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NEDD4 controls the expression of GUCD1, a protein upregulated in proliferating liver cells

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Abbreviations: GUCD1, guanylyl cyclase domain containing 1; NEDD4-1, neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin protein ligase; PH, partial hepatectomy; h, hours; HCC, hepatocellular carcinoma; dbcAMP, dibutyryl-ciclic AMP; ELAVL3, embryonic lethal, abnormal vision, *Drosophila*-like 3

Liver regeneration is a unique means of studying cell proliferation in vivo. Screening of a large cDNA library from regenerating liver has previously allowed us to identify and characterize a cluster of genes encoding proteins with important roles in proliferative processes. Here, by examining different rat and human tissues as well as cell lines, we characterized a highly conserved gene, guanylyl cyclase domain containing 1 (*GUCD1*), whose modulation occurs in liver regeneration and cell cycle progression in vitro. High-level expression of *GUCD1* transcripts was observed in livers from patients with hepatocellular carcinoma. A yeast two-hybrid interaction assay, aimed at identifying any relevant interaction partners of GUCD1, revealed direct interactions with NEDD4-1 (E3 ubiquitin protein ligase neural precursor cell expressed, developmentally downregulated gene 4), resulting in control of GUCD1 stability. Thus, we have characterized expression and function of a ubiquitous protein, GUCD1, which might have a role in regulating normal and abnormal cell growth in the liver.

Introduction

A most fascinating property of the liver is the capacity to regulate its own size and growth.[1] After surgical removal or loss of cells caused by toxic or viral injury, quiescent hepatocytes initiate a proliferative process, so as to restore liver mass. Liver regeneration is considered to be a prototypic model for studying the mechanisms of cell growth and proliferation in vivo.[2,3] The most useful experimental procedure is partial hepatectomy (PH), as described by Higgins and Anderson.[4] After 70% hepatectomy, residual hepatocytes undergo proliferation, with 2 cycles of synchronous cell division, until the original liver mass is reconstituted. The entire process is completed at 10–12 d of surgery.

Many factors contribute to initiating and promoting liver regeneration.[5-7] A series of rapid signaling events are arranged, involving activation and DNA binding of several transcription factors.[8] Hepatocytes become responsive to growth factors and progress through the G_1/S phase, undergoing replication. The cascade of events resulting from G-protein-coupled receptors activation is well characterized.[9] During liver regeneration, hepatic cAMP concentration increases over the first hours (h) of PH, and it is responsible for phosphorylation and activation of many cAMP-regulated transcription factors (cAMP response element-binding [CREB], *ccaat* enhancer binding protein [C/EBP] β, and Jun-B), through protein kinase A (PKA) activation.[10] Inducible cAMP early repressor (ICER) expression is upregulated after PH,[11] and the cAMP response element modulator (CREM) coordinates the regenerative process in hepatocytes.[12,13] Yet, even though many pathways activated by liver regeneration are firmly established, others still need to be clarified.

We constructed and screened a rat regenerating liver cDNA library with the aim of identifying changes in gene expression during G_1–S transition. Screening was conducted by subtracted cDNA probes derived from rat regenerating liver cDNAs (2–18 h after PH). We isolated approximately 40 genes, which were upregulated in liver after PH and in hepatoma cells (H-35). Two of them have previously been described. The first, referred to as liver annexin like-1 (*Lal-1*), encodes a protein that is highly expressed during the proliferative process;[14] the second encodes a nucleo–cytoplasmic shuttling protein, hepatocyte odd protein

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shuttling (HOPS). HOPS is involved in protein synthesis and centrosome assembly regulation and in controlling cell division,[15,16] and it has recently been identified as a component of a trimeric complex with the nucleolar protein nucleophosmin (NPM) and the tumor suppressor $p19^{Arf}$.[17,18]

In this study, we characterized a third gene, guanylyl cyclase domain containing 1 (*Gucd1*). *Gucd1* is highly expressed in liver during regeneration, but it is also abundant in other tissues. We found that *GUCD1* mRNA is upregulated in livers from patients with hepatocellular carcinoma (HCC). In addition, we obtained evidence for physical association of GUCD1 with NEDD4-1, an E3 protein that appears to control GUCD1 degradation through the ubiquitin–proteasome system. These findings may pave the way to unveiling any functional role of GUCD1 in tumorigenesis.

Results

Characterization of the gene *Gucd1*

We used screening of a rat regenerating liver cDNA library to identify genes that are highly expressed in liver regeneration. Screening involved a subtracted probe, derived from hybridization of regenerating liver cDNA with an excess of rat normal liver mRNA. The isolated non-hybridized cDNAs were specific for regenerating liver and were used to screen the library.[14] We isolated a pool of about 40 genes. All of these genes were cloned into plasmid vectors, and the cDNAs were partially sequenced. We focused our interest on one gene, *Gucd1*, which is upregulated early in liver regeneration. The relevant mouse and human *GUCD1* nucleotide sequences, and the deduced amino acid sequences, have previously been included in the NCBI database, under accession numbers NM_175133.1 and NM_031444, respectively. The rat sequence has also been determined and submitted to the NCBI database under accession number KC686830. *GUCD1* seems to be a highly conserved gene, with 99% identity in mouse, rat, and human amino acid sequences (**Fig. 1A**). Human *GUCD1* spans 3619 bp with a +1 ATG sequence at 317 bp, a coding sequence of 723 bp and a 3′UTR region of 2580 bp, with a polyA+ consensus sequence at 3583–3588. The gene is comprised of 5 introns and 6 exons (**Fig. 1B**) and is located on human chromosome 22. The putative GUCD1 protein is composed of a guanylyl cyclase 2 domain, which characterizes a family of proteins catalyzing the conversion of GTP to guanosine 3′,5′-cyclic monophosphate (cGMP) and pyrophosphate. Other functional domains have not been described yet.

***Gucd1* expression during liver regeneration**

To determine the temporal pattern of *Gucd1* expression during liver regeneration and the size of the specific mRNA, we performed northern blot analysis of rat liver *Gucd1* mRNA at different times after PH. As shown in **Figure 1C**, a single specific band of almost 3.2 kb was observed, corresponding in size to the *Gucd1* transcript. During liver regeneration, we observed that *Gucd1* mRNA levels peaked at 2 h of surgery then decreased and started to rise again at 12 h, reaching maximum values at 24–72 h of PH (**Fig. 1C**). No changes in *Gucd1* expression were observed in liver from sham-operated animals (data not shown).

During liver regeneration after PH, cAMP acts rapidly on residual hepatocytes and strongly affects gene expression. Therefore, we tested whether *Gucd1* expression might change after intraperitoneal injection of dbcAMP. As expected, real-time PCR showed that *Gucd1* mRNA expression in rat liver gradually decreased, at 30–120 min of treatment, in contrast to what observed in saline-injected controls (**Fig. 1D**). We wanted to determine whether *Gucd1* is a liver-specific gene by studying its expression in different tissues. Analysis of different rat tissue mRNAs by real-time PCR revealed that *Gucd1* mRNA was most abundant in liver, kidney, and testis, but relatively high levels were also detected in gut, heart, and brain (**Fig. 1E**), suggesting that *Gucd1* is not a tissue-specific gene, its expression being ubiquitous.

Investigation of human *GUCD1* expression

After this initial characterization in rats, we focused our attention on human *GUCD1*. RNA was extracted from various cell lines derived from human cancers, and the abundance of *GUCD1* mRNA was determined by real-time PCR. Increased expression of *GUCD1* mRNA occurred in all cell lines compared with normal livers. The highest values were detected in SK-MEL23, HepG2, Jurkat, and ARO cells, with only a modest increase in other cell types (**Fig. 2A**).

We assessed whether the observed differences in mRNA levels were accompanied by similar changes in protein expression and investigated GUCD1 protein expression in the same cell lines. Western blot analysis of total cell lysates revealed the presence of a specific band of about 27 kDa, corresponding to the endogenous GUCD1 protein (**Fig. 2B**). In some types of cells, GUCD1 expression correlated with mRNA levels (ARO, NT2-D1, HT-29, HEK-293, PFSK-1, MIA PaCa-2). In contrast, in other cells (HepG2, Calu-1, SNKBE, MCF7, Jurkat), a discrepancy was observed between mRNA and protein expression, suggesting that post-translational modifications could occur, affecting stability of the newly synthesized protein (**Fig. 2B**; **Fig. S1A**).

Cellular localization of GUCD1

The full-length cDNA of human *GUCD1* was subcloned into the expression vectors pCS2-MT and pSG5. Overexpression of GUCD1 was obtained in COS-1 cells, and lysates from transfected cells, analyzed by western blot with anti-GUCD1 antibody, revealed the presence of a specific band of 27 kDa in *GUCD1*-pSG5-overexpressing cells, corresponding to the native protein, and a band of 36 kDa, corresponding to the fusion protein, in cells transfected with *GUCD1*-pCS2 MT (**Fig. 2C**). Immunolocalization analysis of COS-1 cells transfected with the same plasmids revealed diffuse distribution, with a prevalent localization within the cytoplasm of both native (**Fig. 2D**) and tagged (**Fig. S1B**) proteins. Further immunolocalization experiments were conducted in HepG2 hepatocarcinoma cells to investigate localization of the endogenous GUCD1 protein. The results confirmed a prevalent cytoplasmic localization of the protein (**Fig. 2E**). Similar results were observed after cell fractionation experiments (**Fig. 2F**).

GUCD1 interacting proteins revealed through two-hybrid system

Based on our results in the liver regeneration model, we searched for any roles of GUCD1 in proliferative events through

the identification of proteins with which GUCD1 would specifically associate. We used mouse fetal library screening by yeast two-hybrid assay. We used GUCD1 as the bait, in order to identify its cellular molecular partners.

Initially, 355 clones were identified and sequentially numbered, based on the day of appearance. A further selection—by increasing the selectivity of growth conditions—reduced clone numerosity to 289. About 100 clones were found to be negative when tested for β-galactosidase activity, which detects false positive samples or too weak interactions. A total of 183 positive yeast clones were then transformed in bacteria, and cDNA was extracted, digested, and sequenced (**Fig. S2**). Up to 10 different families of genes were identified, each containing multiple copies of the same clone. While some genes encoded proteins of unknown function, others had known function and structure.

The most interesting GUCD1-interacting proteins are listed in **Table 1**. All of the clones were also positive for β-galactosidase activity (**Table 1**). Among those proteins were NEDD4-1 and embryonic lethal, abnormal vision, *Drosophila*-like 3 (ELAVL3 or Hu antigen C). NEDD4-1 is one of the most abundant E3 ubiquitin ligases in the central nervous system and is also involved in various basic cellular functions, including endocytosis of transmembrane receptors and ion channels, intracellular vesicle transport, and virus budding.[19,20] ELAVL3 is a neural-specific RNA-binding protein, member of the Hu family, which plays a central role in regulating the stability and translation of

Table 1. Interaction partners of GUCD1

Gene	β-gal	Accession number
Nedd4-1	++	NP_035020.2
Elavl3	++	NP_034617.1
Phosphacan	++	NP_001074775.1
α-globin	+++	NP_032244.2
Cadherin-related neuronal receptor	++	NP_031793.2
EF1	+	NP_034236.2

List of yeast two-hybrid interaction partners of GUCD1 with relative protein sequence database entries. The signal intensities of β-galactosidase activity were rated as very strong (+++), strong (++), or weak (+).

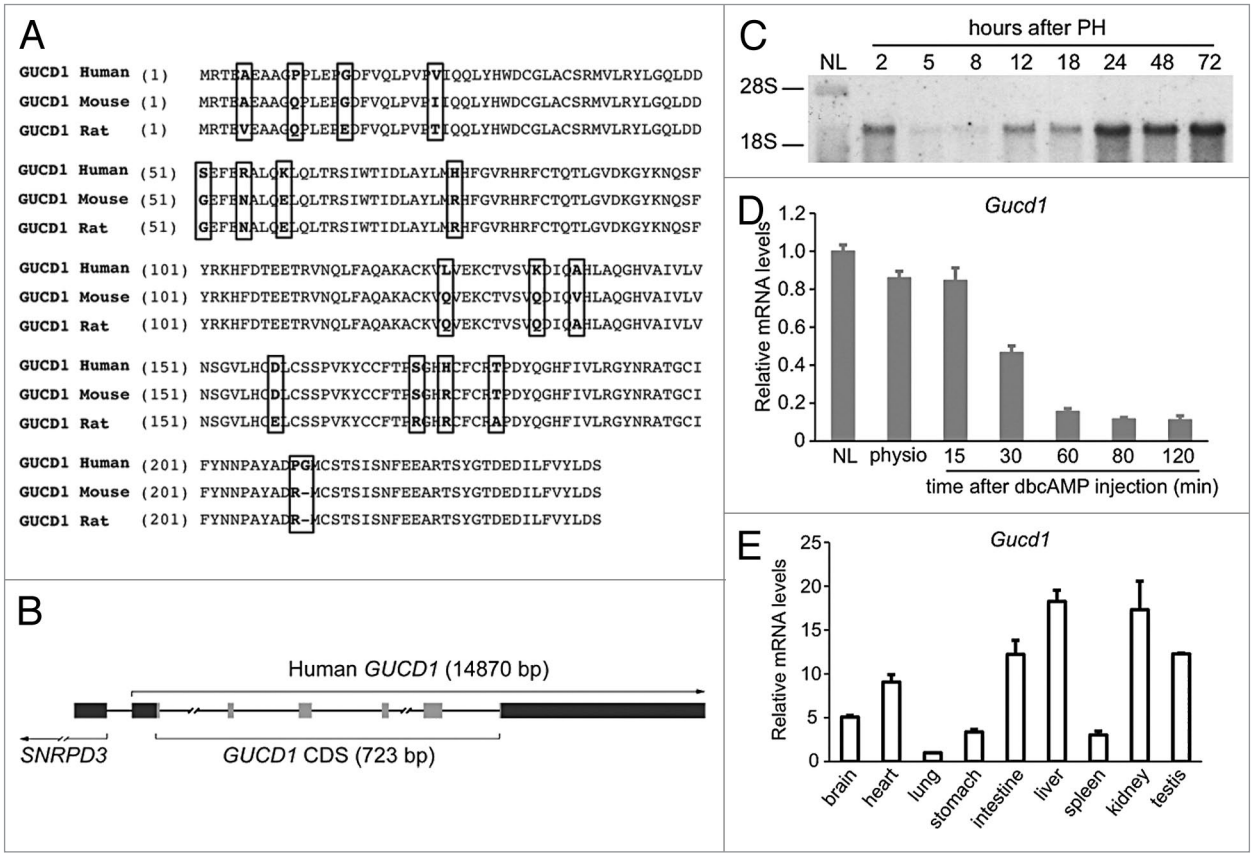

Figure 1. *Gucd1* gene structure and mRNA expression during liver regeneration. (**A**) The deduced amino acid sequences of human, mouse, and rat GUCD1 were aligned and compared. Numbering begins with the first methionine. Bold and boxed letters indicate divergences among species. (**B**) Schematic representation of the intron–exon distribution of the human *GUCD1* gene. The 6 exons are indicated with light gray bars and the 5 introns by the black lines running through them. (**C**) *Gucd1* mRNA extracted from rat at different hours after PH was analyzed by northern blot. Normal liver (NL) was used as a control. (**D**) Quantitative real-time PCR analysis of *Gucd1* from RNA samples prepared from livers of rats at different times after dibutyryl-ciclic AMP (dbcAMP) injection. Normal liver (NL) and liver from rats injected with saline solution (physio) were used as controls. Value in normal liver (NL) was set to 1. All values are the mean ± SD (n = 3). (**E**) *Gucd1* mRNA expression in rats tissues analyzed by quantitative real-time PCR. The values are relative to those of *Gus* mRNA levels in each tissue. *Gucd1* expression in the lung was set to 1. All values are the mean ± SD (n = 3).

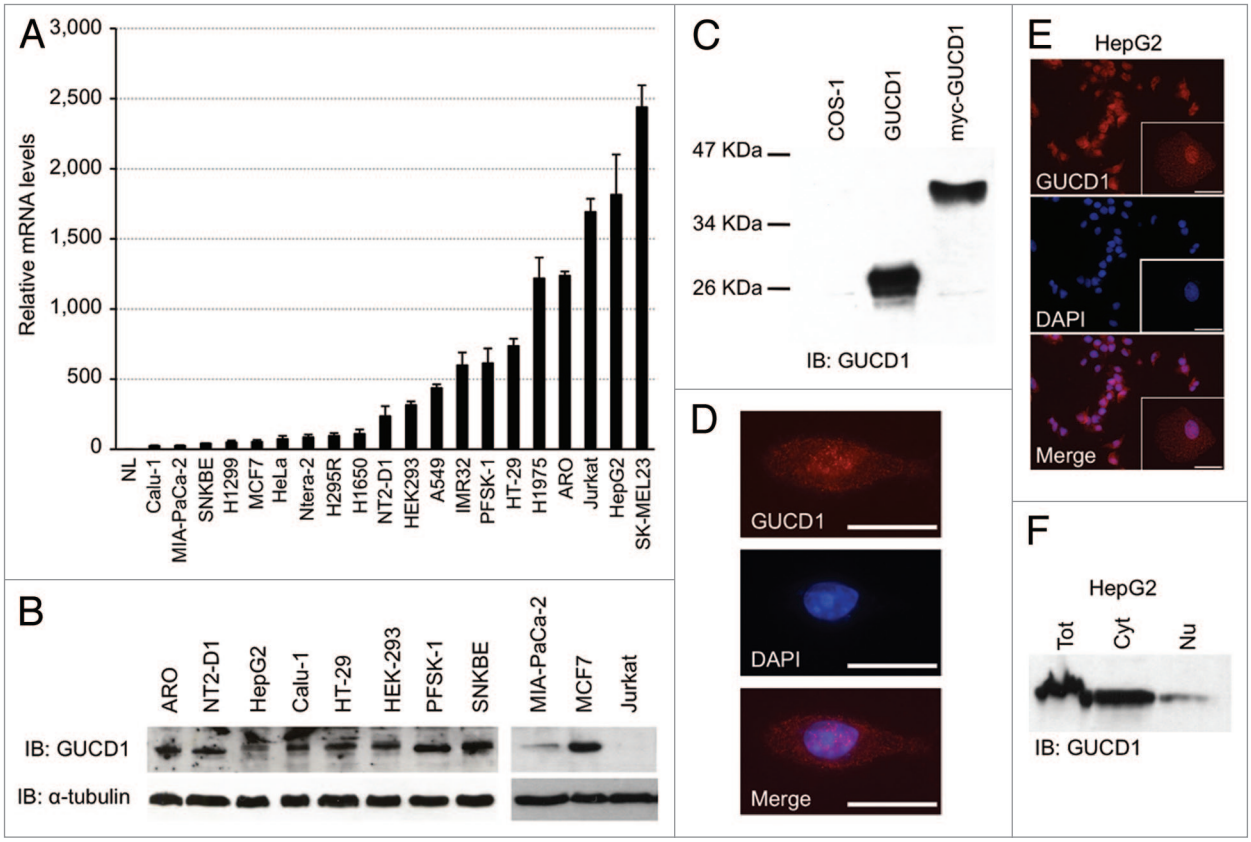

Figure 2. Expression and localization of human GUCD1. (**A**) Quantitative real-time PCR analysis of *GUCD1* from RNA samples prepared from different human cell lines. The values are relative to those of *HPRT* mRNA levels in each cell line. Expression value in Calu-1 cells was set to 1. All values are the mean ± SD (n = 3). (**B**) Immunoblot detection of GUCD1 protein in lysates derived from different human cell lines, analyzed by using anti-GUCD1 antibody. Tubulin was used as loading control. (**C**) Western blot analysis of GUCD1 in COS-1 cells transfected with the full-length cDNA of human *GUCD1* subcloned into expression vectors pSG5 (GUCD1) and pCS2-MT (myc-GUCD1). (**D**) Immunolocalization of GUCD1 in COS-1 cells transfected with *GUCD1*-pSG5, using the anti-GUCD1 antibody. DAPI was used for labeling nuclei (blue). Bars, 10 μm. (**E**) Immunolocalization of endogenous GUCD1 in HepG2 cells, using the anti-GUCD1 antibody. DAPI was used for labeling of nuclei (blue). Bars, 10 μm. (**F**) HepG2 cells were transfected with *GUCD1*-pSG5. Cells were lysate 24 h after transfection and total (Tot), cytosolic (Cyt), and nuclear (Nu) protein extracts were prepared and analyzed by western blot using the anti-GUCD1 antibody.

numerous mRNAs encoding stress-response and proliferative proteins.[21]

Because of the role of NEDD4-1 in regulating protein degradation, we focused our attention on the interaction of GUCD1 with NEDD4-1. Transformants co-expressing GUCD1 and NEDD4-1 would grow on selective medium, unlike yeast strains transformed with empty vectors (pASV3, pGBKT7) (**Fig. 3A**). The same clones also displayed high β-galactosidase activity (**Fig. 3B**; **Table 1**).

To validate the interaction, we used COS-1 cells co-expressing NEDD4-1 and myc-tagged GUCD1. An immunoprecipitation assay with both anti-NEDD4-1 and anti-myc antibodies confirmed that GUCD1 interacts with NEDD4-1 in vitro (**Fig. 3C**). Furthermore, immunolocalization analysis revealed partial cytoplasmic colocalization of GUCD1 and NEDD4-1 in both HEK-293 (**Fig. 3D**) and COS-1 cells (**Fig. S3A**), ectopically overexpressing both plasmids.

The E3 ligase NEDD4 regulates GUCD1 degradation

Because NEDD4-1 is known to promote ubiquitination and subsequent degradation of several target proteins, we wanted to investigate the potential role of NEDD4-1 in GUCD1 degradation. We overexpressed myc-GUCD1 alone or together with NEDD4-1 in COS-1 cells in order to establish any mutual changes in protein levels. Analysis of protein extracts revealed that co-expression of NEDD4-1 together with GUCD1 led to decreased GUCD1 levels (**Fig. S3B**). Moreover, multiple high-molecular mass signals were detected, indicating a possible effect of poly-ubiquitination. This hypothesis is also supported by a previous publication, where GUCD1 was identified as a putative ubiquitin substrate through mass spectrometric analysis.[22]

To confirm our hypothesis, we performed an in vivo ubiquitination assay. HEK-293 cells were transiently transfected with HA-Ubiquitin (HA-Ubi), myc-tagged GUCD1 and NEDD4-1 or empty vector, in the presence or absence of the proteasome inhibitor MG132 (**Fig. 3E**). After immunoprecipitation with anti-myc, immunoblotting with anti-HA revealed that GUCD1 was labeled with HA-Ubi in vivo. Moreover, GUCD1 protein amount was significantly increased in the presence of MG132, suggesting that its degradation is regulated via the ubiquitin-proteasome system.

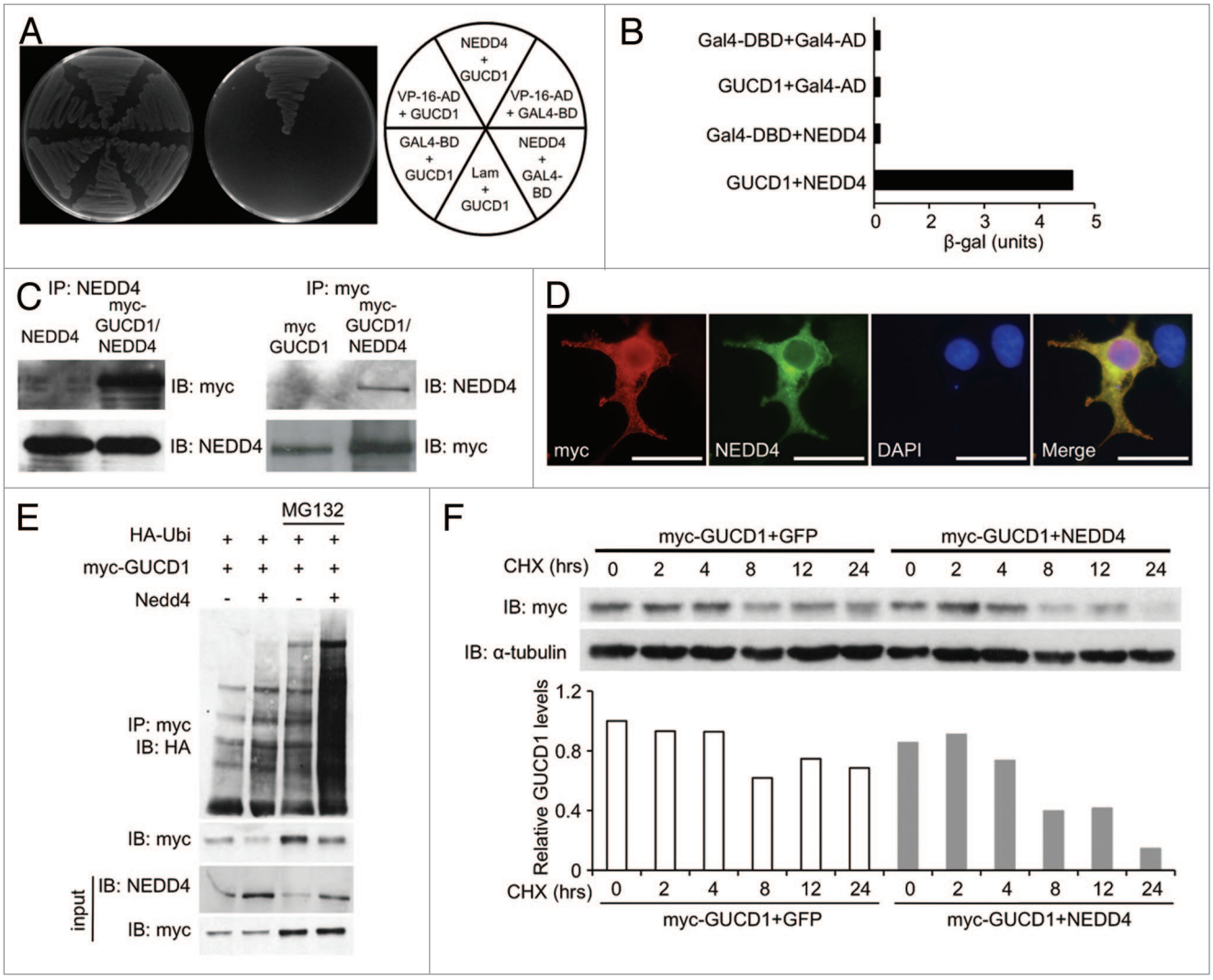

Figure 3. Isolation of NEDD4-1 as GUCD1 binding protein by the yeast two-hybrid system. (**A**) Growth of transformants co-expressing GUCD1 and NEDD4-1 on selective medium. GUCD1-Gal4-DBD (GUCD1) was used as bait; NEDD4-1 (NEDD4) was the clone isolated from the library screening; Gal4-DBD, VP-16-AD, and Lamin (Lam) are negative controls. (**B**) β-galactosidase assay in selected colonies of yeast expressing GUCD1 and NEDD4-1. The results are expressed in Miller units and are the means of triplicate measurements performed using 3 distinct transformations. (**C**) Western blot analysis of protein extracts from COS-1 cells transfected with NEDD4-1 and myc-GUCD1 and immunoprecipitated with anti-NEDD4 (left) or anti-myc (right) antibodies. Abundance of coimmunoprecipitated proteins was determined with anti-myc and anti-NEDD4 antibodies. (**D**) Coimmunolocalization of GUCD1 and NEDD4-1 in HEK-293 cells transfected with myc-GUCD1 (anti-myc) and NEDD4-1 (anti-NEDD4). DAPI was used for labeling of nuclei (blue). Bars, 10 μm. (**E**) HEK-293 cells were transfected with HA-Ubi, myc-GUCD1 and NEDD4-1 or empty vector (pCDNA3.1). Six hours post-transfection 10 μM MG132 was added where indicated for 18 h. Anti-myc immunoprecipitations were immunoblotted with anti-HA and anti-myc. Lower panels show the expression of NEDD4-1 (anti-NEDD4) and GUCD1 (anti-myc) in total cell lysates as an input. (**F**) Lysates from HepG2 cells transfected with myc-GUCD1 and GFP-expressing vector (GFP) or NEDD4-1 treated with 100 μM Cycloheximide (CHX) for the indicated hours. Western blot analysis was performed using anti-myc antibody, and anti-tubulin as an internal control. Bottom, densitometry was shown for myc-GUCD1 expression levels.

Furthermore, co-immunoprecipitation of lysates overexpressing NEDD4-1 as well as myc-tagged GUCD1 in the presence of MG132 demonstrated that NEDD4-1 enhances GUCD1 labeling with HA-Ubi. Of interest, GUCD1 total amounts decreased (**Fig. 3E**), further indicating that NEDD4-1 functions as a ubiquitin ligase for GUCD1. We examined whether NEDD4-1 is also important in determining GUCD1 half-life. HepG2 cells, transfected with myc-tagged GUCD1 in the presence of green fluorescent protein (GFP) (used as a control) or with NEDD4-1, were treated with cycloheximide (CHX) to inhibit de novo protein synthesis and harvested at different times (**Fig. 3F**). Western blot analysis showed a reduction in GUCD1 stability in the presence of increased amounts of NEDD4-1 (**Fig. 3F**; **Fig. S3C**).

Collectively, the results demonstrate a relationship between GUCD1 and NEDD4-1 and establish GUCD1 as a new target of NEDD4-1 E3 function in mammalian cells. Further studies will specifically clarify the role of NEDD4-1 in the proteasome-dependent degradation of GUCD1 during cell cycle progression.

GUCD1 levels are regulated in a cell cycle-dependent fashion

Based on the results obtained from liver regeneration experiments, we investigated the expression of GUCD1 in proliferating hepatocellular carcinoma cells. HepG2 cells were synchronized using the double thymidine arrest technique. GUCD1 protein levels—at the end of the double thymidine arrest (time 0) and at different times after release—were monitored by western blot and compared with the expression levels in asynchronous cells (AS). GUCD1 expression showed a limited increase at time 0 in arrested cells, then its expression decreased rapidly after release, to rise again commencing on 4 h of release, until 12 h (**Fig. 4A**). Western blot for P(S10)-H3 (**Fig. 4A**) and flow cytometry (**Fig. S4A**) were used as controls of the synchronization. Conversely, NEDD4-1 protein expression decreased after the arrest as compared with asynchronous cells, and then remained fairly stable during cell cycle progression (**Fig. 4A**). Overall, these data demonstrate that GUCD1 protein levels increase during the G_2/M transition, whereas NEDD4-1 expression is downregulated. The data also suggest a possible role of GUCD1 in modulating cell cycle progression.

To better assess the role of NEDD4-1 during liver regeneration, we evaluated whether its expression levels change in regenerating livers. PH was performed in wild-type mice, and livers were collected at different times after surgery. Analysis of *Gucd1* mRNA levels revealed a pattern of expression similar to that observed in rat livers, with a reduction of *Gucd1* levels during the first hours following PH, and a subsequent increase at 48 and 72 h after PH, although changes were less pronounced relative to those observed in rats (**Fig. 4B**). Analysis of *NEDD4-1* mRNA levels under the same conditions showed a peak of mRNA expression 36 h after PH, while only mild variations were observed at other time points. Collectively, these data suggest a potential role of NEDD4-1 in the regulation of GUCD1 stability during cell cycle progression and liver regeneration. Further experiments are needed to clarify whether and how these events play a role in the proliferative process.

***GUCD1* expression in HCC**

Because of the role of GUCD1 in the proliferative process during liver regeneration, we explored whether GUCD1 expression was altered in the proliferation of tumor cells. Real-time PCR analysis on RNAs extracted from 12 HCC samples showed upregulated expression of *GUCD1* transcripts in tumor samples, as compared with normal human liver tissue, although variations did occur between patients' samples (**Fig. 4C**). Because the levels of *GUCD1* mRNA do not always reflect equal amounts of GUCD1 protein in cultured cells (**Fig. 2B**), we examined the possibility that high *GUCD1* mRNA levels in HCC samples could not be associated with increased amounts of GUCD1 protein. Immunohistochemistry was done on sections derived from HCC samples. GUCD1 protein levels were slightly reduced in HCC as compared with normal liver tissue surrounding the tumor (**Fig. 4D**; **Fig. S4B**), suggesting the possibility that a fine modulation of GUCD1 expression and stability might occur in vivo. Overall, these data reinforce the hypothesis that GUCD1 could play an important role during both normal and abnormal proliferative processes.

Discussion

Several sets of genes are activated during the first hours following partial hepatectomy. Some of these genes, such as those transcribing for cyclins, are well characterized, while others still need to be defined. After construction and screening of a rat regenerating liver cDNA library, we successfully isolated and analyzed many upregulated genes. One of these was *Gucd1*, and its mRNA expression was monitored in regenerating liver after PH. The observation that *Gucd1* transcription increases 2 h after PH, at a time when several immediate early genes are likewise upregulated—and between 24 and 72 h, corresponding to the hepatocyte proliferative phase—prompted us to speculate that GUCD1 could take part in the molecular and biochemical changes inducing hepatocytes proliferation. In the first hours after PH, stimulation of the adenylate cyclase signaling pathway activates transcription of the family of cAMP-responsive nuclear factors, including CREB and CREM, positively regulating gene expression.[9] Analysis of *Gucd1* mRNA levels showed that its transcription decreases following stimulation with cAMP, an effect that could be mediated by pathways other than PKA activation.[23] High expression of *GUCD1* mRNA in many cell lines derived from human cancers prompted us to hypothesize a well-defined role in the mechanisms regulating cell growth.

Several proteins have been identified as GUCD1-binding partners from a yeast two-hybrid assay of a mouse fetal cDNA library. For many of these proteins, studies are ongoing to confirm the interaction. One of these is NEDD4-1, a protein of about 120 kDa in weight, localized in the cytoplasm, mainly in the perinuclear region and in the cytoplasm periphery.[24] *NEDD4-1* was initially identified by Kumar et al. through a subtraction cloning approach as a developmentally regulated mouse gene highly expressed in the early embryonic central nervous system.[25] NEDD4 is an HECT domain containing ubiquitin–protein ligase, responsible of the selective ubiquitination of some regulatory proteins involved in transcription and membrane transport.[26] *NEDD4-1* has been recently found to be an important proto-oncogene, through its E3 function on the tumor suppressor gene phosphatase and tensin homolog (PTEN). When NEDD4-1 levels increases in the cell, the amount of PTEN decreases because of its degradation through the ubiquitin-proteasome system, thus promoting cell survival.[27,28] Although NEDD4-1 and GUCD1 appear to co-localize only partially into the cell, they physically interact, probably when GUCD1 needs to be degraded.

Our in vitro experiments demonstrated that GUCD1 expression decreases when the two proteins are both present at high levels in the cell, because of a reduction of its stability. At the same time, many polyubiquitinylated species of GUCD1 appeared as high molecular weight forms, suggesting that GUCD1 is degraded by the proteasome, after polyubiquitin chain formation, in the presence of NEDD4-1. These data indicate that GUCD1 might play a role in triggering hepatocytes proliferation, and that ubiquitination by NEDD4-1 in vivo might serve as an active mechanism to downregulate its expression at the end of the process and adjust its concentration into the cell.

Furthermore, NEDD4 family proteins contains cAMP- and cGMP-dependent protein-kinase phosphorylation sites in their sequences, and NEDD4-2 is directly phosphorylated by PKA;[29] further studies are required to understand whether this event could explain the modulation of *Gucd1* mRNA levels through the cAMP pathway. Also, the upregulation of *Gucd1* mRNA observed in 12 cases of HCC suggests that it might be a valuable prognostic and therapeutic biomarker in liver cancer.

The imbalance between GUCD1 mRNA and protein levels in liver cancer cells might be explained by an active role of NEDD4-1 as a controller of cell proliferation. At the present time only a limited number of papers explored the function of NEDD4-1 in the liver. A recent publication shows how NEDD4-1 is directly involved in the proteasomal degradation of its target protein Sprouty2 in HCC, with an inverse correlation between NEDD4-1 and Sprouty2 protein levels.[30] Intriguingly, the study also shows that HCC samples, where Sprouty2 protein expression is downregulated, display an increased *Sprouty2* mRNA expression.[30] We cannot exclude that a similar regulation occurs for *Gucd1*. Our experiments suggest that transcriptional mechanisms increase *Gucd1* mRNA expression in all human HCC samples that we analyzed, possibly because of feedback compensatory mechanisms that are activated following downregulation of GUCD1 expression via NEDD4-1, or through other unknown mechanisms (**Fig. 5**). This interpretation is in line with findings describing high levels of expression of NEDD4 and different NEDD4-like E3 ubiquitin ligases in various human cancers.[31] Future studies will shed light on the mechanism how NEDD4-1 regulates GUCD1 expression in human hepatocarcinogenesis. In addition to NEDD4-1, another protein isolated in our two-hybrid assay deserves special attention. This protein, ELAVL3 or HuC, might be important in regulating *GUCD1* mRNA stability in the nervous system and in other tissues. The Hu family of proteins is also comprised of the primarily neuronal HuB and HuD and the ubiquitous HuR. Interestingly, a microarray screening of ribonucleoproteins, immunoprecipitated by HuR, identified *GUCD*1 transcript as an HuR-associated mRNA.[32] Future studies exploring the functional role of HuC interaction with GUCD1 may clarify the mechanisms presiding over *Gucd1* mRNA levels in the proliferative process.

In summary, we have described a protein that interacts with the E3 ubiquitin ligase NEDD4-1, whose mRNA is highly

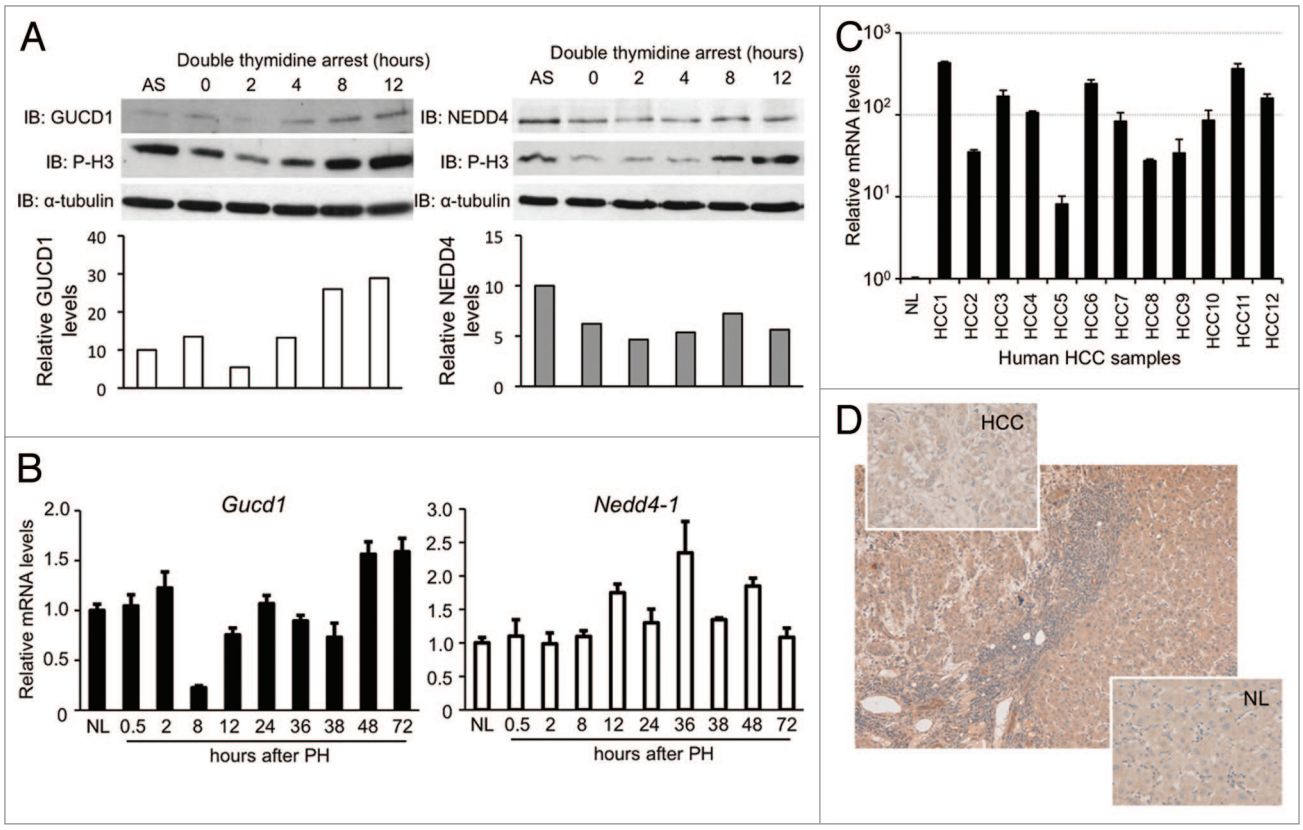

Figure 4. Analysis of GUCD1 and NEDD4-1 expression during cell cycle progression and in human hepatocellular carcinoma (HCC) tumor samples. (**A**) Protein expression of GUCD1 and NEDD4-1 in HepG2 cells arrested by double thymidine excess, evaluated by western blot. GUCD1 and NEDD4-1 proteins were monitored using anti-GUCD1 (left) and anti-NEDD4 (right) antibody, respectively. P-H3 was used as control of synchronization. Tubulin was used as loading control. Bottom, densitometry was shown for GUCD1 (left) and NEDD4 (right) expression levels. (**B**) *Gucd1* and *Nedd4-1* mRNA expression profile in mouse regenerating liver was analyzed by quantitative real-time PCR. The values are relative to those of *Gus* mRNA levels at each time. Value in normal liver (NL) was set to 1. All values are the mean ± SD (n = 3). (**C**) *GUCD1* relative mRNA expression from 12 human HCC tumor tissues was analyzed by quantitative Real-Time PCR. The values are relative to those of *18S* mRNA levels in each sample. The average *GUCD1* expression in normal liver (NL) tissues (n = 3) was arbitrarily set to 1. The expression in each tumor sample is the mean ± SD of technical triplicates. (**D**) Immunohistochemical staining shows GUCD1 expression in HCC and in the surrounding normal liver (NL). Representative images (10× and 40× magnification) are shown.

expressed in regenerating liver after PH and in several cancer cell lines, as well as in 12 cases of HCC. These results represent a first step toward the characterization of a potentially important gene, and future studies will provide a better understanding of its cellular function and its exact role in the proliferative process with specific relevance to hepatocellular carcinoma.

Materials and Methods

Animals

This study was performed according to national laws and authority guidelines, using 3-mo-old male Sprague–Dawley rats and SVJ-129 mice. The animals were purchased from Harlan-Nossan and received human care according to NIH guidelines. Animals used for liver regeneration experiments were maintained in a temperature-controlled room under a 12:12 h light/dark cycle and food and water were provided ad libitum. Liver resection of the left lateral and median lobes was performed according to the Higgins and Anderson's procedure[4] between 8 and 12 AM, after induction of deep anesthesia with isofluorane, by exerting a classical sub-xiphoid incision, which allows extrusion, extra-abdominal ligation of the lobes, and removal of 2/3 of the liver.[33] A sham operation, consisting of transverse abdominal incision followed by digital manipulation of the liver, was performed as the appropriate control. After the surgical procedure, animals were placed in a warmed cage to recover from surgery. Rats and mice were sacrificed at the indicated times after PH. Three to four animals per time point were used, and livers were pooled prior to analysis. In selected experiments, after intraperitoneal injections of dibutyryl-cAMP (dbcAMP), animals were sacrificed at the indicated times. Rats receiving saline injection were used as controls. Regenerating liver and other tissues were frozen in liquid nitrogen and stored at −80 °C for further processing.

Real-time quantitative PCR

Total isolated RNA was retrotranscribed using random hexamer primers and RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, Thermo Fisher Scientific), according

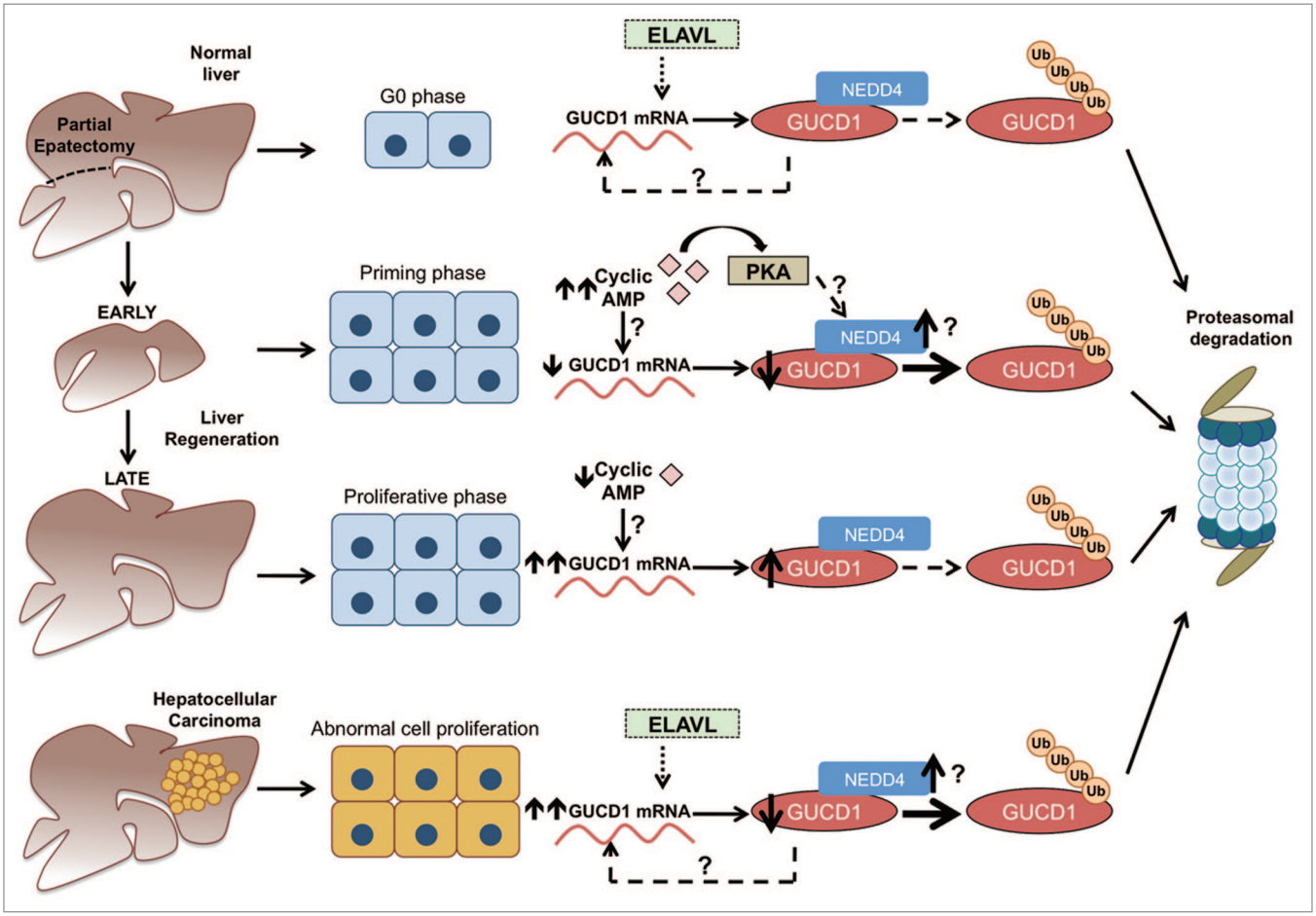

Figure 5. Schematic representation of GUCD1 regulation in normal and regenerating liver, and in HCC. In quiescent hepatocytes, GUCD1 expression is kept at relatively low levels owing to NEDD4-mediated ubiquitylation. During the priming phase after partial hepatectomy, elevated cAMP levels repress *Gucd1* gene expression, possibly through transcriptional mechanisms. At the same time, PKA might induce NEDD4-1 phosphorylation and modulate its activity, thus influencing GUCD1 protein levels. In the late phase of liver regeneration, when hepatocytes undergo through G_2/M phase and levels of cAMP decreases, *Gucd1* mRNA and protein rises up at its highest levels. In HCC, *GUCD1* mRNA is strongly upregulated, while its protein levels remain low, possibly because of the increased activity or expression of NEDD4, which acts as a proto-oncogene. ELAVL proteins might act by modulating *GUCD1* mRNA stability.

to the manufacturer's instructions. Quantitative PCR was performed by monitoring in real-time the increase in fluorescence on an Mx3000P™ Real-Time PCR detector system, using Brilliant SYBR® Green QPCR Master Mix (Agilent Technologies) and ROX as reference dye. Results were analyzed using Mx3000P™ System Software (Stratagene). The primers for rat (r) *Gucd1*, r *Gus*, mouse (m) *Gucd1,* m *Nedd4-1*, m *Gus,* human (h) *GUCD1,* h *HPRT, 18S rRNA* are as follows: r *Gucd1*: Forward 5′- ATGAGAACGG AGGTAGAG - 3′, Reverse 5′ - ACAGTCCCAG TGATACAG - 3′; r *Gus*: Forward 5′- GCCTGTCTCT TCTCTGAAAC CTG - 3′, Reverse 5′ - TGTCTGCGTC ATATCTGGTA TTGC - 3′; m *Gucd1*: Forward 5′- CTGCTCCAGG ATGGTGCT - 3′, Reverse 5′ - TAGGCCAGGT CGATGGTC - 3′; m *NEDD4-1*: Forward 5′- AGACCAGGCT GAGGAGTTAG - 3′, Reverse 5′ - GGTGGATGCG GCAAATGA - 3′; m *Gus*: Forward 5′-TCACTTCGGC ACCACCTAG - 3′, Reverse 5′- CCACAGACCA CATCACAACC - 3′; h *GUCD1*: Forward 5′- AGGAGCATCT GGACCATC- 3′, Reverse 5′ - GTTCTTGTAG CCCTTGTCG - 3′; h *HPRT*: Forward 5′- CCTCCGCCTC CTCCTCTG - 3′, Reverse 5′- CTCGGTTCAT CATCACTAAT CACG - 3′; *18S rRNA*: Forward 5′- CGGACACGGA CAGGATTGAC AG - 3′, Reverse 5′- ATCGCTCCAC CAACCAAGAA CGG - 3′ (Invitrogen, Life Technologies).

Two-hybrid screening and analysis

The full-length of mouse *Gucd1* cloned into the GAL4 binding domain expression vector pGBKT7 and an E9.5–12.5 mouse embryo cDNA library cloned into the VP16 activation domain vector pASV3 were used to transform AH109 yeast cells, according to the MATCHMAKER two-hybrid system protocol (Takara Bio Europe/Clontech).[34] The transformants were plated onto appropriate selective medium (lacking tryptophan, leucine, and histidine) supplemented with 5 mM 3-aminotriazole and incubated for 10–25 d at 30 °C. β-galactosidase assays were performed on isolated clones; the results are expressed in Miller units and are the means of triplicate measurements performed using 3 distinct transformations. The plasmids were extracted by lysing cells with acid-washed beads and transformed in *E.coli* bacterial strain HB101. After growth in M9 (–Leu) medium, the identified clones were sequenced using the Sanger method.

Cell cycle synchronization

HepG2 cell cycle was synchronized in G_1/S phases by the double thymidine method, as previously described.[35] The time point, corresponding to the G_1/S transition, was considered time 0. The percentage of HepG2 cells at different phases of the cell cycle was determined by flow cytometry.

Immunohistochemistry analysis

Four (4) µm sections from paraffin-embedded samples were cut on polarized slides. The immunohistochemistry was performed using the polyclonal antibody against GUCD1 (dilution 1:100) and the polyclonal antibody against NEDD4-1 protein (dilution 1:350). The primary antibody was detected using a biotin-free polymeric-horseradish peroxidase (HRP)-linker antibody conjugate system (Bond Polymer Refine Detection, Leica BioSystems, 2009) with a heat-induced epitope retrieval, using the Bond Enzyme Pretreatment Kit (Leica Biosystems) conducted with the Bond III automated immunostainer (Leica BioSystems). The section was incubated with the primary antibody for 15 min. Representative images (10× and 40× magnification) are shown.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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