepatocytic and biliary/progenitor signature. This underscores its value as a genetically defined hepatoma model that combines preserved hepatocyte identity with cancer-associated dedifferentiation.

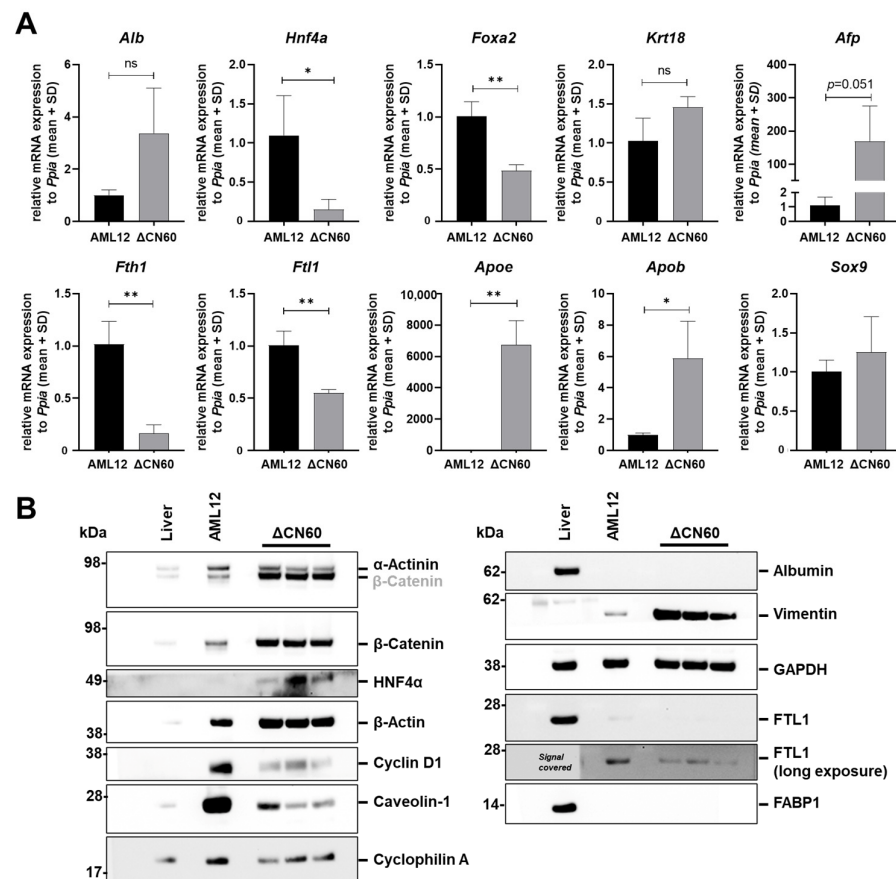


Figure 6. Transcript and protein expression analysis of Δ CN60 cells compared with AML12 hepatocytes. **(A)** Comparison of gene expression in Δ CN60 and AML12 cells by RT-qPCR. RT-qPCR was conducted for albumin (*Alb*), hepatocyte nuclear factor 4- α (*Hnf4a*), forkhead box A2 (*Foxa2*), keratin 18 (*Krt18*), alpha-fetoprotein (*Afp*), ferritin heavy polypeptide 1 (*Fth1*), ferritin light polypeptide 1 (*Ftl1*), apolipoprotein E and B (*Apoe*, *Apob*), and SRY (sex determining region Y)-box 9 (*Sox9*). Data are presented as fold-change normalized to peptidylprolyl isomerase A (*Ppia*), with Δ CN60 normalized to AML12 expression. Statistical analysis was performed using an unpaired *t*-test, as all data were normally distributed. Significant differences between the groups are denoted by asterisks (* $p \leq 0.05$, ** $p < 0.01$, ns = not significant). Data are displayed as the mean $\pm$ SD. **(B)** Validation of next-generation sequencing (NGS) data in Δ CN60 cells by Western blot analysis. Western blot analysis was conducted on whole-cell protein lysates from Δ CN60 cells (40 μ g) using antibodies against α -actinin, β -catenin, HNF4 α , cyclin D1, caveolin-1, albumin, vimentin, ferritin light chain (FTL1), and fatty-acid-binding protein 1 (FABP1). Hepatic AML12 cells (40 μ g) and murine liver extracts (80 μ g) were included as controls. β -actin, cyclophilin A, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading controls. To visualize the FTL1 signal in Δ CN60 cells, the membrane was exposed for a longer period. Therefore, the portion of the membrane containing liver protein samples was covered during signal development to prevent signal saturation. The signal marked in gray in the α -actinin panel resulted from previous probing of the membrane with the β -catenin antibody.

4. Discussion

In this study, we established Δ CN60, a DEN-induced murine hepatoma cell line derived from *Casp8^{f/f}/Nemo^{f/f}* mice that preserves floxed alleles of both genes and enables efficient Cre-mediated generation of *Casp8 Δ Nemo Δ* derivatives in an isogenic hepatocyte-derived background. This genetic configuration allows Δ CN60 to bridge observations from *Casp8/Nemo* conditional mouse models with mechanistically tractable in vitro hepatocyte systems. Functionally, combined *Casp8* and *Nemo* deletion in Δ CN60 reproduced key features of hepatocyte responses observed in *Casp8*- and *Nemo*-deficient

mouse livers [14,17,18,27]. *Casp8^ΔNemo^Δ* ΔCN60 cells showed reduced proliferation, impaired cell-cycle progression, and decreased expression of cyclin A2 (*Ccna2*) and cyclin D1 (*Ccnd1*), supporting non-apoptotic roles of Caspase-8 and NEMO in hepatocyte proliferation, consistent with previous links between Caspase-8, RIPK1, NF-κB, and JNK during liver regeneration [18,27,28]. In parallel, loss of *Casp8* and/or *Nemo* increased AFP and CD133 expression while reducing albumin levels, indicating enhanced dedifferentiation and acquisition of progenitor-like features.

ΔCN60 also recapitulates central aspects of TNF-α signaling, including NF-κB activation and TNF-induced cell death responses [29,30]. In *Casp8^{f/f}Nemo^{f/f}* ΔCN60 cells, TNF-α induced nuclear accumulation of NF-κB p65, whereas *Casp8^ΔNemo^Δ* cells showed markedly impaired p65 nuclear translocation, confirming the importance of NEMO for canonical TNF-α-induced NF-κB activation in this model. The increased sensitivity of *Casp8/Nemo*-deficient ΔCN60 cells to prolonged TNF-α exposure is consistent with reports linking loss of Caspase 8 or NEMO to RIPK1/RIPK3-dependent cell death signaling in hepatocytes [3,31,32]. However, because RIPK1/RIPK3 activation and downstream necroptotic effectors were not directly analyzed, these findings indicate potential susceptibility to necroptosis-associated signaling rather than definitive proof of necroptotic cell death. Future studies should therefore assess TNF-α-induced necroptosis using dedicated assays, including Annexin V/propidium iodide-based cell-death analyses, RIPK1/RIPK3/MLKL activation, and genetic or pharmacological inhibition of necroptotic signaling components.

Morphological, ultrastructural, and cytoskeletal analyses confirmed the hepatocytic origin and epithelial organization of ΔCN60 while revealing features of a partially dedifferentiated hepatoma. ΔCN60 cells formed adherent polygonal epithelial-like monolayers with occasional spindle-shaped cells at colony margins, consistent with phenotypic heterogeneity. TEM showed euchromatic nuclei, abundant mitochondria, well-developed rough endoplasmic reticulum, canaliculus-like lumina with microvilli, and junction-like contacts, indicating preserved hepatocyte-like organelle architecture and partial polarization. F-actin staining further demonstrated a prominent cortical actin network supporting epithelial cell–cell adhesion.

A major strength of ΔCN60 is its favorable genetic profile. Inverted DAPI banding, mFISH, and mcb analyses revealed an apparently unaltered diploid complement of 40 murine chromosomes without detectable structural rearrangements, gains, losses, or aneuploidy, contrasting with the chromosomal complexity frequently reported for cancer cell lines [33,34]. STR profiling further demonstrated a distinct genetic signature separating ΔCN60 from AML12 [21] and Hepa1-6 [25], excluding cross-contamination with these commonly used murine liver cell lines. Together, the diploid karyotype in the analyzed passages and unique STR profile support the reliability and reproducibility of the model, although stability should not be overgeneralized beyond the examined culture conditions and passages.

Bulk RNA-seq and RT-qPCR showed that ΔCN60 retains a core hepatocyte identity while displaying partial metabolic dedifferentiation and a strong biliary/progenitor signature. ΔCN60 expressed hepatocyte-associated regulators and products, including *Hnf4a*, *Foxa1-3*, *Hhex*, *Prox1*, *Alb*, *Apob*, *Cp*, *Ambp*, and *Gc*, as well as genes involved in lipid, amino acid, and urea metabolism, such as *Acadm*, *Acat2*, *Acly*, *Hmgcs1*, *Msmo1*, *Glul*, *Ass1*, and *Asl*. These data, supported by Western blot validation of markers including *Alb*, *Hnf4a*, *Foxa2*, *Krt18*, *Apob/Apoe*, *Fabp1*, and β-catenin, confirm a hepatocyte-derived protein profile. At the same time, low or absent expression of adult hepatocyte markers, such as *G6pc*, *Pck1*, *Gck*, *Cyp7a1*, *Slc10a1*, and *Slc2a2*, indicates loss of mature gluconeogenic and bile acid-related functions, consistent with a transformed hepatoma phenotype. Expression of *Afp*, *Gpc3*, *Fth1*, and *Fhl1* further supports a fetal/progenitor-like and iron-responsive state.

Beyond its hepatocytic core, ΔCN60 showed high expression of biliary/progenitor-associated genes, including *Krt19*, *Spp1*, *Sox9*, *Hnf1b*, *Onecut2*, *Aqp1*, *Itgb4*, *Slc4a2*, *Elf3*, *Muc5b*, *Tff1*, and *Tff2*, whereas markers of mature large bile ducts, such as *Agr2* and *Kcnn2* were absent, and *Krt7* and *Cldn4* remained low. This indicates a partial hybrid hepatocyte-cholangiocyte or liver-progenitor-like state rather than full cholangiocyte differentiation. In addition, ΔCN60 expressed matricellular and stromal factors, such as *Ccn1/Cyr61*, *Sparc*, *Ctgf/Ccn2*, *Igfbp3*, and *Igfbp7*, which are unusual for fully differentiated hepatocytes but relevant to liver cancer and apoptosis [32,35–38]. The absence of canonical myofibroblastic stellate cell markers (*Acta2*, *Des*, *Gfap*, *Tagln*, *Pnpla3*, *Fap*, *Hgf*) [39,40] and Kupffer cell-specific genes (*Adgre1*, *Clec4f*, *Marco*, *Cd163*, *C1qa-c*) [41,42] argues against contamination by hepatic stellate cells or resident macrophages. Instead, expression of shared inflammatory and iron-regulatory genes, such as *Cd14*, *Tlr4*, *Slc40a1*, *Ftl*, *Mmp13*, *Ppard*, and *Hfe* is consistent with hepatocyte-intrinsic inflammatory and iron-sensing functions. Overall, ΔCN60 represents a hepatocyte-derived, progenitor-biased tumor line combining hepatocytic, biliary, stromal-like, and inflammatory traits, reminiscent of ductular reaction or combined HCC-biliary phenotypes observed in chronic liver injury [43].

Compared with commonly used murine hepatocytic lines such as AML12 [8,21] and Hepa1-6 [9] and human hepatoma lines including HepG2 [4], Hep3B [4], Huh7 [5,6], and HepaRG [7], ΔCN60 offers the advantage of direct derivation from a defined conditional *Casp8^{fl/fl}Nemo^{fl/fl}* mouse model. This facilitates integration of in vitro experiments with in vivo data from the same genetic background. Its authenticated STR profile, diploid karyotype in the passages analyzed, TNF-α-responsive NF-κB axis, conditional Caspase-8/NEMO/RIPK1/RIPK3 signaling context, and intrinsic biliary/progenitor signature distinguish it from many long-established hepatic tumor lines with complex or incompletely defined chromosomal alterations [6,21]. Although primary hepatocytes remain the gold standard for fully differentiated liver functions [44], their limited lifespan, rapid dedifferentiation, and resistance to stable genetic manipulation make ΔCN60 useful for gain- and loss-of-function studies, high-content signaling analyses, and chronic stimulation experiments. Thus, ΔCN60 complements existing liver cell models and supports the 3R principles by enabling mechanistic in vitro studies that may reduce and refine subsequent animal experiments [45].

At the same time, ΔCN60 should not be considered a universal surrogate for fully differentiated murine hepatocytes. Its partial loss of adult metabolic functions limits its suitability for studies requiring precise modeling of gluconeogenesis, xenobiotic metabolism, bile acid synthesis, or bile acid transport. Such studies should be complemented by primary hepatocytes or more differentiated models. Conversely, the progenitor-like and plastic phenotype of ΔCN60 makes it particularly suitable for investigating how inflammatory cues, Caspase-8/Nemo signaling, and necroptosis-associated pathways influence hepatocyte identity, ductular reactions, and tumor progression.

In summary, ΔCN60 combines an authenticated genetic background, a preserved but partially dedifferentiated hepatocytic identity, a pronounced biliary/progenitor and inflammatory transcriptional profile, and a conditional *Casp8/Nemo* system that reproduces key aspects of TNF-α-induced NF-κB activation, cell-cycle regulation, and cell-death signaling. These features establish ΔCN60 as a versatile in vitro platform for studying Caspase-8/NEMO/RIPK1/RIPK3 signaling, hepatocyte plasticity, and TNF-α-mediated liver injury while supporting the reduction and refinement of in vivo experimentation.

5. Limitations of This Study

Several limitations should be considered when using ΔCN60. First, all functional conclusions are based on a single murine DEN-induced HCC-derived cell line from a

Casp8^{f/f}Nemo^{f/f} background, and the *in vivo* tumorigenicity of ΔCN60 after establishment in culture was not assessed by transplantation or comparable tumor-forming assays. Therefore, although ΔCN60 is referred to as a DEN-induced HCC-derived hepatoma cell line based on its tumor origin, epithelial hepatoma-like morphology, tumor/progenitor marker expression, and hepatocyte-derived transcriptional program, the capacity of cultured ΔCN60 cells to form tumors *in vivo* remains to be experimentally demonstrated. Consequently, the term “hepatoma cell line” in this study denotes the DEN-induced HCC-derived origin and molecular phenotype of the cells rather than direct confirmation of transplantation-based tumorigenicity. The findings may therefore not be directly generalizable to other tumor etiologies, mouse strains, donor sexes, or human hepatocellular carcinoma.

Second, ΔCN60 was established as a serially passaged adherent tumor-derived cell population and was not generated by single-cell cloning. Although STR profiling and cytogenetic analyses support a distinct and stable identity in the passages examined, subclonal heterogeneity and long-term genetic or epigenetic drift cannot be excluded.

Third, ΔCN60 is maintained as a two-dimensional (2D) monoculture and does not reproduce the full liver microenvironment, including immune, stromal, endothelial, and vascular signals. Fourth, the pronounced fetal/biliary-progenitor signature and partial loss of mature adult hepatocyte metabolic functions limit its use for studies requiring precise modeling of gluconeogenesis, xenobiotic metabolism, bile acid synthesis, or bile acid transport. Fifth, although *Casp8/Nemo*-deficient ΔCN60 cells show altered TNF-α responsiveness and increased sensitivity to prolonged TNF-α exposure, necroptosis was not directly demonstrated in this study. Future experiments should assess RIPK1/RIPK3/MLKL activation, Annexin V/propidium iodide profiles, and the effects of pharmacological or genetic inhibition of necroptotic signaling.

Another limitation is that recombination efficiency was assessed only at the bulk population level, and future studies should quantify or enrich successfully recombined cells using approaches such as single-cell cloning, digital PCR, flow cytometry-based enrichment, or allele-specific quantitative PCR.

Finally, the RNA-seq analysis was performed as a bulk transcriptomic characterization and does not resolve possible subpopulations within the culture. Single-cell transcriptomics, systematic low- and high-passage comparisons, and *in vivo* transplantation assays would further strengthen the characterization of ΔCN60. Nevertheless, the current data establish ΔCN60 as a genetically defined and experimentally useful hepatoma model for investigating Caspase-8/NEMO-dependent TNF-α signaling and hepatocyte plasticity *in vitro*.

6. Conclusions

ΔCN60 is a murine DEN-induced HCC-derived hepatoma cell line established from *Casp8^{f/f}Nemo^{f/f}* mice. It retains floxed *Casp8* and *Nemo* alleles and can be converted by adenoviral Cre treatment into *Casp8/Nemo*-deficient derivatives at the bulk population level. Combined *Casp8/Nemo* deletion reduces proliferation, impairs TNF-α-induced NF-κB p65 nuclear translocation, and promotes a more fetal/progenitor-like marker profile. Together with its distinct STR identity, diploid karyotype in the passages examined, and mixed hepatocytic/biliary-progenitor phenotype, ΔCN60 represents a useful *in vitro* platform for studying Caspase-8/NEMO-dependent TNF-α signaling and hepatocyte plasticity. The model may support future reduction and refinement of animal experiments by enabling focused mechanistic studies in cell culture before selected hypotheses are tested *in vivo*. However, because systematic long-term comparisons between low- and high-passage ΔCN60 cultures were not performed, conclusions regarding genetic and

phenotypic stability should be restricted to the passages analyzed in this study and should not be overgeneralized to extended culture periods.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15151325/s1>, Figure S1: Testing for *Mycoplasma* spp. contamination in murine ΔCN60 cells; Figure S2: Microvilli-like structures in ΔCN60 cells; Figure S3: STR profiling chromatograms of ΔCN60 cells. Table S1: Primers used in this study; Table S2: Antibodies used in this study; Table S3: Gene expression in ΔCN60 cells under standard culture conditions; Table S4: Expression of hepatocyte-specific markers in ΔCN60 cells; Table S5: Expression of hepatic stellate cell-specific genes in ΔCN60 cells; Table S6: Expression of cholangiocyte-specific genes in ΔCN60 cells; Table S7: Expression of Kupffer cell-specific genes in ΔCN60 cells.

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Abbreviations

The following abbreviations are used in this manuscript:

3R	Replacement, reduction and refinement
CLASTR	Cellosaurus STR Similarity Search Tool
Cre	Cre recombinase
DEN	Diethylnitrosamine
ΔCN60	DEN-induced murine hepatoma cell line derived from <i>Casp8^{f/f}Nemo^{f/f}</i> mice
HCC	Hepatocellular carcinoma
IKKγ	Inhibitor of κB kinase gamma
mcb	Multicolor banding

mFISH	Multicolor fluorescence in situ hybridization
NEMO	NF- κ B essential modulator (also known as IKK γ)
RNA-seq	RNA sequencing
RT-PCR	Reverse transcription polymerase chain reaction
SKY	Spectral karyotyping
STR	Short tandem repeat
TBP	TATA-box binding protein
TEM	Transmission electron microscopy
TPM	Transcripts per million
TRAIL	TNF-related apoptosis-inducing ligand

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