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Tumor suppressive role of the antimicrobial lectin REG3A targeting the *O*-GlcNAc glycosylation pathway

Nicolas Moniaux[1,2] | Nicolas Geoffre[1,2] | Alice Deshayes[1,2] | Alexandre Dos Santos[1,2] | Sylvie Job[1,2] | Claire Lacoste[1,2] | Tung-Son Nguyen[1,2] | Marion Darnaud[1,2] | Mélanie Friedel-Arboleas[3] | Catherine Guettier[1,2,4] | Janne Purhonen[5,6] | Jukka Kallijärvi[5,6] | Gilles Amouyal[7] | Paul Amouyal[7] | Christian Bréchot[8] | Romain R. Vivès[3] | Marie Annick Buendia[1,2] | Tarik Issad[9] | Jamila Faivre[1,2,10]

[1]INSERM, U1193, Paul-Brousse University Hospital, Hepatobiliary Centre, Villejuif, France

[2]Faculté de Médecine, Université Paris-Saclay, Le Kremlin-Bicêtre, France

[3] Université Grenoble Alpes, CNRS, CEA, IBS, Grenoble, France

[4]Assistance Publique-Hôpitaux de Paris (AP-HP), Université Paris-Saclay, Hôpital Bicêtre, Laboratoire Anatomie Pathologique, Le Kremlin Bicêtre, France

[5]Folkhälsan Research Center, Helsinki, Finland

[6]Stem Cells and Metabolism Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland

[7]Alfact Innovation, Paris, France

[8]USF Health, University of South Florida, Tampa, Florida, USA

[9]Institut Cochin, Université de Paris, CNRS, INSERM, Paris, France

[10]Assistance Publique-Hôpitaux de Paris (AP-HP), Medical-University Department (DMU) Biology Genetics, Université Paris-Saclay, Paul-Brousse Hospital, Villejuif, France

Correspondence
Nicolas Moniaux, U1193 INSERM (French National Institute of Health and Medical Research)/University Paris-Saclay, Paul-Brousse University Hospital, Hepatobiliary Centre, Villejuif 94800, France.
Email: nicolas.moniaux@inserm.fr

Jamila Faivre, U1193 INSERM/University Paris-Saclay, Paul-Brousse University Hospital, Hepatobiliary Centre, Villejuif 94800, France.
Email: jamila.faivre@inserm.fr

Abstract

Background and Aims: Antimicrobial proteins of the regenerating family member 3 alpha (REG3A) family provide a first line of protection against infections and transformed cells. Their expression is inducible by inflammation, which makes their role in cancer biology less clear since an immune-inflammatory context may preexist or coexist with cancer, as occurs in HCC. The aim of this study is to clarify the role of REG3A in liver carcinogenesis and to determine whether its carbohydrate-binding functions are involved.

Abbreviations: CRB, Centre de Ressources Biologiques (biological resource center); DEN, diethylnitrosamine; EXTL3, exostosin-like glycosyltransferase 3; Fru, fructose; Fru-6P, fructose-6 phosphate; Gal, galactose; GFAT, glutamine fructose-6-phosphate amidotransferase; Glc, glucose; Glc-1P, glucose-1 phosphate; Glc-6P, glucose-6 phosphate; GlcNac, *N*-acetyl glucosamine; GlcNac-6P, *N*-acetyl glucosamine 6 phosphate; gp130, glycoprotein 130; HBP, hexosamine biosynthetic pathway; NT, nontumor; *O*-GlcNAc, *O*-linked *N*-acetylglucosamine; OGA, *O*-linked *N*-acetylglucosaminyl hydrolase; OGT, *O*-linked *N*-acetylglucosaminyltransferase; REG3A, regenerating family member 3 alpha; T, tumor; UDP, uridine diphosphate; WT, wild-type.

Nicolas Moniaux and Jamila Faivre contributed equally to this work.

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Approach and Results: This study provides evidence for a suppressive role of REG3A in HCC by reducing *O*-GlcNAcylation in 2 mouse models of HCC, in vitro cell studies, and clinical samples. REG3A expression in hepatocytes significantly reduced global *O*-GlcNAcylation and *O*-GlcNAcylation of c-MYC in preneoplastic and tumor livers and markedly inhibited HCC development in REG3A-c-MYC double transgenic mice and mice exposed to diethylnitrosamine. REG3A modified *O*-GlcNAcylation without altering the expression or activity of O-linked N-acetylglucosaminyltransferase, O-linked N-acetylglucosaminyl hydrolase, or glutamine fructose-6-phosphate amidotransferase. Reduced *O*-GlcNAcylation was consistent with decreased levels of UDP-GlcNAc in precancerous and cancerous livers. This effect was linked to the ability of REG3A to bind glucose and glucose-6 phosphate, suggested by a REG3A mutant unable to bind glucose and glucose-6 phosphate and alter *O*-GlcNAcylation. Importantly, patients with cirrhosis with high hepatic REG3A expression had lower levels of *O*-GlcNAcylation and longer cancer-free survival than REG3A-negative cirrhotic livers.

Conclusions: REG3A helps fight liver cancer by reducing *O*-GlcNAcylation. This study suggests a new paradigm for the regulation of *O*-GlcNAc signaling in cancer-related pathways through interactions with the carbohydrate-binding function of REG3A.

INTRODUCTION

C-type lectins are a large family of secreted or transmembrane carbohydrate-binding proteins that act as pattern recognition receptors capable of recognizing molecules from pathogens (pathogen-associated molecular patterns) and damaged cells (damage-associated molecular patterns) and thus modulate many essential processes, such as innate and adaptive immune responses and inflammation.[1] They recognize a wide range of carbohydrate and protein ligands and form the first line of protection against infections and transformed cells. They have several distinct functions, including signal transduction, cell adhesion and aggregation, cell proliferation and death, platelet activation, endocytosis, and phagocytosis.[2] C-type lectins are essential for life, and their invalidation in different species generally results in developmental and physiological defects.

Human regenerating family member 3 alpha (REG3A) is a small, secreted C-type lectin composed of a single carbohydrate-binding domain that binds mannose and *N*-acetylglucosamine (GlcNAc) with a preference for long carbohydrate chains.[3] REG3A binds to peptidoglycan and lipopolysaccharides,[4] destabilizes bacterial membranes by forming pores,[5] enhances the survival of highly oxygen-sensitive bacteria, and promotes intestinal barrier function against inflammation and oxidative stress.[4,6,7] In addition to its role in antimicrobial immunity, REG3A also promotes repair and regeneration of injured tissues, such as the liver,[6] heart,[8] skin,[9] pancreas, and nervous system,[10] and improves energy metabolism and insulin resistance.[11] Other members of the REG3 family are REG3G in humans[12] and Reg3a, Reg3b, Reg3g, and Reg3d in mice,[13] all of which share a high degree of sequence similarity. Reg3b and Reg3g are considered orthologs of REG3A in mice. REG3A expression, which is restricted to a small number of healthy tissues and cells, increases sharply in a range of pathological conditions, including infectious, inflammatory, tumor, and autoimmune diseases.[14,15] REG3A is subsequently secreted into extracellular sites and is linked to numerous components and signaling pathways involved in cell survival, proliferation, migration, and differentiation.[9,16,17] The mechanisms by which REG3A triggers intracellular signaling remain poorly defined and involve, depending on the context, tissue, and cell type, carbohydrate ligand recognition, carbohydrate-protein interaction, or protein-protein interaction. REG3A-binding surface receptors and membrane-associated proteins vary according to tissue and cell type. Glycoprotein 130 (gp130), fibronectin, epidermal growth factor receptor, and exostosin-like glycosyltransferase 3 (EXTL3) have been shown to interact with REG3A and transmit the REG3A signal into cells through different signaling cascades.[9,16,17]

We showed that REG3A attenuated oxidative damage to proteins, including gp130, thereby activating gp130-dependent AMP-activated protein kinase in skeletal muscle and improving glucose homeostasis and insulin sensitivity in obese and diabetic mice.[11]

Although members of the REG3 family have been studied in inflammatory settings, their role in cancer biology remains unclear. REG3A expression is usually altered in inflammatory conditions (such as pancreatitis, colitis, hepatitis, and cirrhosis) and in cancers that develop chronic inflammation (such as pancreatic ductal adenocarcinoma, gastrointestinal tract cancers, and primary liver cancers), giving it potential value as a diagnostic or prognostic marker of disease, but raising difficulties in establishing causality between REG3A and cancer.[15,18,19] As homeostatic regulators of cells, REG3 functions can be exploited by cancer and noncancer cells in the tumor ecosystem to interfere with antitumor immunity and malignant phenotypes. As a result, the effects of REG3A on carcinogenesis are not clearly defined and vary according to the methods and models used, tissue of origin, type of cancer, and stage of tumor development, with no clear understanding of phenotypic variations.

O-GlcNAcylation results from the addition of GlcNAc to the serine and threonine residues of cytosolic, nuclear, and mitochondrial proteins. This reversible glycosylation process regulates the activity, stability, and localization of over 5000 proteins. Only 2 enzymes, *O*-linked *N*-acetylglucosaminyltransferase (OGT) and *O*-linked *N*-acetylglucosaminyl hydrolase (OGA), control the level of *O*-GlcNAc on proteins. OGT uses the uridine diphosphate (UDP)-GlcNAc produced from glucose by the hexosamine biosynthetic pathway (HBP) to add GlcNAc to proteins, and OGA removes it.[20] Numerous studies have demonstrated a dynamic interplay between *O*-GlcNAcylation and phosphorylation, enabling fine-tuning of cellular signaling pathways and the regulation of gene expression, cell proliferation, and apoptosis. Increases in protein *O*-GlcNAcylation have been described in many cancers, including HCC.[20]

The present study describes a hitherto unknown mechanism by which REG3A reduces protein *O*-GlcNAcylation in preneoplastic and advanced stages of HCC, markedly influencing tumor phenotype by delaying cancer onset and reducing tumor burden in chemical and genetic mouse models of HCC. This was coupled with a significant decrease in UDP-GlcNAc levels in precancerous and cancerous livers from MYC/REG3A transgenic mice compared to MYC transgenic mice, consistent with the reduced levels of protein *O*-GlcNAcylation that were detected. We show that REG3A, through lectin activity, binds to the main sugar substrates required for UDP-GlcNAc synthesis by HBP, suggesting a change in their balance and availability for *O*-GlcNAcylation. Importantly, higher REG3A expression in the liver of patients with cirrhosis correlates with longer cancer-free survival and lower *O*-GlcNAcylation. By placing REG3A at the interface of carbohydrate motif binding and regulation of key cellular processes, our study suggests a new paradigm whereby REG3A acts as a transducer of glycosylation pathways to regulate cancer cell fate and growth.

METHODS

Human samples

The liver samples used in this study were collected from patients undergoing liver transplantation for HCC at the Paul-Brousse Hospital (Hepatobiliary Center, Villejuif, France), with the approval of the INSERM Institutional Review Board (number 11-047). All research was conducted in accordance with both the Declarations of Helsinki and Istanbul, and written consent was obtained from all subjects. Nontumor (NT)/tumor (T) liver samples from 18 patients aged 59 $\pm$ 3 years with HCC developing cirrhotic fatty liver disease (NASH, n = 7; alcohol-associated liver disease, n = 3; HCV, n = 6, alcohol-associated and HCV, n = 1, HBV, n = 1) were analyzed. Histological analysis was performed by pathologists from the pathology department of Bicêtre Hospital (Kremlin-Bicêtre, France), and frozen tissues were stored at −80°C for research purposes at the Centre de Ressources Biologiques of the AP-HP-Université Paris-Saclay.

Mouse models for liver cancer

Animal studies were performed in accordance with institutional and European Union guidelines for laboratory animal care and were approved by the ethics committee of CE2A-03-CNRS-Orléans (Accreditation No. 01417.01). The number of *Mus musculus* mice used was in accordance with institutional ethical guidelines and standard practice in cancer studies. Two liver cancer models were studied: a model chemically induced by diethylnitrosamine (DEN) in REG3A transgenic mice (REG3A-TG) and a genetic model of MYC-REG3A double homozygous transgenic mice after crossing REG3A-TG mice with mice expressing c-MYC in the liver (MYC-REG3A-TG).

Biochemical analyses

As appropriate, total RNA extracted from liver samples or HuH7 cells expressing or not REG3A was used for transcriptomic analysis by Affymetrix microarray or real-time RT-PCR using the primer sequences for c-MYC, OGT, OGA, GFAT1, glutamine fructose-6-phosphate amidotransferase (GFAT)2, and EXTL3 (Supplementary Table S3, http://links.lww.com/HEP/I531). Protein lysates were prepared from frozen liver samples or HuH7 cells

expressing full-length REG3A or REG3A mutated in the EPN motif of the C-like domain of the lectin and extracts of total, nuclear, and cytoplasmic fractions were immunoblotted against, among others, REG3A, MYC, pT58 c-MYC, *O*-GlcNAc (anti-RL2), GSK3β, pGSK3β, ERK, pERK, OGT, OGA, GFAT1/2, EXTL3, or used for anti-MYC or anti-EXTL-3 immunoprecipitation, succinylated wheat germ agglutin pull-down, wheat germ agglutin and phytohemaglutinin-L lectin blotting, and protein carbonylation analysis. Quantification of UDP-GlcNAc, glycogen, and the enzymatic activities of OGT, OGA, and GFAT was also performed on frozen samples of liver or cells expressing or not REG3A following the appropriate procedures. Quantification of heparan sulfate (HS) and chondroitin disaccharides was performed using ion-pair reversed-phase chromatography.

Statistical analysis

Differences in the number of animals developing liver tumors and the size of tumors formed were estimated using a 1-tailed Fisher exact test applied to the DEN and MYC models. The ratio of liver weight to body weight of the mice was analyzed using the Student *t* test. Differences in RNA, protein, and enzyme activity levels between experimental groups were determined using the Mann-Whitney *U* test. Differences in *O*-GlcNAcylation in human tissues were estimated using a paired Student *t* test, and differences in *O*-GlcNAcylation in Huh7 cells expressing REG3A ± EXTL3, as well as the amount of HS and chondroitin sulfate in the cells using a paired Wilcoxon test with correction for multiple testing. Data are expressed as mean ± SEM or box plots. $p < 0.05$ is considered significant. Kaplan-Meier survival analysis using a log-rank test was applied to compare the survival time of cohorts of c-Myc expressing transgenic and MYC and REG3A double transgenic mice.

RESULTS

REG3A is associated with late onset of HCC in patients with cirrhosis

We assessed REG3A expression in cancer-free cirrhotic livers and livers with HCC and investigated whether REG3A levels were associated with HCC onset or progression using publicly available transcriptomic data from 3 data sets. We used GSE15654, which includes data from 216 liver biopsies taken at the time of cirrhosis diagnosis (Child A) in patients subsequently followed for 20 years,[21] TCGA (286 patients with HCC), and GSE14520 (221 patients with HCC).[22] The follow-up period for patients with cirrhosis in GSE15654 was 20 years. Correlations between REG3A expression and patient clinicopathological characteristics are presented in Supplemental Table S1, http://links.lww.com/HEP/I531 (patients with cirrhosis) and Supplemental Table S2, http://links.lww.com/HEP/I531 (patients with HCC). Figure 1 shows the Kaplan-Meier curves of tumor-free survival and overall survival as a function of REG3A levels in patients with cirrhosis and HCC. We found that patients with cirrhosis in the subgroup with high REG3A expression (n = 45) had significantly longer overall tumor-free survival than patients in the subgroup with low REG3A expression (n = 171; $p = 0.00052$; Figure 1A). Cancer-free survival at 10 years was 92% and 66%, and at 20 years, it was 88.1% and 19.7% for the high and low REG3A expression subgroups, respectively. The median cancer-free survival was not achieved in the high REG3A subgroup and was 12 years in the low REG3A subgroup. Once HCC had formed, no significant correlation was found between REG3A expression in tumor (T) or NT liver and overall survival of patients with HCC in the GSE14520 and TCGA sets (Figures 1B–D). We also found no correlation between REG3A expression and some of the available clinical and pathological features (risk factors, TNM stages, and histological grades) (Supplemental Table S2, http://links.lww.com/HEP/I531). These results suggest that REG3A expression in preneoplastic cirrhotic liver helps delay the onset of HCC but does not affect tumor growth once cancer has formed.

REG3A delays the onset of cancer and slows tumor growth in mice with HCC induced by DEN or c-MYC overexpression

To assess the effects of hepatic REG3A expression on the initiation and fate of HCC, we used previously generated homozygous transgenic mice expressing human REG3A in hepatocytes under the control of the mouse albumin gene promoter (REG3A-TG).[23] Two liver cancer models were studied: a chemical model of a single dose of DEN in 2-week-old REG3A-TG mice,[24] and a genetic model of MYC-REG3A double-homozygous transgenic mice by crossing REG3A-TG mice with mice expressing c-MYC (now called MYC) in the liver following the integration of the woodchuck hepatitis virus (WHV)/MYC oncogene into the mouse genome (MYC-REG3A-TG).[25] Transgenic hepatocytes secrete REG3A into blood vessels through the basolateral membranes, resulting in serum REG3A levels of 346 ± 38 ng/mL in REG3-TG mice, 6 ± 2 ng/mL in MYC-TG mice, and 214 ± 17 ng/mL in MYC-REG3A-TG mice (n = 40). Five months after DEN injection, REG3A-TG mice showed no macroscopic or histological tumor nodules, in contrast to 40% of wild-type (WT) mice ($p = 0.043$) (Figures 2A, B). At 6 months, REG3A-TG mice (40%) had tumor nodules, all of which were small (< 1 mm in diameter), whereas WT mice (60%) developed large tumors, with one-third having 1–5 mm tumors and another third having large

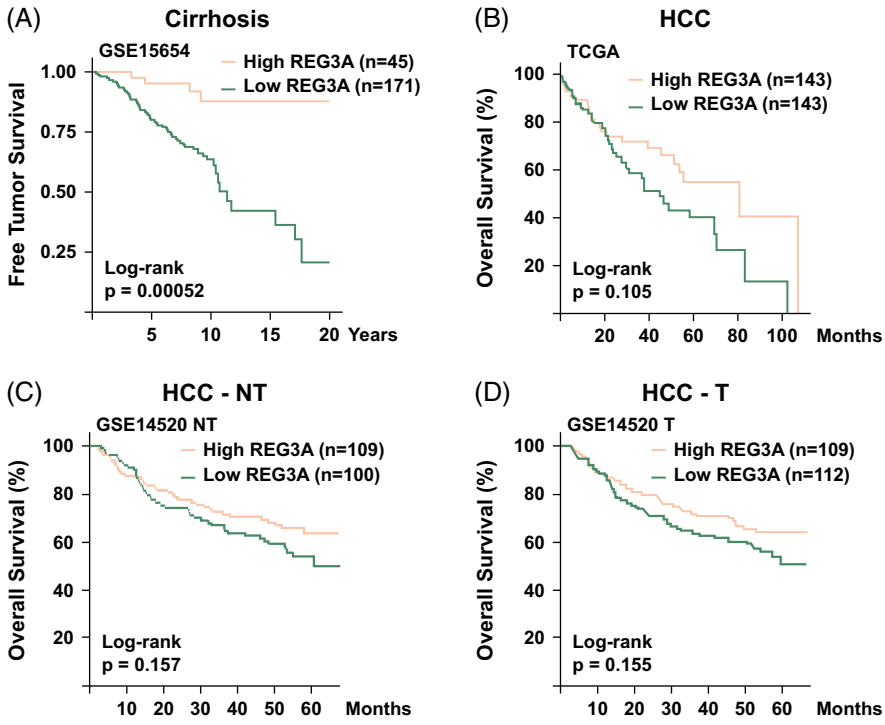

FIGURE 1 Cancer-free survival and overall survival of patients with cirrhosis or HCC, respectively, according to hepatic REG3A expression. (A) Kaplan-Meier curve of tumor-free survival (GSE15654; n = 216 patients with hepatitis C–related early-stage cirrhosis). Patients with high REG3A-expressing cirrhosis have longer tumor-free survival than those with low REG3A-expressing cirrhosis. (B) Overall survival of patients with HCC according to the level of REG3A expression in the tumor (TCGA; n = 286). (C, D) Overall survival of patients with HCC (GSE14520) according to the level of REG3A expression in (C) nontumor area adjacent to the tumor (HCC-NT; n = 209) and (D) in the tumor area (HCC-T; n = 221). No difference in overall survival was found in patients with HCC, whether or not the tumor area expressed REG3A. Abbreviations: NT, nontumor; REG3A, regenerating family member 3 alpha; T, tumor.

tumors of 5–20 mm. At this stage, the liver remained completely healthy in 55% and 33% of the REG3A-TG and WT mice, respectively ($p = 0.009$) (Figure 2B). Thereafter, comparable tumor development in terms of tumor incidence and size was observed in the REG3A-TG and WT mice. Two cohorts of MYC-REG3A-TG transgenic mice were sampled at different time points for a survival study. The ratio of liver weight to body weight was significantly lower in MYC-REG3A-TG mice than in MYC-TG mice, suggesting a reduced tumor burden in MYC-REG3A-TG mice (Figure 2C). In this model, the first macroscopically visible tumors were detected in 6-month-old mice, with a tumor incidence of 40% in MYC-TG and 13% in MYC-REG3A-TG mice ($p = 0.0195$) (Figure 2D). The tumor incidence at 9 months was 82% versus 42% ($p = 0.0018$), at 12 months 90% versus 71% ($p = 0.059$), and at 18 months 81% versus 73% ($p =$ n. s.) for MYC-TG and MYC-REG3A-TG mice, respectively. Tumor mass was significantly lower in the livers of MYC-REG3A-TG mice than in those of MYC-TG mice during the 6–12-month period of carcinogenesis (Figure 2E). After this period, the tumor mass did not differ between the 2 mouse lines, nor were there levels of cell proliferation (Ki67 and cyclin expression), apoptosis (cleaved caspase 3), and DNA damage (histone H2AX phosphorylated at serine 139, γ-H2AX) (Supplemental Figure S1, http://links.lww.com/HEP/I531). Kaplan-Meier analysis showed that overexpression of REG3A resulted in significantly longer survival of MYC-REG3A-TG mice compared to MYC-TG mice, which is likely due to delayed tumor onset ($p = 0.0085$; Figure 2F). These data establish that REG3A overexpression in the liver slows tumor nodule development in the 2 induced models of HCC, suggesting a negative regulatory role for REG3A in tumor initiation.

REG3A overexpression is associated with a decrease in MYC *O*-GlcNAcylation and MYC-driven transcriptome in HCC

MYC expression is regulated at multiple levels, from transcriptional initiation to protein turnover. The latter involves competing posttranslational modifications at threonine 58 (T58), which can be either *O*-GlcNAcylated (*O*-linked *N*-acetylglucosaminylation) or phosphorylated. *O*-GlcNAcylation increases MYC stability, whereas phosphorylation facilitates proteolysis through the ubiquitin-proteasome pathway. We analyzed changes in MYC mRNA and protein expression, as well as the ratio of nuclear (active) to cytoplasmic (inactive) MYC protein, in preneoplastic liver samples

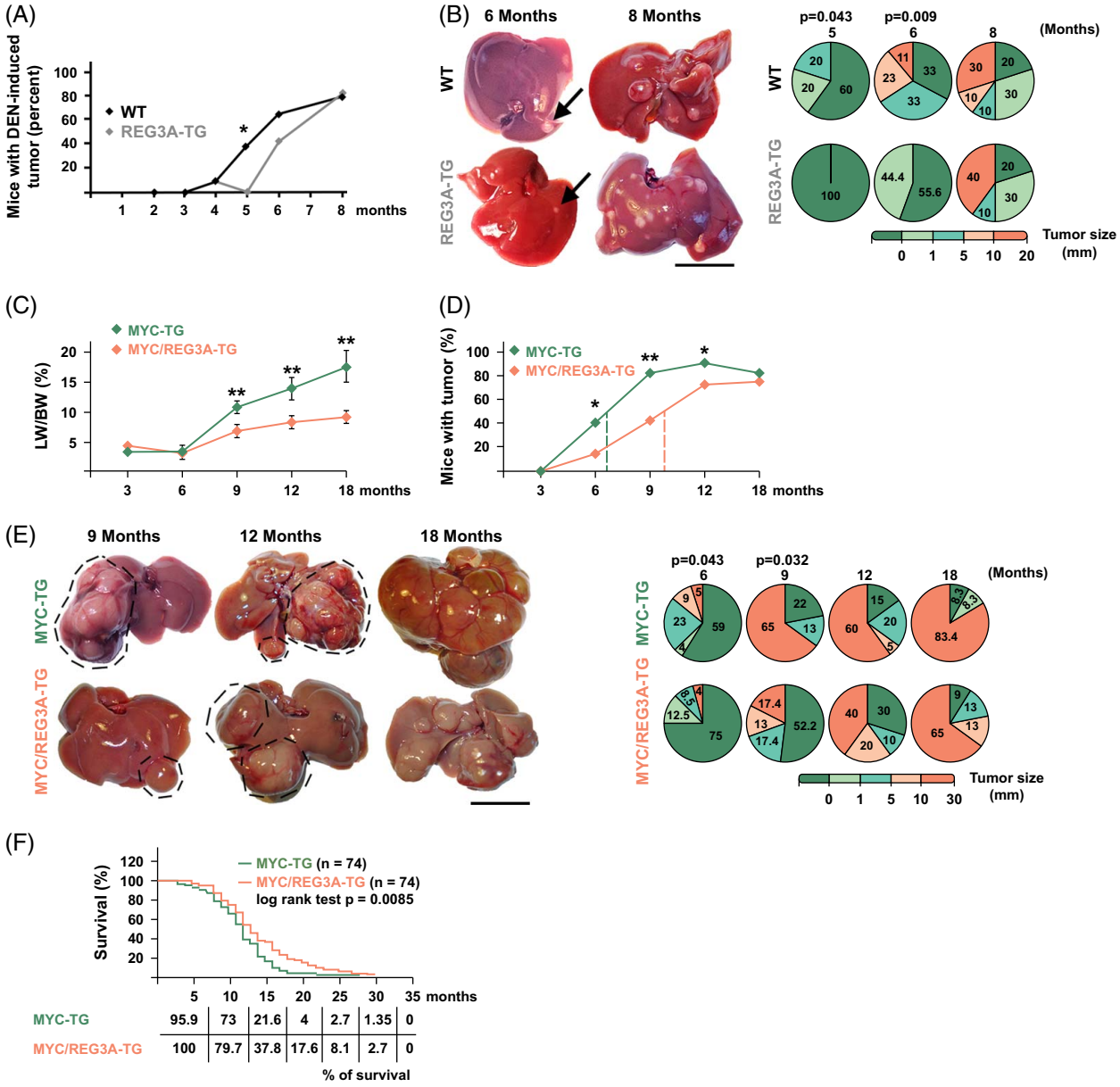

FIGURE 2 REG3A strongly reduced cancer development in 2 mouse models of HCC. (A, B) WT and transgenic mice overexpressing REG3A in hepatocytes (REG3A-TG) received a single injection of the chemotoxic agent DEN at 14 days of age, and then tumor growth was monitored in independent groups of mice monthly for 8 months. (A) Proportion of mice with tumors over time. n = 9–16 mice for WT group and n = 9–14 for REG3A-TG group. (B) Left: Representative macroscopic views. Scale bar: 10 mm. Right: Circle pie charts for counting mice with healthy (dark green) or tumor livers (color-coded by nodule size, measured by the sum of the largest diameters of the nodule). WT livers show multiple nodules of (sub) centimeter size from 6 months of age. Transgenic livers are macroscopically normal over a longer time period or contain much smaller and fewer nodules than WT livers. The value shown in each portion of the pie chart represents the percentage of mice with a given nodule size. Arrows: the smallest tumor nodules detected at 6 months. (C–F) Genetic model for HCC in single transgenic mice for MYC (MYC-TG) and double transgenic mice for MYC and REG3A (MYC/REG3A-TG). (C) Liver weight to body weight (LW/BW). n = 12–20 independent mice per time and per group of mice. (D) Percentage of mice with HCC tumors over time (n = 40). (E) Left: Representative macroscopic views. Scale bar: 10 mm. Dotted lines: delineated tumor mass when possible. Right: Proportion of mice with healthy (dark green) or tumor (other colors of the code) liver. (F) Kaplan-Meier curve of overall survival of mice. Data are means ± SEM. The 1-tailed Fisher exact test was performed for analysis except for (C) (Student *t* test). $*p < 0.05$, $**p < 0.01$. NS or no statistical significance, no significance. Abbreviations: DEN, diethylnitrosamine; REG3A, regenerating family member 3 alpha; WT, wild-type.

(histologically healthy 6-month-old mouse livers) and pairs of NT and tumor (T) livers. As expected, MYC mRNA expression was barely detectable in preneoplastic and NT liver samples (mice aged 3–6 mo) and then increased over time to reach maximal levels in T liver samples from older mice (>12 mo) (Figure 3A). No difference in MYC mRNA levels was observed between MYC-TG and MYC-REG3A-TG mice at any of the time points examined (Figure 3A). Similarly, the levels of total MYC, nuclear MYC, and

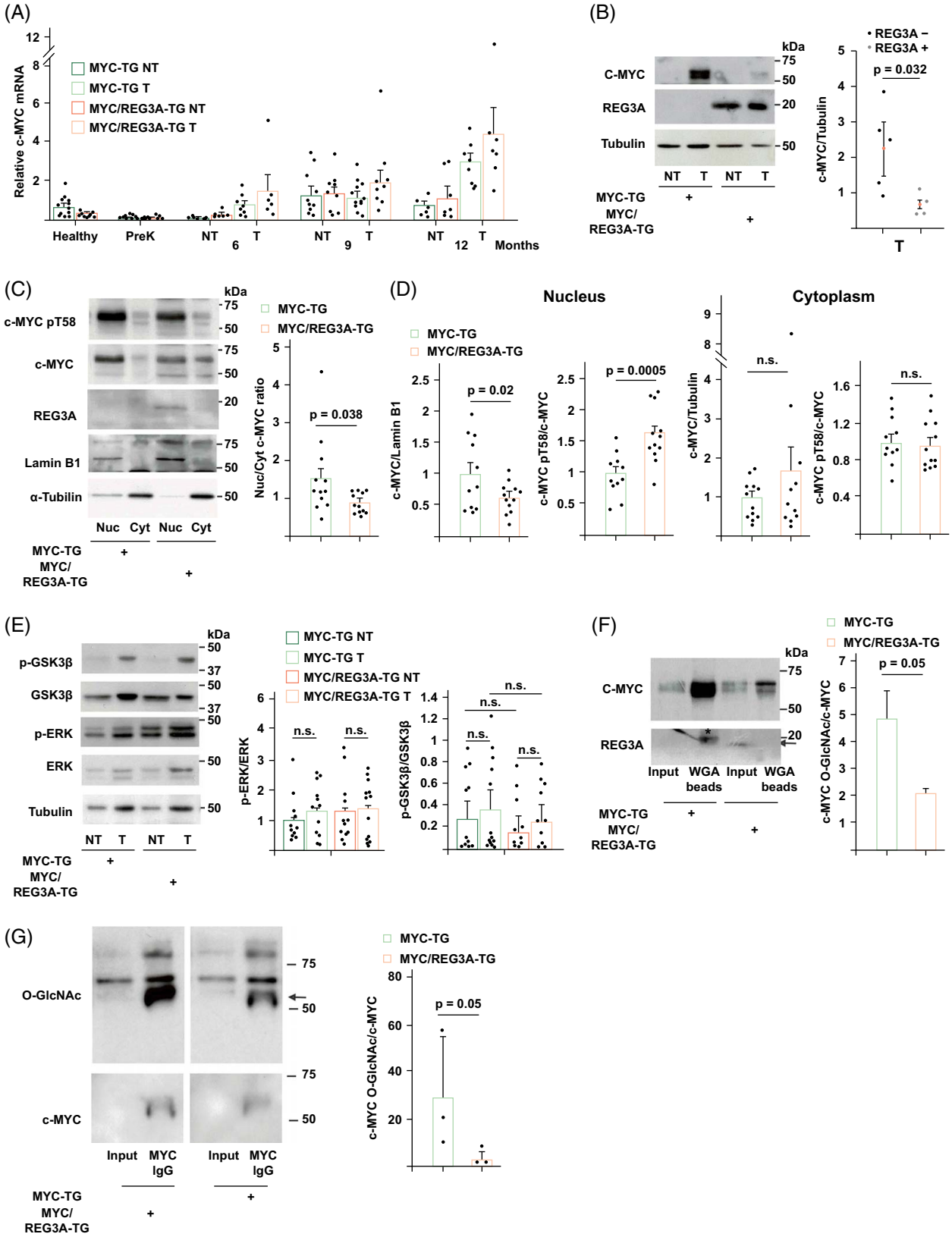

FIGURE 3 REG3A is associated with a decrease in *O*-GlcNAcylation of MYC in mouse liver carcinoma. (A) qRT-PCR analysis of MYC transcript levels in liver samples at the indicated time points and pathological conditions. MYC-TG, transgenic mice homozygous for MYC; MYC/REG3A-TG, transgenic mice double homozygous for MYC, and REG3A. Healthy, normal liver sample from 3-month-old mice; preK, estimated precancerous liver sample from 6-month-old mice, livers in which the absence of tumor nodules was assessed macroscopically and microscopically; nontumor areas (NT); tumor areas (T); n = 6–16 per group. (B) Immunoblots for MYC and REG3A proteins and quantification of MYC from total protein extracts in

REG3A-negative and positive tumors. Each dot represents a tumor sample from an independent mouse. (C) Immunoblots for the indicated proteins after nucleo-cytoplasmic fractionation and quantification of nuclear (Nuc) to cytosolic (Cyt) MYC ratio. (D) Quantification of total MYC protein levels and pT58 to total MYC ratio. (E) Immunoblots of phospho-GSK3β (Ser9), total GSK3β, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), and total Erk1/2, and related densitometry. Each dot represents a sample from an independent mouse. (F) Succinylated WGA lectin pull down to determine the level of *O*-GlcNAcylated MYC in MYC tumors expressing or not REG3A. n = 3 independent experiments. The arrow indicates the REG3A signal. Asterix: nonspecific signal. (G) Immunoblots for *O*-GlcNAc and MYC proteins following immunoprecipitation of MYC from liver extracts of MYC and MYC/REG3A transgenic mice. The arrow indicates the MYC signal. n = 3 independent experiments. Data are averages ± SEM. The Mann-Whitney *U* test was performed for analysis except for (E) (paired Wilcoxon test with correction for multiple testing). NS or no statistical indication, no significance. Abbreviations: qRT-PCR, quantitative real-time PCR; REG3A, regenerating family member 3 alpha; WGA, wheat germ agglutinin.

phosphorylated MYC protein (p-MYCT58) were low and comparable in preneoplastic and NT liver samples from MYC-TG and MYC-REG3A-TG mice (Supplemental Figure S2, http://links.lww.com/HEP/I531). In contrast, the elevation of total and nuclear MYC protein levels in tumor tissue was lower in livers expressing REG3A than in those that did not (Figures 3B–D). Importantly, the ratio of p-MYCT58 to total MYC was higher in the nuclear fractions of MYC-REG3A tumors than in those of MYC tumors, consistent with the shorter MYC half-life in MYC-REG3A tumors. REG3A was associated with high tumor levels of phosphorylated MYC without changes in the expression or phosphorylation of ERK1/2 and GSK-3β, 2 kinases essential for regulating MYC stability (Figure 3E). Given the crucial role of *O*-GlcNAcylation in MYC stability, we assessed *O*-GlcNAcylated MYC levels in tumor samples by pull down with succinylated wheat germ agglutinin, which binds to GlcNAc-containing glycoconjugates (Figure 3F) and by immunoprecipitation of MYC (Figure 3G). A significant decrease in the level of *O*-GlcNAcylated MYC was found in tumor liver samples from MYC-REG3A mice compared to that in MYC mice (Figures 3F, G), consistent with lower MYC accumulation. This was further supported at the gene expression level through an MYC-dependent transcriptome unique to REG3A-positive tumors. We show that HCCs from MYC-REG3A-TG belong to transcriptomic subclasses S3 and G5 of the human HCC classifications proposed by Hoshida et al and Boyault et al, respectively, subclasses that concentrate on well-differentiated HCCs and HCCs with CTNNB1 gene mutations[26,27] (Figure 4A). HCCs from MYC-TG mice exhibited features of the S1 and G2/G3 subclasses (Figure 4B). The results of the GSEA analysis indicated markedly differential gene expression profiles. MYC-REG3A tumors and adjacent liver were enriched in metabolic pathways, including bile acid, xenobiotic and fatty acid metabolism, oxidative phosphorylation, and activation of Wnt/β-catenin signaling, while transcriptional reprogramming of liver from MYC-TG mice resulted in activation of the cell cycle, inflammation, and glycolysis pathways, as expected (Figures 4C, D). Using the TCGA data set, we defined a signature of 100 genes that were most differentially expressed in human HCCs expressing REG3A, applied the GSEA method, and showed that this signature was predominant in MYC-REG3A mouse HCCs, implying that the MYC-REG3A-TG model reproduces the molecular alterations of REG3A-positive human HCCs (Figure 4E). We also assessed the expression of target genes of the MYC core signature (n = 77) proposed by Ji et al[28] and found that this was strongly attenuated in MYC-REG3A tumors compared to MYC tumors (Figure 4F). Overall, these results suggest that the decrease in MYC protein levels in MYC-REG3A tumors, partly correlated with the increase in p-MYCT58 (degradation signal) and partly with the decrease in *O*-GlcNAcylated MYC levels (stabilization signal), helped improve the hepatic differentiation phenotype.

REG3A-expressing HCC livers from patients and mouse models show a strong reduction in protein *O*-GlcNAcylation

To assess whether the decrease in *O*-GlcNAcylation caused by REG3A overexpression was restricted to MYC, we quantified overall hepatic *O*-GlcNAcylation in total cell lysates of preneoplastic liver samples and NT/T pairs from MYC-TG and MYC-REG3A-TG mice using western blotting with an anti-*O*-GlcNAc antibody (Supplemental Figure S3A, http://links.lww.com/HEP/I531v). We showed that hepatic *O*-GlcNAcylation increased from the preneoplastic to tumor state in both mouse lines (Supplemental Figure S3A, http://links.lww.com/HEP/I531), as reported in different cancer types.[20] However, *O*-GlcNAcylation in preneoplastic livers from MYC-REG3A-TG mice was significantly reduced compared to that in MYC-TG mice and was comparable in tumor samples from both mouse lines (Supplemental Figure S3A, http://links.lww.com/HEP/I531). A reduction in O-GlcNAcylation was also observed in the basal state of healthy livers of the transgenic parental line compared to that in syngeneic WT mice. As protein *O*-GlcNAcylation occurs in the cytosol and nucleus, we quantified *O*-GlcNAcylation in the cytosolic and nuclear compartments after subcellular fractionation. A strong reduction in protein *O*-GlcNAcylation was observed in the nuclear (−60 to −70%) and cytosolic (−40%) fractions of preneoplastic and NT (adjacent to T) liver samples in MYC-REG3A-TG mice compared to MYC-TG mice (Figures 5A, B). A decrease in nuclear *O*-GlcNAcylation was observed in MYC-REG3A-TG mouse T samples, albeit

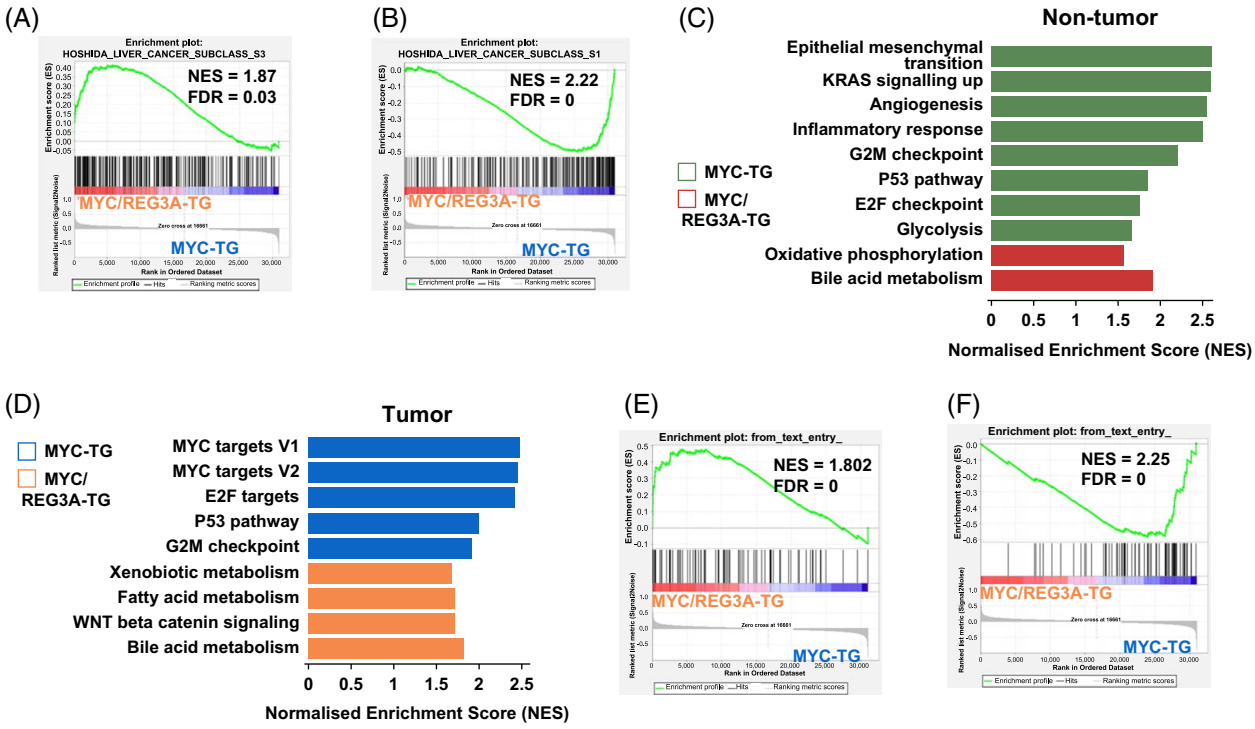

FIGURE 4 REG3A alters the MYC-dependent transcriptional program in mouse HCC. (A–F) Differentially expressed genes identified by microarray analysis of mouse liver specimens expressing MYC (MYC-TG) or MYC and REG3A (MYC/REG3A-TG). n = 5 mice per group. Enrichment plots of genes related to the S3 subclass (A) or S1 subclass (B) of the molecular classification of Hoshida et al[26] in murine MYC tumors expressing REG3A or not, respectively. (C, D) Pathway enrichment analysis showing the different molecular pathways from enrichment analysis of differentially expressed genes in nontumor area (C) and tumor area (D) of MYC-REG3A mouse livers compared with MYC mouse livers. (E) Enrichment plots of the 100 most expressed genes in human HCCs that express endogenous REG3A (TCGA data set). The top 100 signatures is enriched in HCCs from MYC/REG3A-TG mice compared to MYC-TG mice. (F) Enrichment plot of an MYC core signature (n = 77) described by Ji et al[28] that is strongly attenuated in MYC tumors expressing REG3A compared to MYC tumors not expressing REG3A. Data are averages ± SEM. The Mann-Whitney *U* test was performed for analysis. NS or no statistical indication, no significance. Abbreviation: REG3A, regenerating family member 3 alpha.

less pronounced (−20%) (Figure 5C). We also observed a decrease in nuclear *O*-GlcNAcylation in the DEN-induced HCC model, with a 30% reduction in liver cancer in REG3A-TG mice compared to WT mice (Figure 5D). We analyzed global *O*-GlcNAcylation in 18 pairs of NT/T human HCC samples according to whether they expressed REG3A. Although sampling was limited, an increase in *O*-GlcNAcylation was detected in T samples compared with NT samples ($p = 0.0047$) (Figure 5E), which is in agreement with reports on human HCC.[29] Interpatient variability in *O*-GlcNAcylation in T and NT is, however, noteworthy, with approximately two-thirds of T samples showing a strong apparent elevation in *O*-GlcNAc levels compared to matched NT samples. When the level of *O*-GlcNAc was analyzed as a function of REG3A, we found that NT samples expressing REG3A had significantly lower levels of *O*-GlcNAc than NTs not expressing REG3A, whereas this difference was not observed between T samples, regardless of the presence of REG3A (Figure 5F). These data indicate that the REG3A-mediated reduction of *O*-GlcNAcylation is a common feature in several experimental and human models of HCC. They further suggested that the lower level of *O*-GlcNAcylation in NT areas of REG3A-positive compared to REG3A-negative human livers may be involved in the delayed onset of HCC in REG3A-expressing cirrhotic livers, as shown in Figure 1A.

REG3A alters global *O*-GlcNAcylation without disrupting *O*-GlcNAc processing enzymes

Protein *O*-GlcNAcylation is regulated by 2 enzymes, OGT and OGA (*O*-GlcNAcase; *N*-acetyl-β-D glucosaminidase), which catalyze the addition or removal of *O*-GlcNAc from target proteins, respectively, thereby regulating protein function, interactions, and subcellular localization. To better understand how REG3A-related signaling negatively regulates global *O*-GlcNAcylation, we analyzed OGT and OGA RNA and protein levels and their enzymatic activities in preneoplastic livers that exhibited the greatest decreases in *O*-GlcNAcylation. We found no difference in OGT and OGA transcript levels (Supplemental Figure S3B, http://links.lww.com/HEP/I531) or OGA protein levels when we compared the livers of 6-month-old MYC-TG and MYC-REG3A-TG mice (Supplemental Figure S3C, http://links.lww.com/

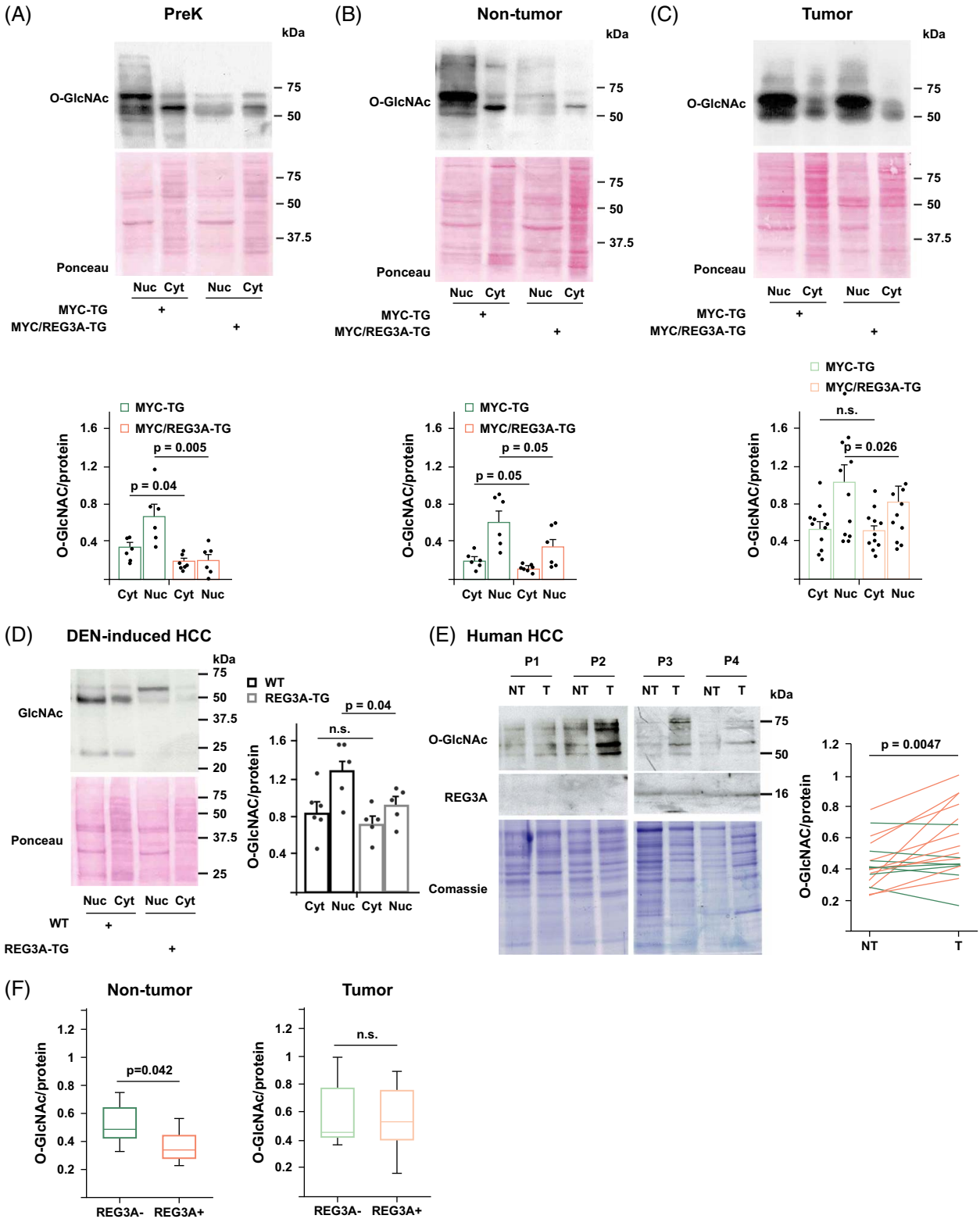

FIGURE 5 Preneoplastic and tumor livers of HCC expressing REG3A show substantial reduction of *O*-GlcNAcylation in mice and humans. Anti-RL2 immunoblots for *O*-GlcNAc. Proteins in nuclear (Nuc) and cytosolic (Cyt) fractions of (A) preneoplastic (preK) livers (n = 6), (B) in the nontumor area (n = 6) and (C) the tumor area (n = 12) of HCC from transgenic mice homozygous for MYC (MYC-TG) and transgenic mice double homozygous for MYC and REG3A (MYC/REG3A-TG). Quantification by densitometry below western blots. Each dot represents a sample from an independent mouse. (D) Western blots for *O*-GlcNAc proteins in livers of WT (n = 6) and transgenic mice overexpressing REG3A in the liver (REG3A-TG; n = 4) with DEN-induced HCC. (E) Western blots for *O*-GlcNAc proteins and endogenous REG3A protein in human HCC. Quantification of *O*-GlcNAc proteins in 18 NT and T matched samples. P1 = patient 1. Increased *O*-GlcNAcylation in 13 T samples (red line) and no detectable real change in *O*-GlcNAcylation in 5 T samples (green line) compared with matched NT samples ($p = 0.0047$). (F) *O*-GlcNAcylation

in human HCC samples as a function of whether they express or not endogenous REG3A in the NT and T areas. Ponceau S and Coomassie blue staining were used as loading controls. Data are averages ± SEM. The Mann-Whitney *U* test was performed except for (E) (paired Student *t* test) and (F) (Student *t* test). NS or no statistical indication, no significance. Abbreviations: DEN, diethylnitrosamine; NT, nontumor; REG3A, regenerating family member 3 alpha; T, tumor; WT, wild-type.

HEP/I531). A slight increase in OGT protein was noted in MYC-REG3A-TG mice (Supplemental Figure S3C, http://links.lww.com/HEP/I531). Equivalent levels of OGA and OGT activity were detected in both types of mice (Supplemental Figure S3D, http://links.lww.com/HEP/I531). OGT uses UDP-GlcNAc as a sugar-donor substrate produced by glucose metabolism by means of the HBP. HBP activity is controlled by the rate-limiting activity of GFAT, which is the first step of this pathway. A significant increase in GFAT1 mRNA expression, but not GFAT2, was observed in the livers of REG3A-positive MYC tumors (Supplemental Figure S3E, http://links.lww.com/HEP/I531). However, similar levels of GFAT1/2 protein expression (Supplemental Figure S3F, http://links.lww.com/HEP/I531) and activity (Supplemental Figure S3G, http://links.lww.com/HEP/I531) were observed in the livers of MYC-REG3A-TG and MYC-TG mice. We speculated that the increase in OGT and GFAT1 mRNA expression in preneoplastic livers expressing REG3A may reflect positive feedback in response to reduced *O*-GlcNAcylation, a phenomenon that has been described for homeostatic regulation of OGT mRNA expression in response to cellular *O*-GlcNAc levels.[30] These results indicate that the decrease in *O*-GlcNAcylation associated with REG3A cannot be attributed to the reduced expression or activity of the HBP rate-limiting enzyme (GFAT), nor to alterations in the *O*-GlcNAc processing enzymes, OGT, and OGA. The link between *O*-GlcNAcylation and cellular stress response pathways,[31] including oxidative stress signaling,[32] has been widely documented. Thus, an increase in *O*-GlcNAcylation is known to have protective effects against oxidative stress,[32] whereas a reduction in oxidative stress is associated with a decrease in protein *O*-GlcNAcylation.[33] We reasoned that a reduction in oxidative stress through the reactive oxygen species scavenging activity of REG3A could lead to a decrease in global *O*-GlcNAcylation, thereby reducing liver carcinogenesis. We quantified protein carbonylation levels in preneoplastic liver samples and NT/T pairs and detected no differences in protein oxidation levels between MYC-TG and MYC-REG3A-TG mice (Supplemental Figure S3H, http://links.lww.com/HEP/I531). These results suggested that the decrease in hepatic *O*-GlcNAcylation does not rely on the interactions between REG3A and its antioxidant activity.

To gain further insight, REG3A was overexpressed in the HuH7 HCC cell line, and analyses were performed 48 hours after transfection (Supplemental Figure S4, http://links.lww.com/HEP/I531). REG3A overexpression, in line with what was observed in mouse tissues, resulted in no change in endogenous MYC mRNA levels, a marked reduction in total MYC protein levels, and an increase in pT58-MYC in the nuclear fraction of HuH7 cells compared to control cells (Supplemental Figures S4A, B, http://links.lww.com/HEP/I531). The increase in pT58-MYC expression was not related to the altered phosphorylation of GSK3β and ERK (Supplemental Figure S4C, http://links.lww.com/HEP/I531). The level of global *O*-GlcNAcylation was significantly reduced in REG3A-expressing cells compared to that in control cells ($p = 0.0004$; Supplemental Figure S4D, http://links.lww.com/HEP/I531), and this was not associated with a reduction in glucose uptake (Supplemental Figure S4E, http://links.lww.com/HEP/I531). Decreased *O*-GlcNAcylation was associated with decreased proliferation of REG3A-expressing cells compared to those not expressing REG3A, consistent with positive correlations described in previous studies between protein *O*-GlcNAcylation and cancer cell proliferation (Supplemental Figure S4F, http://links.lww.com/HEP/I531). Similar to the liver tissue from MYC-REG3A-TG mice, the decrease in *O*-GlcNAcylation in HuH7 cells overexpressing REG3A was not accompanied by a change in the expression of OGT, OGA, or GFAT1/2 (Supplemental Figure S4G, http://links.lww.com/HEP/I531). Furthermore, the treatment of HuH7 cells with the OGA inhibitor Thiamet G (at a maximum inhibitory concentration of 10 μM) did not compromise the effect of REG3A, confirming that REG3A did not reduce *O*-GlcNAcylation by stimulating OGA enzymatic activity (Supplemental Figure S4H, http://links.lww.com/HEP/I531). As REG3A is a secreted protein that acts through both paracrine and autocrine pathways, we assessed whether the extracellular addition of REG3A to HuH7 cells could mimic the effect of REG3A overexpression. The addition of 3 μg of recombinant REG3A protein to the culture medium of HuH7 cells did not alter MYC expression levels, global *O*-GlcNAcylation, or the rate of cell proliferation (Supplemental Figure S5, http://links.lww.com/HEP/I531). These results indicate that cells expressing REG3A contain sufficient substrate (increased glucose uptake) and enzymes (GFAT1/2) for the production of UDP-GlcNAc used in the glycosylation processes, including *O*-GlcNAcylation.

REG3A regulates carbohydrate metabolism by binding to several metabolic intermediates of glucose

The exostosin (EXT) gene family encodes glycosyltransferases involved in the initiation of HS biosynthesis and HS chain elongation, a process that consumes UDP-GlcNAc. We wondered whether the interaction reported in

nonhepatic cells between REG3A and the EXTL3 receptor was found in hepatic cells and whether it could affect the glycosyltransferase activity of EXTL3. Cotransfection of HuH7 cells with REG3A-GFP and EXTL3-OFP constructs resulted in colocalization of both proteins with the Golgi marker (Supplemental Figure S6A, http://links.lww.com/HEP/I531). EXTL3 was immunoprecipitated with REG3A, indicating an interaction between REG3A and EXTL3 (Supplemental Figure S6B, http://links.lww.com/HEP/I531). Coexpression of the 2 proteins did not alter the HS content detected compared to REG3A or EXTL3 expressed alone or with empty vectors (Figure 6A), suggesting that REG3A, by binding to EXTL3, does not interfere with the glycosyltransferase activity of EXTL3 in liver cells. Nor does the REG3A/EXTL3 interaction appear to affect the synthesis of chondroitin sulfate, which requires the same tetrasaccharide linker as HS to initiate its biosynthesis (Figure 6A). We next examined the binding capacity of REG3A to phosphorylated and nonphosphorylated metabolic intermediate ligands involved in the glucose metabolism pathway as a function of an increasing range of glycolytic intermediate concentrations. Overall, in sugar slot-blot experiments, REG3A binds glucose (Glc), glucose-6 phosphate (Glc-6P), GlcNAc, and GlcNAc-6P, but not glucose-1 phosphate (Glc-1P), fructose-6 phosphate (Fru-6P), UDP, or UDP-GlcNAc, at the highest concentration used (12.8 μmol) (Figure 6B). The series also included several proven REG3A carbohydrate ligands (galactose, lactose [Glc-Gal], and mannan)[34,35] (Figure 6B). Preincubation of recombinant REG3A protein with lactose inhibited REG3A binding to glucose (Figure 6C), suggesting that REG3A binding to mono- and polysaccharide targets may be affected by REG3A (local concentration, occupied binding sites) as well as carbohydrate type and concentration. We analyzed the impact of REG3A as a lectin on *O*-glcNAcylation (construction of a REG3A mutant labeled $REG3A^{EPN/GPG}$ unable to bind carbohydrate ligands following substitution of the EPN (glutamic acid-proline-glutamine) motif for a GPG (glycine-proline-glycine) motif in the C-type lectin-like domain (CTLD).[36] We report that the $REG3A^{EPN/GPG}$ protein is unable to bind glucose (Glc) and Glc-6P (Figure 6D) and that protein *O*-GlcNAcylation was not altered by $REG3A^{EPN/GPG}$ expression as it was by full-length $REG3A^{WT}$, remaining at levels similar to those of control Huh7 cells transfected with the empty vector (Figure 6E). This suggests that the lectin function of REG3A is important for its ability to modulate *O*-GlcNAcylation. Two REG3A substrate sugars, Glc and Glc-6P, are used for glycogen synthesis, the pentose phosphate pathway, glycolysis, and the de novo production of UDP-GlcNAc (the end product of the hexosamine pathway), which serves as a substrate for *O*-GlcNAcylation, *N*- and *O*-linked glycosylation and proteoglycans. Assuming that REG3A binds sugars in the first step of carbohydrate metabolism and that this binding disrupts the availability of glucose and some intracellular intermediate sugars, we examined the impact of REG3A on changes in UDP-GlcNAc and carbohydrate metabolism pathways. UDP-GlcNAc levels were quantified in preneoplastic (preK) and tumor liver samples from MYC and MYC/REG3A transgenic mice. Levels were 0.9 ± 0.11 versus 1.6 ± 0.12 nmol/mg protein ($p = 0.0043$) in preneoplastic livers and 1.4 ± 0.10 versus 1.97 ± 0.2 nmol/mg protein ($p = 0.035$) in NT (adjacent to T) livers of MYC/REG3A-TG mice compared to MYC-TG mice, respectively (Figure 6F). No difference in the level of UDP-GlcNAc was observed in the tumor setting between the 2 mouse lines. This result is consistent with reduced levels of protein *O*-GlcNAcylation when REG3A is involved. Other pathways of carbohydrate metabolism are affected to a variable degree when REG3A is expressed in liver cells, namely, a marked reduction in glycolysis (reduced ATP production; Figure 6G), complex *N*-glycosylation (Figure 6H), and glycogen storage (Figures 6I, J). In contrast, liver cells expressing the mutant $REG3A^{EPN/GPG}$ lacking lectin function showed unchanged glycolysis and glycogen storage profiles similar to control cells (Figures 6G, J). Regarding *O*-glycosylation, we found no difference in whether REG3A was present or not (Figure 6K). These observations, therefore, suggest that REG3A, by binding to the substrates (Glc, Glc-6P, and Fru-6P) required for de novo synthesis of UDP-GlcNAc, negatively regulates *O*-GlcNAcylation and, to a lesser extent, *N*-linked glycosylation by modulating substrate availability (Figure 6L).

DISCUSSION

Well known as the carbohydrate-binding activity of members of the Reg gene family, the interaction of lectin REG3A with glycans and glucose metabolic pathways remains largely unknown, as does their impact on cellular signaling and regulation. Our results provide an example of the modulation of protein glycosylation by a C-type lectin, REG3A (an innate immunity molecule with numerous regulatory effects in prokaryotes and eukaryotes), and the phenotypic consequences thereof. This study substantiates the suppressive role of REG3A in HCC by modifying *O*-GlcNAcylation in experimental mouse models of HCC (MYC-induced or DEN-induced HCC), in vitro cell studies, and human samples. REG3A, when expressed in hepatocytes, significantly reduced liver *O*-GlcNAcylation in all systems studied and delayed the onset of HCC in murine livers. In patients with cirrhosis, higher REG3A expression correlates with longer cancer-free survival and lower protein *O*-GlcNAcylation. We show that REG3A reduced *O*-GlcNAcylation without disrupting the expression or activity of the 2 *O*-GlcNAc processing enzymes, OGT and OGA, and without restricting the activity of the rate-limiting enzymes (GFAT1 and 2) of the HBP, which provides the sugar-

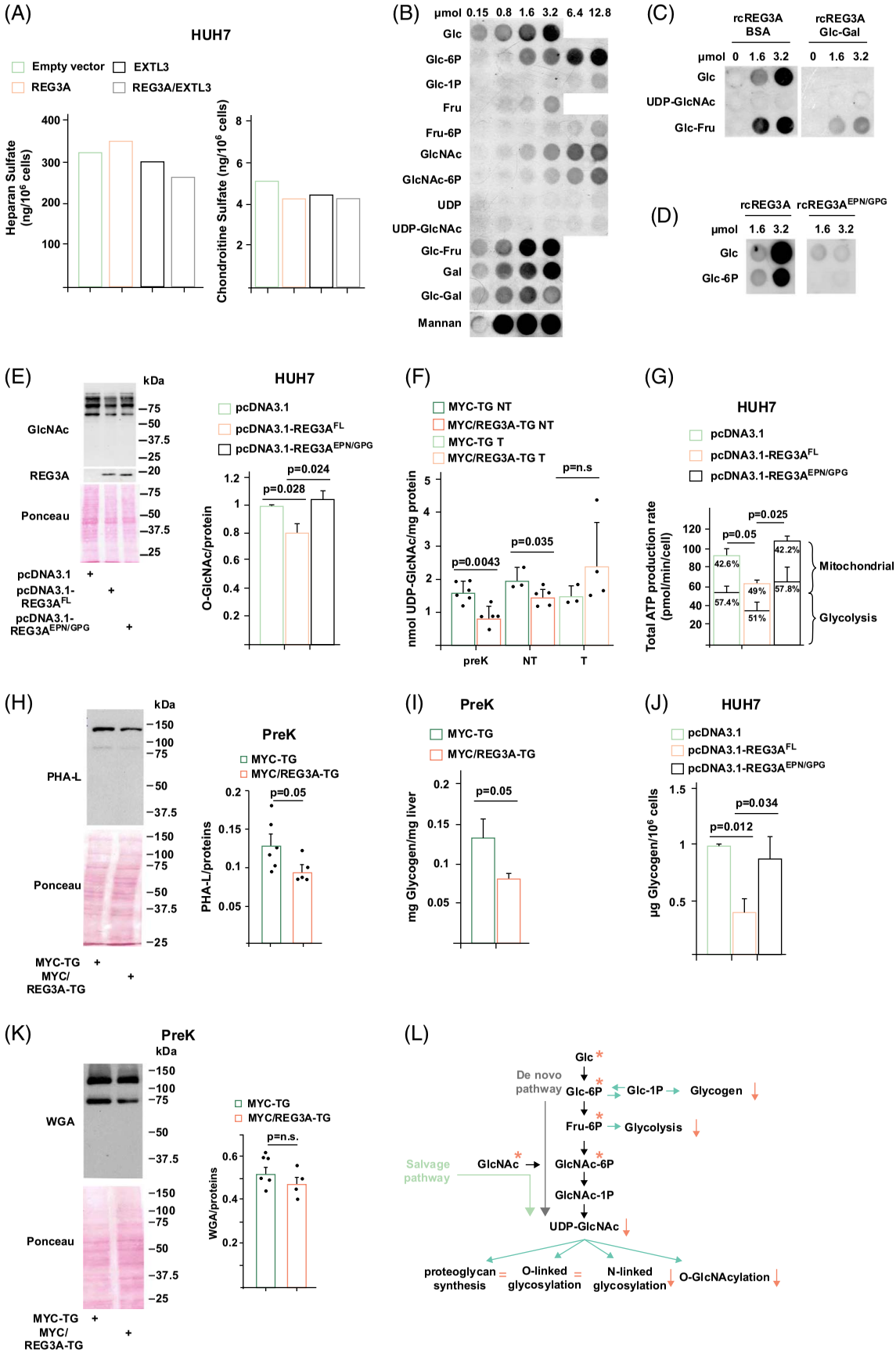
(A)
HUH7
Empty vector
EXTL3
REG3A
REG3A/EXTL3
Heparan Sulfate (ng/10^6 cells)
Chondroitine Sulfate (ng/10^6 cells)
(B)
µmol 0.15 0.8 1.6 3.2 6.4 12.8
Glc
Glc-6P
Glc-1P
Fru
Fru-6P
GlcNAc
GlcNAc-6P
UDP
UDP-GlcNAc
Glc-Fru
Gal
Glc-Gal
Mannan
(C)
rcREG3A BSA
rcREG3A Glc-Gal
µmol 0 1.6 3.2 0 1.6 3.2
Glc
UDP-GlcNAc
Glc-Fru
(D)
rcREG3A
rcREG3A^EPN/GPG
µmol 1.6 3.2 1.6 3.2
Glc
Glc-6P
(E)
kDa
GlcNAc
REG3A
Ponceau
pcDNA3.1 +
pcDNA3.1-REG3A^FL +
pcDNA3.1-REG3A^EPN/GPG +
HUH7
pcDNA3.1
pcDNA3.1-REG3A^FL
pcDNA3.1-REG3A^EPN/GPG
p=0.024
p=0.028
O-GlcNAc/protein
(F)
MYC-TG NT
MYC/REG3A-TG NT
MYC-TG T
MYC/REG3A-TG T
p=n.s
p=0.035
p=0.0043
nmol UDP-GlcNAc/mg protein
preK
NT
T
(G)
HUH7
pcDNA3.1
pcDNA3.1-REG3A^FL
pcDNA3.1-REG3A^EPN/GPG
p=0.025
p=0.05
42.6%
57.4%
49%
51%
42.2%
57.8%
Mitochondrial
Glycolysis
Total ATP production rate (pmol/min/cell)
(H)
kDa
PHA-L
Ponceau
MYC-TG +
MYC/REG3A-TG +
PreK
MYC-TG
MYC/REG3A-TG
p=0.05
PHA-L/proteins
(I)
PreK
MYC-TG
MYC/REG3A-TG
p=0.05
mg Glycogen/mg liver
(J)
HUH7
pcDNA3.1
pcDNA3.1-REG3A^FL
pcDNA3.1-REG3A^EPN/GPG
p=0.034
p=0.012
µg Glycogen/10^6 cells
(K)
PreK
kDa
WGA
Ponceau
MYC-TG +
MYC/REG3A-TG +
MYC-TG
MYC/REG3A-TG
p=n.s.
WGA/proteins
(L)
Glc
De novo pathway
Glc-6P
Glc-1P
Glycogen
Fru-6P
Glycolysis
Salvage pathway
GlcNAc
GlcNAc-6P
GlcNAc-1P
UDP-GlcNAc
proteoglycan synthesis
O-linked glycosylation
N-linked glycosylation
O-GlcNAcylation

FIGURE 6 REG3A bound to glucose and glucose intermediates is a regulator of protein glycosylation. (A) Quantification of HS and CS disaccharides by ion-pair reversed-phase chromatography in HuH7 cells expressing REG3A or EXTL3 alone, or both, or appropriate empty vectors. (B) Representative sugar slot blot of mono- and polysaccharides deposited at the indicated doses and hybridized with recombinant REG3A lectin. Lactose, mannan: positive control for REG3A binding. (C) Slot blot of indicated sugars in the presence of a full-length human recombinant REG3A protein (rcREG3A) preincubated with BSA or BSA + lactose (Glc-Gal). (D) Slot blot of indicated sugars in the presence of rcREG3A or a mutant recombinant REG3A protein (rcREG3A$^{EPN/GPG}$) proteins. (E) Immunoblots for *O*-GlcNAc (anti-RL2) and REG3A in nuclear fractions of HuH7 cells expressing REG3A, REG3A$^{EPN/GPG}$ or empty vector. Densitometry quantification (n = 3). (F) Enzymatic quantification of cellular UDP-GlcNAc content in preneoplastic (preK), nontumor (NT adjacent to a tumor), and tumor (T) liver samples from MYC/REG3A and MYC-TG transgenic mice. Each dot represents a sample of an individual mouse. (G) Rates of cellular ATP production in HuH7 cells expressing REG3A, REG3A$^{EPN/GPG}$, or the empty vector, showing significant changes in total, mitochondrial, and glycolytic ATP production under the action of the full-length REG3A protein (n = 3). (H) Lectin blot for biotin-labeled PHA-L (phaseolus vulgaris leucoagglutinin) lectin on preneoplastic liver extracts from mice transgenic for MYC (MYC-TG) and for MYC and REG3A (MYC/REG3A-TG). Densitometry quantification (n = 6). (I) Glycogen concentrations in preneoplastic liver extracts (preK; left) from the mice shown (n = 6) and (J) in HuH7 cells expressing full-length REG3A or the mutant REG3A$^{EPN/GPG}$ (right) (n = 4). (K) Lectin blot for biotin-labeled WGA in extracts of preneoplastic (PreK) liver from MYC and MYC/REG3A transgenic mice and quantification by densitometry (n = 6). (L) A schematic view of carbohydrate metabolism from glucose and its intermediates. Gray = de novo synthesis of UDP-GlcNAc from glucose through the hexosamine synthesis pathway; Blue: salvage of GlcNAc through the metabolic processes that produce UDP-GlcNAc. *sugars bound to REG3A. Red arrows: carbohydrate pathways downregulated by REG3A. Equal symbols: glucose pathways not modulated by REG3A. Data are averages ± SEM. The Mann-Whitney *U* test was used for analysis, except for (E), (F), (G), and (J) (ANOVA test followed by a post hoc test). NS or no statistical indication, no significance. Abbreviations: CS, chondroitin sulfate; EXTL3, exostosin-like glycosyltransferase 3; HS, heparan sulfate; PHA-L, phytohemaglutinin-L; REG3A, regenerating family member 3 alpha; WGA, wheat germ agglutinin.

donor substrate UDP-GlcNAc, used for protein *O*-GlcNAcylation and other glycosylations. Our results show that REG3A reduces UDP-GlcNAc levels, *O*-GlcNAc glycosylation, and other types of glycosylation, such as glycolysis, gluconeogenesis, and complex *N*-glycosylation. This effect may be linked to the ability of REG3A to bind 2 sugars, Glc and Glc-6P, which are at the crossroads of numerous carbohydrate pathways, as shown by the failure of a REG3A mutant to bind Glc and Glc-6P and to modify *O*-GlcNAcylation, glycolysis, and glycogen storage.

O-GlcNAcylation is unique because it dynamically links cellular responses to nutrient availability. Therefore, it is considered a nutrient sensor that regulates transcription, translation, signaling, the cell cycle, differentiation, and metabolism.[37] Thousands of proteins are subjected to variation by *O*-GlcNAcylation, which regulates their stability, activation, localization, and interaction in physiological and pathological states. Elevated *O*-GlcNAcylation and altered expression of OGT and OGA have been reported in a wide range of cancers, including primary liver cancers.[38–40] Cancer cells positively regulate glucose uptake and HBP flux, and consequently, the level of available UDP-GlcNAc, leading to elevated *O*-GlcNAcylation and activation of multiple signaling pathways, including oncogenic and stress tolerance pathways.[41,42] Several *O*-GlcNAc–regulated proteins are involved in cancer biology, such as MYC,[43] YAP,[44] c-Jun,[45] RACK1,[46] ChK2,[47] FOXA2,[48] and HDAC1,[49] which promote HCC growth, invasion, and metastasis. Conversely, glucose deprivation or inhibition of HBP or OGT results in reduced tumor cell growth. Clinically, *O*-GlcNAcylation has been shown to correlate with an aggressive malignant phenotype and a higher risk of tumor recurrence in patients with HCC undergoing liver transplantation.[50] In the present study, a decrease in MYC *O*-GlcNAcylation (indicating less MYC accumulation) was observed in murine preneoplastic and tumor livers co-expressing MYC and REG3A, leading to a decrease in the expression of MYC target genes involved in cell cycle regulation and proliferation, and an increase in the expression of target genes involved in metabolism and differentiation.

O-GlcNAcylation is more sensitive to changes in UDP-GlcNAc than complex glycosylation. The weak impact on complex glycosylation after significant changes in UDP-GlcNAc levels compared with profound alterations in *O*-GlcNAcylation has been demonstrated in studies other than the present one.[51,52] Reduced availability of UDP-GlcNAc after invalidation of glucosamine 6-phosphate acetyltransferase (which converts GlcN-6P to GlcNAc-6P) had a limited effect on complex glycosylation compared with a major reduction in *O*-GlcNAcylation.[51] It is not yet understood why complex glycosylation, which consumes a large quantity of UDP-GlcNAc, is maintained after de novo reduction of the HBP, whereas *O*-GlcNAcylation, which requires a single UDP-GlcNAc per modification, is profoundly altered. No mechanism has yet been established for this phenomenon, but it may lie partly in the sugar recovery pathway. Reduction of de novo HBP by glutamine deprivation leads to recovery of GlcNAc (derived from glycoprotein recycling) and overexpression of *N*-acetylglucosamine kinase, which converts salvaged GlcNAc to GlcNAc-6P and replenishes the pool of UDP- GlcNAc preferentially used for complex sugar synthesis.[52] The hypothesis proposed is that because UDP-GlcNAc is actively transported into the ER/Golgi compartments to fuel complex glycosylation at a concentration 20 times higher than in the cytosol,[53] limiting the UDP-GlcNAc available in the cytosol for *O*-GlcNAcylation, the recovery of GlcNAc combined with the active transport of UDP-GlcNAc to ER/Golgi may explain the sensitivity of *O*-GlcNAcylation to variations in UDP-GlcNAc compared with complex glycosylation.

As previously mentioned, REG3A responses in cancer are debated and contradictory, and the promoter or suppressor activity of REG3A has been suggested for the same cancer type. In gastric and colorectal cancers, REG3A is an oncogenic or tumor suppressor factor depending on the study and the different cellular and preclinical models used, and REG3A overexpression in pathological tissues has been associated with better or worse patient survival.[54–60] In head and neck squamous cell carcinoma, REG3A expression is associated with better survival, chemosensitivity, and radiosensitivity, consistent with in vitro data showing a lower proliferation rate and better response to treatment with REG3A-positive head and neck squamous cell carcinoma cells.[61,62] In the pancreas, a number of experimental animal studies support a procarcinogenic effect in the context of pancreatic inflammation, notably under the effect of IL6 and IL17.[17] In mice, although the expression of REG3b (1 of 2 murine orthologs of human REG3A) appears to be associated with the development of a greater number of preneoplastic lesions when pancreatitis is induced by cerulein in C57BL/6 and RIP-I/Reg3B[63] or Pdx1-cre; KrasG12D expressing REG3b,[16] these studies have not established that these lesions give rise to a tumor. No data have been reported on pancreatic cancer in humans.

As far as HCC is concerned, to our knowledge, no in vivo experience has yet been reported. The studies in the literature concern the prognosis of patients with established HCC, not the prospect of HCC arising in a cirrhotic liver. Increased REG3A has been associated with good or poor clinicopathological features in patients with HCC[64,65] and molecularly with an HCC subtype mutated for the gene encoding β-catenin (CTNNB1) and a favorable differentiation phenotype. Our study, therefore, investigates the effect of REG3A on the progression of pretumor lesions in an inflammatory context (cirrhosis) in humans and shows that REG3A slows down liver carcinogenesis in 2 genetic and toxic models of cancer in mice. The mechanism of action of REG3A described in this study is likely to be involved in other types of solid carcinogenesis.

In addition to the fact that cancer is a complex dynamic ecosystem linking a multitude of biological processes that are difficult to reproduce in experimental models, the contradictory observations on REG3A in murine experiments may, in part, be explained by different dynamic profiles of *O*-GlcNAc proteins in diseased structures and by the poorly understood nature of the specific *O*-GlcNAc-dependent proteins and pathways that are altered. We believe that a more complete definition of the sugar ligands recognized by REG3A and the resulting consequences for tissue-specific glucose metabolism pathways are needed to clarify its role in carcinogenesis. Overall, given that *O*-GlcNAcylation contributes to significant changes in gene expression and signaling in cancer cells, our data on the crosstalk between REG3A and *O*-GlcNAc through sugar binding offers new insights into the potential regulatory mechanisms of *O*-GlcNAcylation during oncogenesis.

AUTHOR CONTRIBUTIONS

Nicolas Moniaux and Jamila Faivre conceived the project and designed the research. Nicolas Moniaux, Nicolas Geoffre, Marion Darnaud, Alice Deshayes, Tung-Son Nguyen, Claire Lacoste, Mélanie Friedel-Arboleas, Janne Purhonen, Jukka Kallijärvi, and Romain R. Vivès performed experiments. Nicolas Moniaux, Nicolas Geoffre, Marion Darnaud, Alexandre Dos Santos, Sylvie Job, Claire Lacoste, Catherine Guettier, Janne Purhonen, Jukka Kallijärvi, Gilles Amouyal, Paul Amouyal, Christian Bréchot, Romain R. Vivès, Marie Annick Buendia, Tarik Issad, and Jamila Faivre analyzed the data. Catherine Guettier provided clinical samples. Nicolas Moniaux and Jamila Faivre provided funding and drafted the manuscript. All the authors approved the final version of the manuscript.

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CONFLICTS OF INTEREST

Gilles Amouyal is employed by Alfact Innovation. He owns stock in The Healthy Aging Company. Paul Amouyal is employed by and owns stock in Alfact Innovation. Christian Bréchot owns stock and holds intellectual property rights with The Healthy Aging Company. The remaining authors have no conflicts to report.

ORCID

Nicolas Moniaux https://orcid.org/0000–0001–9718–1386

Claire Lacoste https://orcid.org/0000–0001–7013–2533
Marion Darnaud https://orcid.org/0000–0002–0919–8303
Janne Purhonen https://orcid.org/0000–0002–0635–4075
Jukka Kallijärvi https://orcid.org/0000–0003–3773–7025
Christian Bréchot https://orcid.org/0000–0002–2353–9362
Tarik Issad https://orcid.org/0000–0002–2638–6330
Jamila Faivre https://orcid.org/0000–0002–1846–9224

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