Loss of lysosomal membrane protein NCU-G1 in mice results in spontaneous liver fibrosis with accumulation of lipofuscin and iron in Kupffer cells

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ABSTRACT
Human kidney predominant protein, NCU-G1, is a highly conserved protein with an unknown biological function. Initially described as a nuclear protein, it was later shown to be a bona fide lysosomal integral membrane protein. To gain insight into the physiological function of NCU-G1, mice with no detectable expression of this gene were created using a gene-trap strategy, and Ncu-g1gtgt mice were successfully characterized. Lysosomal disorders are mainly caused by lack of or malfunctioning of proteins in the endosomal-lysosomal pathway. The clinical symptoms vary, but often include liver dysfunction. Persistent liver damage activates fibrogenesis and, if unremedied, eventually leads to liver fibrosis/cirrhosis and death. We demonstrate that the disruption of Ncu-g1 results in spontaneous liver fibrosis in mice as the predominant phenotype. Evidence for an increased rate of hepatic cell death, oxidative stress and active fibrogenesis were detected in Ncu-g1gtgt mice as the predominant phenotype. Evidence for an increased rate of hepatic cell death, oxidative stress and active fibrogenesis were detected in Ncu-g1gtgt liver. In addition to collagen deposition, microscopic examination of liver sections revealed accumulation of autofluorescent lipofuscin and iron in Ncu-g1gtgt Kupffer cells. Because only a few transgenic mouse models have been identified with chronic liver injury and spontaneous liver fibrosis development, we propose that the Ncu-g1gtgt mouse could be a valuable new tool in the development of novel treatments for the attenuation of fibrosis due to chronic liver damage.

KEY WORDS: NCU-G1, Lysosome, Fibrosis

INTRODUCTION
Human kidney predominant protein, NCU-G1, is a protein whose function is only beginning to emerge. First reported in 1994 (Eskild et al., 1994), mouse NCU-G1 was cloned in 2001 (Kawamura et al., 2001). Initial studies described NCU-G1 as a nuclear protein with gene regulatory properties (Steffensen et al., 2007), whereas more recent reports have shown that NCU-G1 is a bona fide lysosomal type I integral membrane protein (Sardiello et al., 2009; Schieweck et al., 2009; Schröder et al., 2010). NCU-G1 is highly conserved, proline-rich and ubiquitously expressed; however, it shows no sequence homology to other known proteins.

The endosomal-lysosomal pathway is sensitive to protein dysfunctions, and alterations in protein composition and function can lead to a group of metabolic diseases categorized as lysosomal disorders (Puterman and van Meer, 2004; Parkinson-Lawrence et al., 2010). Intralysosomal accumulation of unmetabolized substrates can result from malfunction of any endosomal-lysosomal protein, but the severity and clinical manifestations vary owing to secondary alterations in other biochemical and cellular pathways (Puterman and van Meer, 2004), such as apoptosis and elevated levels of reactive oxygen species due to impaired lysosomal clearance of damaged organelles through autophagy (Tardy et al., 2004; Kisl’yov et al., 2007; Kurz et al., 2008; Cox and Cachón-González, 2012). Certain cell types are more sensitive to lysosomal dysfunction than others, especially cells with low proliferation capacity (Kisl’yov et al., 2007; Kurz et al., 2008; Cox and Cachón-González, 2012). This is reflected in the vast frequency of neurological symptoms in different lysosomal disorders (Parkinson-Lawrence et al., 2010; Cox and Cachón-González, 2012; Platt et al., 2012). For some disorders, however, other symptoms like liver damage are also a consistent clinical finding (Platt et al., 2012).

Persistent injury to the liver eventually leads to liver fibrosis and deposition of excess connective tissue (Bataller and Brenner, 2005; Friedman, 2008; Gieling et al., 2008; Kisseleva and Brenner, 2011; Ramachandran and Iredale, 2012). The development of liver fibrosis involves several liver cell types and immune cells, including hepatocytes, hepatic stellate cells (HSCs), Kupffer cells (KCs), endothelial cells and infiltrating leukocytes (Friedman, 2008; Pinzani and Macias-Barragan, 2010; Hernandez-Gea and Friedman, 2011). Dying cells secrete reactive oxygen species, and release necrotic and apoptotic fragments, leading to activation of KCs and recruitment of inflammatory cells to the site of injury (Bataller and Brenner, 2005; Friedman, 2008). Activated KCs and recruited leukocytes contribute to the activation of HSCs into myofibroblast-like cells by secreting pro-fibrogenic cytokines, including transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (Bataller and Brenner, 2005; Friedman, 2008). When activated, HSCs start secreting high amounts of extracellular matrix (ECM) proteins that differ from those normally expressed, including fibrillar collagen, thus replacing dead parenchymal cells with fibrous ECM, ultimately compromising the organ function if untreated (Gressner et al., 2007; Mormone et al., 2011).
TRANSLATIONAL IMPACT

Clinical issue
Lysoosomal storage disorders are a group of inherited metabolic disorders that are caused by malfunction of the endosomal-lysosomal pathway, often with devastating consequences for patients. Most lysosomal disorders exhibit neurological symptoms, but internal organ injury and liver damage are consistent clinical findings in several lysosomal disorders. Indeed, recent research has uncovered a direct link between abnormal lysosomal function and liver fibrosis – scarring of the liver in response to chronic injury that eventually leads to liver cirrhosis. Currently, due to a shortage of more suitable models, the development of liver fibrosis is often studied in animal models that involve experimental induction of liver damage, which leads to acute fibrosis. An animal model of chronic fibrosis is needed to help improve our understanding of this condition.

Results
Transgenic mouse models of lysosomal disorders are valuable tools for characterization of the molecular mechanisms behind the broad spectrum of clinical symptoms, including liver fibrosis. In this study, the authors develop a novel mouse model with no detectable expression of the lysosomal membrane protein, NCU-G1, using a gene-trap strategy; NCU-G1 is highly expressed in liver, kidney, lung and prostate and might be crucial for normal lysosomal function. The authors report that disruption of Ncu-g1 in mice does not interfere with fertility, normal embryonic development or growth. They show that the predominant phenotype of Ncu-g1−/− mice is spontaneous development of liver fibrosis, accompanied by splenomegaly. Using gene expression analyses, the authors show that Ncu-g1−/− mice exhibit all the hallmarks of well-established liver fibrosis by the age of 6 months. Finally, they report that NCU-G1 deficiency leads to accumulation of lipofuscin and iron in Kupffer cells in the liver.

Implications and future directions
These results indicate that disruption of NCU-G1 function leads to chronic liver injury and activation of fibrogenesis in mice. Ncu-g1−/− mice therefore represent a potential animal model for studies of these conditions and for the development of treatments for liver fibrosis. Further studies using this model will enable a more extensive characterization of the biochemical and molecular pathways that are affected during chronic liver damage and fibrogenesis, and will determine whether abnormal storage of lipofuscin and iron in Kupffer cells is a primary or secondary effect of NCU-G1 ablation. Finally, these findings suggest that disruption of Ncu-g1 might be causative for a previously undescribed lysosomal disorder that has liver fibrosis as its predominant phenotype.

Several rodent models for inducible liver fibrosis are available (Weller-Normann et al., 2007; Starkel and Leclercq, 2011), but only a few transgenic mouse models have been identified that develop spontaneous liver fibrosis (Fickert et al., 2002; Takehara et al., 2004; Wandziach et al., 2004; Warskulat et al., 2006). Mouse models are widely used in the study of lysosomal disorders and the impact of lysosomal protein deficiency (Parkinson-Lawrence et al., 2010; Platt et al., 2012). However, the physiological consequences of loss of NCU-G1 function have never been characterized before. The lysosomal localization suggests some alterations in the endosomal-lysosomal pathway following Ncu-g1 gene disruption. In this article, we present a new mouse model, created by using a gene-trap strategy to introduce a transcriptional stop codon to intron 1 of the Ncu-g1 gene (Ncu-g1−/−). Ncu-g1−/− mice have no detectable expression of NCU-G1, display normal growth and fertility, and have a life expectancy up to at least 18 months. The predominant phenotype is a spontaneous development of liver fibrosis by the age of 6 months. Analyses of Ncu-g1−/− liver indicate that absence of this lysosomal membrane protein leads to an increased rate of hepatic cell death, oxidative stress, activation of the fibrogenic response, and accumulation of lipofuscin and iron in KCs.

RESULTS
Successful generation of Ncu-g1−/− mice
Breeding of heterozygous mice carrying a gene-trap cassette inserted into intron 1 of the Ncu-g1 gene (Fig. 1A) resulted in three expected genotypes (Ncu-g1wt/wt, Ncu-g1−/−, and Ncu-g1−/+) (Fig. 1B). Expression of mRNA for Ncu-g1 was analyzed to assess any transcription leakage from the gene-trap. Fig. 1C shows a total elimination of Ncu-g1 mRNA in homozygous gene-trap mice compared with wild-type mice in all organs studied. Lysosome-enriched fractions from mouse liver after tyloxapol treatment (Schieweck et al., 2009) were used to confirm the loss of NCU-G1 at the protein level as shown in Fig. 1D. Genotype and sex distributions for a total of 364 pups generated by four heterozygous breeding pairs were analyzed and found to be in accordance with Mendelian distributions (P<0.001), indicating that NCU-G1 was not essential for mouse embryonic development (data not shown). Heterozygotes and homozygotes had similar growth rates as their wild-type siblings. When mated, homozygous Ncu-g1−/− mice produced litters at the same frequency and of a similar litter size as wild-type and heterozygote mating (data not shown).

Phenotype of Ncu-g1−/− mice
At birth, Ncu-g1−/− mice were indistinguishable from wild-type siblings, and displayed no obvious behavioral differences. At the age of 6 months, lack of NCU-G1 resulted in a fibrotic appearance of the liver, with large nodules on the surface (Fig. 2A). However, the liver:body weight ratio was unaffected (Fig. 2A). Splenomegaly was observed in Ncu-g1−/− mice with a nearly doubled spleen:body weight ratio (Fig. 2B). This is probably a result of portal vein hypertention caused by the liver fibrosis. Measurements of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities showed moderate but significant increases in Ncu-g1−/− mice compared with wild-type animals (Fig. 2C). Other organs (kidney, lung, heart, brain) appeared normal and were not further evaluated in this study. Interestingly, Ncu-g1−/− mice were kept until the age of 18 months before termination, indicating that the liver fibrosis does not progress to a lethal condition during this period.

Inflammation and excess collagen in Ncu-g1−/− liver
In order to gain insight into the underlying mechanisms behind the Ncu-g1−/− mouse phenotype, qPCR arrays were used to identify changes in gene expression in groups of genes known to be involved in liver fibrogenesis. This initial screening suggested that genes associated with fibrosis, hepatotoxicity and oxidative stress responses were regulated in Ncu-g1−/− liver (supplementary material Table S1). In addition, a screening of different signal transduction pathways suggested a strong upregulation of genes encoding chemokines and proteins involved in control of the cell cycle (supplementary material Table S1). A selection of these genes was verified by qPCR (see below).

Light-microscopic examination of hematoxylin and eosin (H&E)-stained liver sections from Ncu-g1−/− mice showed infiltration of mononuclear leukocytes and granulocytes in the portal areas with some piecemeal necrosis (Fig. 3A). Widespread deposition of collagen was revealed in sections stained with Masson-Goldner’s trichrome (Fig. 3B). This observation was supported by electron microscopy, showing thick collagen fibers in the space of Disse (Fig. 3C) and sirius-red quantification of total collagen content in liver homogenates (Fig. 3D). In affected areas, loss of hepatocytes and bridging fibrosis resulted in disruption of the normal tissue architecture (Fig. 3A,C). Expression of genes such as Vcam1, Ccl2
and Nfk1, involved in leukocyte recruitment (Fig. 3E), and Mmp2, Mmp9, Timp1 and Col1a2, involved in ECM remodeling (Fig. 3F,G), were found to be elevated in Ncu-g1gt/gt liver, as detected by qPCR analysis, supporting the microscopy findings.

**Increased rate of cell death and oxidative stress in Ncu-g1gt/gt liver**

In the Ncu-g1gt/gt livers, scattered acidophilic hepatocytes were seen (Fig. 3A, insert). Immunoreactivity for active caspase 3, a specific marker for apoptosis, showed an increased number of apoptotic cells in Ncu-g1gt/gt mouse liver (Fig. 4A). Increased levels of active caspase 3 were also confirmed by western blot (Fig. 4B). Leukocyte infiltration and damage to hepatocytes are often associated with increased oxidative stress (Friedman, 2008), as already indicated by qPCR array analyses. Fig. 4C shows the increased levels of expression of a selection of genes associated with oxidative stress: iNos2, Gpx3, Sod3 and Noxa1.

**Active fibrogenesis in Ncu-g1gt/gt liver**

Injury to hepatic cells and oxidative stress are inducers of liver fibrogenesis, a process that involves several cell types including KCs, HSCs and recruited leukocytes (Bataller and Brenner, 2005; Friedman, 2008; Pinzani and Macias-Barragan, 2010; Hernandez-Gea and Friedman, 2011). KCs can be activated upon liver injury by phagocytosis of apoptotic bodies (Canbay et al., 2003a). Analyses of frozen sections from wild-type and Ncu-g1gt/gt livers showed increased staining for CD68, a marker for activated KCs (Kinoshita et al., 2010), in Ncu-g1gt/gt livers (Fig. 5A). To further characterize the KCs, staining for F4/80 revealed swollen and hypertrophic macrophages (Fig. 5B), displaying a post-phagocytic phenotype. Because inflammatory macrophages have been shown to switch to a restorative subset after phagocytosis (Ramachandran et al., 2012), we investigated the expression of an inflammatory (Cd86) and a restorative (Cd81) marker (Ramachandran et al., 2012) by qPCR. Fig. 5C shows a significant increase in Cd86 expression, suggesting that the activated macrophages in Ncu-g1gt/gt livers belong to a pro-inflammatory subtype (Ramachandran et al., 2012).

Activated KCs stimulate HSC activation by secreting reactive oxygen species and pro-fibrotic cytokines, such as TNF-α (Canbay et al., 2003a), TGF-β and PDGF (Bataller and Brenner, 2005; Friedman, 2008). Fig. 5D,E show increased expression of mRNA for all three cytokines in addition to a receptor for TGF-β, thus confirming activation of KCs. When HSCs are activated by pro-fibrotic cytokines, they evolve into myofibroblast-like cells and start expressing α-Sma, Vim, Igfbp3 and collagen (predominantly type I), characteristic for their activation (Boers et al., 2006; Friedman,
individual lobes, whereas the Ncu-g1gt/gt KCs and HSCs in (Fig. 5G). Taken together, these results point to activation of both presence of star-shaped α and Igfbp3 mice (Fig. 2. Loss of NCU-G1 leads to liver fibrosis and splenomegaly in 6-month-old mice. (A) Representative livers from wild-type (WT) and Ncu-g1gt/gt liver. When activated, these cells recruit white blood cells to sites of lesion, depicted in Fig. 3A and immunostaining of liver sections from Ncu-g1gt/gt mice (supplementary material Fig. S2B). Storage in KCs was confirmed by electron microscopy, where large storage vacuoles were observed in this cell type in Ncu-g1gt/gt liver (Fig. 6B). Such structures were not obviously seen in other cell types (data not shown). Affected KCs were identified based on morphology after immunoreactivity for F4/80 (supplementary material Fig. S2B). These cells were also positively stained with Perls’ Prussian blue (Fig. 6C) and supplementary material Fig. S2B), indicating storage of iron in Ncu-g1gt/gt liver KCs. Gene expression analyses showed that Ncu-g1 mRNA was expressed in isolated parenchymal as well as non-parenchymal cells from wild-type liver, ruling out the possibility that storage in KCs, but not hepatocytes, was a result of a cell-type-specific lack of Ncu-g1 expression (data not shown). In support of these findings, the mRNA levels of Hepcidin1 and L-Ferritin, proteins involved in intracellular iron accumulation (Ganz, 2011), were shown to be elevated in Ncu-g1gt/gt liver (Fig. 6D). In addition, the expression of the iron transporter Ferroportin was increased (Fig. 6D).

Accumulation of autofluorescent material in Ncu-g1gt/gt Kupffer cells Dysfunction of lysosomal proteins is often associated with intralysosomal storage of undigested material due to impaired hydrolytic or transport activity (Futerman and van Meer, 2004). Hence, we investigated the possibility of storage in Ncu-g1gt/gt liver lysosomes. Fig. 6A shows immunofluorescence staining for Lamp1, a marker for lysosomes. Lamp1-positive vesicles were massively enlarged. Fluorescence examination revealed that autofluorescent material was present in these enlarged Lamp1-coated vesicles, and colocalized with CD68-positive staining, indicating activated KCs (supplementary material Fig. S2A). Storage in KCs was confirmed by electron microscopy, where large storage vacuoles were observed in this cell type in Ncu-g1gt/gt liver (Fig. 6B). Such structures were not obviously seen in other cell types (data not shown). Affected KCs were identified based on morphology after immunoreactivity for F4/80 (supplementary material Fig. S2B). These cells were also positively stained with Perls’ Prussian blue (Fig. 6C) and supplementary material Fig. S2B), indicating storage of iron in Ncu-g1gt/gt liver KCs. Gene expression analyses showed that Ncu-g1 mRNA was expressed in isolated parenchymal as well as non-parenchymal cells from wild-type liver, ruling out the possibility that storage in KCs, but not hepatocytes, was a result of a cell-type-specific lack of Ncu-g1 expression (data not shown). In support of these findings, the mRNA levels of Hepcidin1 and L-Ferritin, proteins involved in intracellular iron accumulation (Ganz, 2011), were shown to be elevated in Ncu-g1gt/gt liver (Fig. 6D). In addition, the expression of the iron transporter Ferroportin was increased (Fig. 6D).

Next, we investigated the expression and activity of a selection of lysosomal enzymes known to be causative for lysosomal disorders. Immunofluorescence studies of cathepsin D expression showed colocalization with CD68 in liver sections from wild-type and Ncu-g1gt/gt mice (supplementary material Fig. S3). However, the overall expression of cathepsin D was reduced in Ncu-g1gt/gt liver (supplementary material Fig. S3). This finding was confirmed by western blot analyses where the expression of full-length cathepsin D was significantly reduced (Fig. 7A). Cathepsin D reduction has been shown during the process of tissue regeneration (Suleiman et al., 1980; Fernández et al., 2004). Chronic liver injury leads to activation of oval cells after hepatocytes have exhausted their proliferative capacity (Conigliaro et al., 2010), and might contribute to the observed increase in Mmp2 and Mmp9 expression as shown (Fig. 3G). We examined the oval cell compartment by immunofluorescence labeling for the specific marker, A6 (Engelhardt et al., 1993). Supplementary material Fig. S4 shows strongly increased staining for A6 in Ncu-g1gt/gt liver. Further fluorescence studies indicated that the reduced levels of cathepsin D in Ncu-g1gt/gt liver are partially due to the increased number of oval cells, which do not express cathepsin D (Fig. 7B).
DISCUSSION

In this study, we have shown that Ncu-g1<sup>gt/gt</sup> mice were viable and indistinguishable from wild-type siblings with regard to growth and fertility. However, lack of NCU-G1 expression led to spontaneous development of liver fibrosis within 6 months. The Ncu-g1<sup>gt/gt</sup> mice showed elevated serum ALT and AST activity levels, increased deposition of extracellular collagen, activated KCs and HSCs, infiltrating leukocytes and apoptotic cells. In support of these findings, the expression of marker genes for oxidative stress and fibrosis were increased in the Ncu-g1<sup>gt/gt</sup> liver. These phenotypic findings are all key elements in liver fibrogenesis (Bataller and Brenner, 2005; Friedman, 2008; Kisseleva and Brenner, 2008).

Furthermore, autofluorescent lipofuscin and iron accumulated in Ncu-g1<sup>gt/gt</sup> liver KCs. Despite ubiquitous expression of Ncu-g1 (Steffensen et al., 2007; Schieweck et al., 2009) and the verified loss of transcript in all assayed organs, this study suggested that lack of NCU-G1 predominantly affects the liver. The observed splenomegaly is likely a secondary effect due to portal vein hypertension.

Lysosomes are present in nearly every cell of the body, but pathological manifestations of lysosomal disorders can be restricted to specific organs as shown for mice with disrupted lysosomal integral membrane protein 1 (Limp1). These animals are both fertile and viable and do not present any overt phenotype, but they suffer from a disturbed water balance, and hence a kidney pathology (Schröder et al., 2009). Limp1 is ubiquitously expressed, so the kidney pathology could be a result of an organ-specific function of...
Lysosomal cathepsins play a pivotal role in the execution of lysosomal cell death (reviewed in detail in Aits and Jäättelä, 2013). A direct link between hepatocyte cell death and cathepsin B and D leakage into the cytosol is well established (Roberg et al., 1999; Guicciardi et al., 2000; Kågedal et al., 2001; Canbay et al., 2003b). However, in Ncu-g<sup>1<sup>gt/gt</sup></sup> liver the protein levels of cathepsin B and D are not elevated, indicating that the increased level of cell death and liver fibrosis observed in Ncu-g<sup>1<sup>gt/gt</sup></sup> mice is not a result of an increased expression of these proteases. Decreased expression of cathepsin D has been reported for regenerating liver after partial heptectomy, where the hepatocytes proliferate and restore liver volume within ~10 days (Saleim et al., 1980; Fernández et al., 2004). In Ncu-g<sup>1<sup>gt/gt</sup></sup> liver the reduced level of cathepsin D might result from the observed lack of cathepsin D expression in oval cells. Oval cells contribute to liver regeneration when the proliferative capacity of hepatocytes is exhausted or compromised (Coniglio et al., 2010), as often seen in conjunction with chronic liver injury (Roskams et al., 2003; Coniglio et al., 2010), and their activation has been shown to be closely related to inflammation and cytokine production (Knight et al., 2005). Activation of the oval cell compartment, as seen in the Ncu-g<sup>1<sup>gt/gt</sup></sup> liver, indicates a suppressed hepatocyte proliferation at 6 months of age. The transgenic insults in the Ncu-g<sup>1<sup>gt/gt</sup></sup> mouse are chronic and accompanied by massive infiltration of inflammatory cells, increased apoptosis and elevated expression of cytokines, yet these mice maintain their liver:body weight ratio and sufficient liver function to avoid progression to cirrhosis and death.

Spontaneous development of liver fibrosis in combination with a similar longevity as for the Ncu-g<sup>1<sup>gt/gt</sup></sup> mice has been demonstrated in only a few transgenic mouse models. Among these are the Taut<sup>−/−</sup> mouse model and the hepatocyte-specific deletions of Bcl-xL or Mcl-1, all displaying elevated rates of hepatocyte apoptosis as the fibrogenic inducer (Takehara et al., 2004; Warskulat et al., 2006; Vick et al., 2009; Weber et al., 2010). Interestingly, the liver integrity in the Taut<sup>−/−</sup> mouse is maintained by oval cell proliferation (Warskulat et al., 2006). The ability of Ncu-g<sup>1<sup>gt/gt</sup></sup> mice to maintain sufficient liver function and integrity with older age is currently under investigation.

With regard to putatively affected pathways in which NCU-G1 might be involved, the detection of autofluorescent lipofuscin might give some clues. Intralysosomal accumulation of unmetabolized substrates is often caused by deficiencies of soluble enzymes, but some are caused by dysfunction or absence of transmembrane proteins, some of which act as exporters of metabolites through the lysosomal membrane (Futerman and van Meer, 2004). Whether NCU-G1 with its single transmembrane region (Sardielli et al., 2009; Schieweck et al., 2009; Schröder et al., 2010) might serve any export function similar to the Lamp2-mediated cholesterol export from lysosomes remains to be determined (Schneede et al., 2011). Lipofuscin is an intralysosomal polymeric material, undegradable by lysosomal enzymes. It originates from oxidative damage to macromolecules, often associated with degradation of iron-containing proteins (Terman and Brunk, 2004). KCs contribute to the iron metabolism through phagocytosis of erythrocytes (Graham et al., 2007) and excess storage of heme in KCs has been shown to promote oxidative stress, increase hepatic inflammation, induce hepatocyte apoptosis and stimulate fibrogenesis (Otogawa et al., 2007). In support of the hypothesis that storage material in phagolysosomes of KCs can be causative for the inflammation observed in Ncu-g<sup>1<sup>gt/gt</sup></sup> liver, the threefold increase of CD86 expression suggests that the Ncu-g<sup>1<sup>gt/gt</sup></sup> hepatic macrophage subset is pro-inflammatory (Ramachandran et al., 2012). However, this
result needs to be interpreted with caution, because liver sinusoidal endothelial cells are also known to express this marker (Knolle et al., 1998; Knolle and Limmer, 2003).

Ncu-g1gt/gt KCs also show accumulation of iron, as detected by Perls' Prussian blue staining, coinciding with an elevated hepatic expression of the iron storage protein L-Ferritin (Recalcati et al., 2008). The plasma membrane iron exporter, Ferroportin, is expressed exclusively by KCs in liver (Knutson et al., 2003), and its expression is induced by erythrophagocytosis and iron loading (Knutson et al., 2003). During a state of inflammation, elevated Hepcidin1 levels target Ferroportin for degradation (Collins et al., 2008; Ganz, 2011). In agreement with the observed iron accumulation in KCs, the expression of Hepcidin1 and Ferroportin was increased in Ncu-g1gt/gt liver. These results also suggest a possible accumulation of erythrocyte-derived storage material in the phagolysosomes of Ncu-g1gt/gt KCs. An essential heme transporter from the phagolysosomes of macrophages (HRG1) has recently been described (White et al., 2013). Whether the glycosylated luminal tail of NCU-G1 might interact directly with HRG1 or offer protection against lysosomal proteases (Schieweck et al., 2009) remains to be elucidated. However, we cannot finally rule out that lipofuscin and iron deposition are rather a consequence than the cause of liver fibrosis.

In summary, we have created a viable Ncu-g1gt/gt mouse with spontaneous liver fibrosis as the predominant phenotype. The condition progresses slowly, but displays all hallmarks of fibrosis at 6 months of age. Elevated markers for oxidative stress and inflammation were detected in Ncu-g1gt/gt liver, in addition to increased accumulation of lipofuscin and iron in KCs. Fibrosis has many etiologies and consists of acute as well as chronic liver damage. It is well known that reversal of fibrosis is possible if the cause is remedied; however, that is often not possible. In the constant search for improved drugs to treat this condition, various animal models are essential tools. We propose that the Ncu-g1gt/gt mouse would be a valuable model for the study of fibrosis.

Fig. 5. Activated fibrogenic response in Ncu-g1gt/gt liver. (A,B) Immunofluorescence image shows CD68- and F4/80-stained KCs (green). Liver sections were counterstained with DAPI (blue). Infiltrating leukocytes can be seen in Ncu-g1gt/gt liver section (white arrow). CD68-positive cells seem to congregate at the site of leukocyte infiltration. In Ncu-g1gt/gt liver sections, F4/80-positive cells appear swollen and hypertrophic. Scale bars: 50 μm. (C) qPCR analyses of the restorative (Cd81) and the pro-inflammatory (Cd86) macrophage markers show elevated expression of only Cd86 in Ncu-g1gt/gt liver. (D,E) Activated KCs produce pro-fibrogenic cytokines. qPCR analyses show elevated expression of genes characteristic for activated fibrogenesis (Tnf, Pdgbf, Tgfb1, Tgfb2). (F) Presence of activated HSCs was indicated by increased expression of genes characteristic for activated HSCs (α-Sma, Igfbp3, Vim). (G) This was supported by α-Sma staining in liver sections from wild-type (WT) and Ncu-g1gt/gt mice. Note the positive labeling of α-Sma indicating activated HSCs (arrows) in affected areas. Liver sections were counterstained with hematoxylin. Scale bar: 50 μm. (H) Immunofluorescence imaging shows increased levels of myeloperoxidase at the protein level in Ncu-g1gt/gt liver (green). Liver sections were counterstained with DAPI (blue). Note the granular staining pattern of myeloperoxidase, clustering of myeloperoxidase-positive cells and increased levels of invading polymorphonuclear leukocytes with small nuclei. Scale bar: 50 μm. (I) qPCR analyses supported the elevated myeloperoxidase (Mpo) expression in Ncu-g1gt/gt liver. (J) The expression of S100 calcium binding protein A8 (S100a8) was also elevated in Ncu-g1gt/gt liver. (C-F,J) n=4, *P<0.05, **P<0.005, ***P<0.001. Values are expressed as mean ± s.e.m.
mouse could be useful in the development of treatments for attenuation of fibrosis due to chronic liver damage.

MATERIALS AND METHODS

Generation of Ncu-g1gt/gt mice
Embryonic stem cells (ES cells 129S2, P084H04) carrying a gene-trap in the first intron of the Ncu-g1 gene (RIKEN cDNA 0610031J06 gene) were purchased from The German Gene-Trap Consortium (Neuherberg, Germany). The gene-trap leads to a stop in transcription at this point. In collaboration with the Norwegian Transgenic Centre (Oslo, Norway), ES cells were injected into C57BL/6 blastocysts and chimeric mice were obtained. Five chimeric males were mated with C57BL/6 females. The first litters consisted of 29 pups out of which nine were positive for neomycin and seven of these were shown to carry the gene-trap by PCR. Two females and one male were chosen for further breeding and pups were analyzed for presence of the gene-trap by PCR, using 200 ng of ear genomic DNA and the primer pairs shown in supplementary material Table S2 to differentiate between wild-type and gene-trap-carrying mice: FlpROSA, yielding a 811 bp product when the gene-trap cassette is present; and NCUG1, yielding a 562 bp product when the gene-trap cassette is absent. Founders were selected and mated to yield the F1 generation. Mating resulted in mice heterozygous for the Ncu-g1 gene modification. Heterozygotes were mated, leading to the birth of homozygotes. Mice were maintained in an approved animal facility (National Lab Animal Center, The Norwegian Institute of Public Health, Oslo, Norway) with access to food and water ad libitum. Animal handling was according to national laws and regulations.

Analysis of gene expression
RNA extractions from mouse liver, kidney, lung and spleen (n=3) were carried out according to the manufacturer using RNeasy Plus kit from
Qiagen (Hilden, Germany). Expression of Ncu-g1 mRNA was assessed using TaqMan gene expression assays for detection of mouse Ncu-g1: Mm00658309_g1, and mouse Gapdh: Mm99999915_g1 (Life Technologies, Carlsbad, CA, USA), and LightCycler® 480 Probes Master (Roche Applied Science, Mannheim, Germany). Signals below the threshold value (cp >40) were manually set to 40 in the calculations. RNA was extracted from livers of wild-type and Ncu-g1gt/gt mice (n=3), and gene expression was analyzed using the Fibrosis (PAMM-120A, Qiagen), Hepatotoxicity (PAMM-093F, Qiagen), Signal Transduction Pathway Finder (PAMM-014A, Qiagen) and Oxidative Stress and Antioxidant Defense RT2 ProfilerTM Array (PAMM-065A, Qiagen), following the manufacturer’s instructions. To verify array results, and to further elucidate the fibrogenic response, RNA from livers of wild-type and Ncu-g1gt/gt mice (n=4, age 6 months) was analyzed for relative gene expression of selected mRNA transcripts by qPCR (supplementary material Table S3). Analysis was performed using LightCycler® SYBR Green I Master Mix (Roche Applied Science). Relative gene expression was calculated using β-actin and eukaryotic translation elongation factor 2 as reference genes.

Isolation of mouse liver cells from wild-type mice was carried out by the two-step perfusion method as described (Hansen et al., 2002). Liver parenchymal and non-parenchymal cells were separated by differential centrifugation and Pronase E (Merck, Darmstadt, Germany) treatment as described elsewhere (Berg and Boman, 1973). The expression of Ncu-g1 was analyzed as described above.

**Histochemistry**

Livers from 6-month-old Ncu-g1gt/gt (n=6) and wild-type (n=3) mice were collected and fixed in 4% formaldehyde in 0.1 M PBS. Representative tissue blocks (5 mm thick) were embedded in paraffin, sliced in 4-μm sections and stained with H&E, Masson-Goldner’s trichrome staining for collagen, or Perl’s Prussian blue staining for iron according to standard procedures.

**Immunohistochemistry**

For labeling of α-Sma and active caspase 3, formalin-fixed paraffin-embedded sections were deparaffinized, rehydrated and demasked in a microwave oven for 24 minutes in Tris/EDTA buffer (pH 9.1). Rabbit polyclonal anti-α-Sma (1:500, ab5694, Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-active caspase 3 (1:500, G7481, Promega, Madison, WI, USA) were used as antibodies. For labeling of F4/80, formalin-fixed paraffin-embedded sections were deparaffinized, rehydrated and demasked in a microwave oven for 24 minutes in Target retrieval solution (pH 6.0-6.2). Rat monoclonal anti-F4/80 (1:100, 14-4801, eBioscience, San Diego, CA, USA) and peroxidase-conjugated AffiniPure F(ab′)2 fragment mouse anti-rat IgG (H+L) (1:100, 212-036-168, Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, UK) were used as primary and secondary antibodies, respectively.

The antigen-antibody complexes were visualized with Dako Cytomation Envision+ System-HRP (K4007, DAKO North America Inc., CA, USA) using 3,3′-diaminobenzidine as the chromogen. The sections were counterstained with Mayer’s hematoxylin solution.

**Immunofluorescence**

Formalin-fixed livers were sectioned using a Leica 9000s sliding microtome (Wetzlar, Germany) into 40-μm-thick free-floating sections. After blocking with 4% normal goat serum and permeabilization with 0.5% Triton X-100 in 0.1 M phosphate buffer (PB), sections were incubated with appropriate primary antibodies overnight. Antibodies used for immunofluorescence were: rat monoclonal anti-CD68 (1:500, FA-11, AbD Serotec, Oxford, UK);
rabbit polyclonal anti-myeloperoxidase (1:300, Millipore, Billerica, MA, USA); rat monoclonal anti-F4/80 (1:25 cell culture supernatant). The monoclonal antibody against mouse Lamp1 (clone 1D4B) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Rat monoclonal anti-A6 (cell culture supernatant; 1:25) was a kind gift from Valentina Factor and described previously (Engelhardt et al., 1990). Cathepsin D antibody was described previously (Claussen et al., 1997). After washing with 0.25% Triton X-100 in PB and incubation with Alexa-Fluor-488 or -633 secondary antibodies (Molecular Probes, Eugene, OR, USA), sections were counterstained with DAPI and mounted with Mowiol/DABCO. Confocal laser scanning microscopy was performed on a Leica TCS SP2 microscope (Wetzlar, Germany).

Transmission electron microscopy (TEM)

Ncu-g1gt/gt and wild-type mice (n=3, age 6 months) were subjected to perfusion fixation using HEPES buffer (0.1 M, pH 7.2-7.4) with 4% formaldehyde and 2.5% glutaraldehyde. Livers were cut into tissue blocks of 1 mm³, transferred to new fixative solution and kept at 4°C overnight. The samples were rinsed 2-10 minutes in 0.1 M sodium cacodylate buffer prior to post-fixation (2% OsO₄, in 0.1 M sodium cacodylate buffer) for 1 hour, and rinsed 5-10 minutes in distilled water before bulk staining with 1.5% uranyl acetate [(CH₃COO)₂UO₂.2H₂O] in distilled water for 30 minutes in the dark. Fixed tissue samples were dehydrated in a graded series of ethanol for 10 minutes each (70%, 80%, 90%, 96%), 4×15 minutes in 100% ethanol and finally 2-10 minutes in propylene oxide. Following dehydration, tissue samples were infiltrated with epoxy-propylene oxide (1:1) for 30-60 minutes each, then 2×15 minutes in propylene oxide. Following dehydration, tissue samples were infiltrated with epoxy-propylene oxide (1:1) for 30-60 minutes each on a rotary shaker, evaporated overnight, and the following day incubated 1 hour in pure epoxy before embedding in plastic capsules and polymerization at 60°C. Five tissue blocks were selected per individual mouse. Ultrathin sections were obtained using a Leica Ultracut S microtome (Leica, Wetzlar, Germany) with diamond knife. The sections were collected on copper-coated grids and stained with lead citrate for 20 seconds. All electron micrographs were obtained with a CM200 transmission microscope (Philips, Amsterdam, The Netherlands) at 80 kV and at least 20 unique micrographs were analyzed in order to produce representative figures.

Sirius-red quantification of total liver collagen

Quantification of total collagen content from mouse liver tissue was carried out by a sirius-red colorimetric plate assay as described (Klimek et al., 2011). Briefly, livers were homogenized in 10 volumes of CHAPS buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 10 mM CHAPS and protease inhibitors) using an Ultra-Turrax. The samples were diluted using PBS, dried at 37°C overnight on a microtiter plate, and total liver collagen content was determined colorimetrically using 0.1% sirius-red stain (Direct red 80, Sigma-Aldrich, St Louis, MO, USA) in saturated picric acid, and rat tail collagen (Sigma-Aldrich) as standards.

Western blotting

Liver tritosomes were isolated as described elsewhere (Schieweck et al., 2009). Tritosome preparations (20 μg protein) were electrophoresed on NuPAGE® 4-12% Bis-Tris Mini Gels (Life Technologies), and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking, membranes were incubated overnight at 4°C with rabbit anti-NCU-G1 serum (Schieweck et al., 2009) or rabbit anti-Lamp1 (1:1000, C54H11, Cell Signaling, Beverly, MA, USA). This was followed by 1 hour incubation with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:4000, 65-6120, Life Technologies). For liver homogenates, samples were homogenized in 20 volumes of lysis buffer (Tris-buffered saline with protease inhibitors) using an Ultra-Turrax. The samples were diluted using PBS, dried at 37°C overnight on a microtiter plate, and serum ALT and AST activities for 20 minutes, and serum ALT and AST activities were measured at The Central Laboratory, Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science.

Serum analysis

Coagulated blood samples from wild-type and Ncu-g1gt/gt mice (n=4) were centrifuged at 1500 g for 20 minutes, and serum ALT and AST activities were measured at The Central Laboratory, Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science.

Transmission electron microscopy (TEM)

Ncu-g1gt/gt and wild-type mice (n=3, age 6 months) were subjected to perfusion fixation using HEPES buffer (0.1 M, pH 7.2-7.4) with 4% formaldehyde and 2.5% glutaraldehyde. Livers were cut into tissue blocks of 1 mm³, transferred to new fixative solution and kept at 4°C overnight. The samples were rinsed 2-10 minutes in 0.1 M sodium cacodylate buffer prior to post-fixation (2% OsO₄, in 0.1 M sodium cacodylate buffer) for 1 hour, and rinsed 5-10 minutes in distilled water before bulk staining with 1.5% uranyl acetate [(CH₃COO)₂UO₂.2H₂O] in distilled water for 30 minutes in the dark. Fixed tissue samples were dehydrated in a graded series of ethanol for 10 minutes each (70%, 80%, 90%, 96%), 4×15 minutes in 100% ethanol and finally 2-10 minutes in propylene oxide. Following dehydration, tissue samples were infiltrated with epoxy-propylene oxide (1:1) for 30-60 minutes on a rotary shaker, evaporated overnight, and the following day incubated 1 hour in pure epoxy before embedding in plastic capsules and polymerization at 60°C. Five tissue blocks were selected per individual mouse. Ultrathin sections were obtained using a Leica Ultracut S microtome (Leica, Wetzlar, Germany) with diamond knife. The sections were collected on copper-coated grids and stained with lead citrate for 20 seconds. All electron micrographs were obtained with a CM200 transmission microscope (Philips, Amsterdam, The Netherlands) at 80 kV and at least 20 unique micrographs were analyzed in order to produce representative figures.

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Canbay, A., Feldstein, A. E., Higuchi, H., Wenerburg, N., Grambihler, A., Bronk, S. F. and Gores, G. J. (2003a). Kupfer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression. Hepatology 38, 1188-1198.
Canbay, A., Guicciardi, M. E., Higuchi, H., Feldstein, A., Bronk, S. F., Rydzewska, R., Tanial, M. and Gores, G. J. (2003b). Cationic B-activation attenuates hepatic apoptosis and collagen synthesis in murine cholangitis, Swiss Mouse. J. Hepatol. 112, 152-159.
Claussen, M., Kübler, B., Wendland, M., Neifer, K., Schmidt, B., Zapf, J. and Braulke, T. (1997). Proteolysis of insulin-like growth factors (IGF) and IGF binding proteins by cathepsin D. Endocrinology 138, 3797-3803.
Collins, J. F., Wessling-Resnick, M. and Knutson, M. D. (2008). Hepcidin regulation of iron transport. J. Nutr. 138, 2284-2288.
Conigliaro, A., Brenner, D. A. and Kisseleva, T. (2010). Hepatic progenitors for liver disease: current position. Stem Cells Cloning 3, 39-47.
Cox, T. M. and Cachexinat, M. B. (2012). The cellular pathology of lysosomal diseases. J. Pathol. 226, 241-254.
Desai, P. K., Ahrin, K., Thung, S. N., Gordon, R. E., Short, M. P., Coates, P. M., Desminck, R. J. and Reynolds, J. F. (1981). Cholesterol storage disease: pathogenesis in an affected fetus. Am. J. Med. Genet 26, 689-698.
Engelhardt, N. V., Factor, V. M., Yasova, A. K., Polorotina, V. S., Baranov, V. N. and Lasareva, M. N. (1990). Common antigens of mouse oval and biliary epithelial cells. Expression on newly formed hepatocytes. Differentiation 45, 29-37.
Engels, H., Uckun, V. M., Medvinsky, A. L., Baranov, V. N., Lazareva, M. N. and Polorotina, V. S. (1993). Common antigen of oval and biliary epithelial cells (A6) is a differentiation marker of epithelial and erythroid cell lineages in early development of the mouse. Differentiation 55, 19-26.
Eskold, M. G., Mellgren, S., Follert, P., Donskov, S. S. and A˚ hren, B. (1994). Binding of a member of the Fn1 family of transcription factors to two distinct cis-acting elements in the promoter and 5'-flanking region of the human cellular retinol binding protein 1 gene. Mol. Endocrinol. 8, 732-745.
Fernández, M. A., Turró, S., Ingelmo-Torres, M., Enrich, C. and Pol, A. (2004). Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J. Leukoc. Biol. 75, 425-433.
Fickert, P., Zollner, G., Fuchsbichler, A., Stumptner, C., Weiglein, A. H., Lammert, F., Claussen, M., Kübler, B., Wendland, M., Neifer, K., Schmidt, B., Zapf, J. and Kurz, T., Terman, A., Gustafsson, B. and Brunk, U. T. (2008). Lysosomes and oxidative stress in aging and apoptosis. Biochim. Biophys. Acta 1780, 1291-1303.
Fotyma, R. W., Hight, D. G., Lomas, D. J., Fisher, N. C., Schofield, J. P., Ellis, E. E. and Cox, T. M. (2003). Massive hepatic fibrosis in Gaucher’s disease: clinico-pathological and radiological features. QJM 93, 237-244.
Maher, J. J. (2001). Interactions between hepatic stellate cells and the immune system. Semin. Liver Dis. 21, 417-426.
Moles, A., Tarrats, N., Fernández-Checa, J. C. and Mari, M. (2009). Cathepsins B and D drive hepatic stellate cell proliferation and promote their fibrogenic potential. Hepatology 49, 1297-1307.
Moles, A., Tarrats, N., Morales, A., Domínguez, B., Bataller, R., Caballería, J., García-Ruiz, C., Fernández-Checa, J. C. and Mari, M. (2010). Acidic sphingomyelinase controls hepatic stellate cell activation and in vivo liver fibrogenesis. Am. J. Pathol. 177, 1214-1224.
Moles, A., Tarrats, N., Fernández-Checa, J. C. and Mari, M. (2012). Cathepsin B overexpression disrupts autophagic flux and promotes liver fibrosis in Niemeyer’s-Pick disease. J. Cell. Biochem. 107, 1187-1188.
Mormone, E., George, J. and Nieto, N. (2011). Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. Chem. Biol. Interact. 193, 225-231.
Ototaga, K., Kinosita, K., Fuji, H., Sakabe, M., Shiga, R., Nakatani, Ikeda, K., Nakajima, Y., Ikura, Y., Ueda, M. et al. (2007). Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. Am. J. Pathol. 170, 987-990.
Parkinson-Lawrence, E. J., Shandala, T., Prodöehl, M., Plew, R., Börlace, G. N. and Brooks, D. A. (2010). Lysosomal storage disease: revealing lysosomal function and physiology. Physiology (Bethesda) 25, 102-115.
Pham Van, T., Couchie, D., Martin-García, N., Laperche, Y., Zafar, E. S. and Mayfield, R. (2008). Expression of matrix metalloproteinase-2 and -9 and tissue inhibitor of matrix metalloproteinase-1 in liver regeneration from oval cells in rat. Matrix Biol. 27, 674-681.
Pinzani, M. and Macias-Barragán, J. (2010). Update on the pathophysiology of liver fibrosis. Expert Rev. Gastroenterol. Hepatol. 4, 499-472.
Platt, F. M., Boland, B. and van der Spooel, A. C. (2012). The cell biology of disease: lysosomal storage disorders: the cellular impact of lysosomal dysfunction. J. Cell. Biochem. 119, 723-734.
Ramachandran, P. and Iredale, J. P. (2012). Liver fibrosis: a bidirectional model of fibrogenesis and resolution. QJM 105, 813-817.
Ramachandran, P., Pellicoro, A., Vernon, M. A., Boulter, L., Aucott, R. L., Ali, A., Hartland, S. N., Snowden, V. K., Cappon, A., Gordon-Walker, T. T. et al. (2012). Differential expression of matrix metalloproteinase-2 and -9 and tissue inhibitor of matrix metalloproteinase-1 in liver regeneration from oval cells in rat. J. Cell. Biochem. 109, 109-122.
Roberg, K., Johansson, U. and Ollinger, K. (1999). Lysosomal release of cathepsin D precedes relocation of cathepsin c and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress. Free Radic. Biol. Med. 27, 1228-1237.
Roskams, T., Lammers, B. and Desmet, V. J. (2003). Progenitor cells in diseased human liver. Semin. Liver Dis. 23, 385-396.
Ryckman, C., Vandal, K. L., Rouleau, P., Talbot, M. and Tessier, P. A. (2003). Proangiogenic activities of prolyl oxygenases: proteins S100A8, S100A9, and S100A8/S100A9 induce neutrophil chemotaxis and adhesion. J. Immunol. 170, 3233-3242.
Polds, J. M., Palmieri, M., di Ronza, A., Medina, D. L., Valenza, M., Gennarino, V., Al, A., di Malta, C., Donadu, F., Embrione, V., Polichshuk, R. S. et al. (2009). A gene network regulating lysosomal biogenesis and function. Science 325, 473-477.
Rózewska, T., Schülke, C. K., Hiltt-Vuori, M., Heerinen, J., Willenborg, M., Blanz, J., Domansky, M., Breiden, B., Brodesser, S., Landgrebe, J. et al. (2011). Role for LAMP-2 in endosomal cholesterol transport. J. Cell. Mol. Med. 15, 280-295.
Schröder, J., Lüllmann-Rauch, R., Himmerkus, N., Pleines, I., Nieswander, B., Ornath, U., Kofler, K., Köhl, B., Bleich, M. and Safogt, P. (2009). Deficiency of the tetraspan C6D3 associated with kidney pathology but normal lysosomal function. Mol. Cell. Biol. 29, 1083-1094.
Schröder, B. A., Wrocklage, C., Hasslik, A. and Safogt, P. (2010). The proteome of lysosomes. Proteomics 10, 4053-4076.
Starkel, P. and Leclercq, I. A. (2011). Animal models for the study of hepatic fibrosis. Best Pract. Res. Clin. Gastroenterol. 25, 319-333.

Steffensen, K. R., Bouzga, M., Skjeldal, F., Kasi, C., Karahasan, A., Matre, V., Bakke, O., Guérin, S. and Easkild, W. (2007). Human NCU-G1 can function as a transcription factor and as a nuclear receptor co-activator. BMC Mol. Biol. 8, 106.

Suleiman, S. A., Jones, G. L., Singh, H. and Labrecque, D. R. (1980). Changes in lysosomal cathepsins during liver regeneration. Biochim. Biophys. Acta 627, 17-22.

Takahashi, T., Akiyama, K., Tomihara, M., Tokudome, T., Nishinomiya, F., Tazawa, Y., Horinouchi, K., Sakiyama, T. and Takada, G. (1997). Heterogeneity of liver disorder in type B Niemann-Pick disease. Hum. Pathol. 28, 385-388.

Takehara, T., Tatsumi, T., Suzuki, T., Rucker, E. B., I, Hennighausen, L., Jinushi, M., Miyagi, T., Kanazawa, Y. and Hayashi, N. (2004). Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 127, 1199-1199.

Tardy, C., Andrieu-Abadie, N., Salvayre, R. and Levade, T. (2004). Lysosomal storage diseases: is impaired apoptosis a pathogenic mechanism? Neurochem. Res. 29, 871-880.

Terman, A. and Brunk, U. T. (2004). Lipofuscin. Int. J. Biochem. Cell Biol. 36, 1400-1404.

Vick, B., Weber, A., Urbanik, T., Maass, T., Teufel, A., Kramer, P. H., Opferman, J. T., Schuchmann, M., Galle, P. R. and Schulze-Bergkamen, H. (2009). Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes. Hepatology 49, 627-636.

Wandzioch, E., Költerud, A., Jacobsson, M., Friedman, S. L. and Carlsson, L. (2004). Lhx2−/− mice develop liver fibrosis. Proc. Natl. Acad. Sci. USA 101, 16549-16554.

Warskulat, U., Borsch, E., Reinehr, R., Heller-Stilb, B., Mönnighoff, I., Buchczyk, D., Donner, M., Flögel, U., Kappert, G., Soboll, S. et al. (2006). Chronic liver disease is triggered by taurine transporter knockout in the mouse. FASEB J. 20, 574-576.

Weber, A., Boger, R., Vick, B., Urbanik, T., Haybaeck, J., Zoller, S., Teufel, A., Krammer, P. H., Opferman, J. T., Galle, P. R. et al. (2010). Hepatocyte-specific deletion of the antiapoptotic protein myeloid cell leukemia-1 triggers proliferation and hepatocarcinogenesis in mice. Hepatology 51, 1225-1236.

Weiler-Normann, C., Herkel, J. and Lohse, A. W. (2007). Mouse models of liver fibrosis. Z. Gastroenterol. 45, 43-50.

White, C., Yuan, X., Schmidt, P. J., Bresciani, E., Samuel, T. K., Campagna, D., Hall, C., Bishop, K., Calicchio, M. L., Lapiere, A. et al. (2013). HRG1 is essential for heme transport from the phagolysosome of macrophages during erythrophagocytosis. Cell Metab. 17, 261-270.

Zhan, S.-S., Jiang, J. X., Wu, J., Halsted, C., Friedman, S. L., Zern, M. A. and Torok, N. J. (2006). Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology 43, 435-443.