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1 **Downregulation of Gadd45 Beta Expression by Hepatitis C**  
2 **Virus Leads to Defective Cell Cycle Arrest**

3  
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26

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28

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30

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32  
33

34 **ABSTRACT**

35

36 Members of the Gadd45 family play central roles in the cellular response to  
37 genotoxic stress, and have been implicated in several human cancers including  
38 hepatocellular carcinomas. Chronic infection by hepatitis C virus (HCV) is a major  
39 risk factor for the onset and development of primary hepatocellular tumors, although  
40 the underlying mechanisms are unclear. Here, we demonstrate a novel link between  
41 diminished Gadd45 $\beta$  expression and HCV infection. Inhibited Gadd45 $\beta$  expression  
42 was observed in both non-tumoral and tumoral tissues from infected individuals, and  
43 in cell lines harboring an HCV replicon and the infectious HCV strain JFH1.  
44 Decreased Gadd45 $\beta$  expression was confirmed *in vivo* in a transgenic murine model  
45 expressing the entire HCV open reading frame. Mechanistically, hypermethylation of  
46 the Gadd45 $\beta$  promoter in the presence of HCV is responsible for this defect.  
47 Diminished Gadd45 $\beta$  expression leads to aberrant cell cycle arrest and diminished  
48 DNA excision repair. Together, these results provide a novel insight into the  
49 mechanisms involved in HCV-associated hepatocellular carcinomas, showing that  
50 reduced Gadd45 $\beta$  expression may play a contributory role to this process, and  
51 providing evidence that HCV may interfere with epigenetic gene expression by  
52 altering promoter methylation.

53

54

## 55 INTRODUCTION

56

57 Infection by the hepatotropic hepatitis C virus (HCV) is a leading cause of  
58 chronic liver disease, with more than 170 million chronically infected individuals  
59 worldwide. Chronic HCV infection is associated with the development of chronic  
60 hepatitis, fibrosis and cirrhosis, and is a major risk factor for the onset and  
61 progression of hepatocellular carcinoma (HCC) (1, 2). The molecular mechanisms  
62 surrounding liver tumorigenesis in HCV-infected patients remain unclear, although  
63 cirrhosis appears to be an important, but non-specific, determinant of HCC  
64 occurrence (3). Nevertheless, several studies have suggested that HCV itself (i.e.  
65 HCV protein expression in hepatocytes) plays a role in hepatocarcinogenesis. For  
66 example, the HCV core protein has been reported to interact with the p53 and  
67 promyelocytic leukemia (PML) tumor suppressors in hepatoma cell culture, to  
68 facilitate transformation of murine fibroblasts, and to promote oncogenesis in mice (4-  
69 9). However, the reported effects of the core-p53 interaction are inconsistent.  
70 Another HCV protein, nonstructural protein 5A (NS5A), has been reported to interact  
71 with p53 and the DNA damage effector ataxia telangiectasia mutated (ATM) kinase in  
72 hepatoma cell lines (10-12). Moreover, the expression of HCV proteins has been  
73 linked with up-regulated production of reactive oxygen species (ROS) (13, 14), and a  
74 consequent increase in levels of ROS-specific DNA lesions (15). Despite these  
75 findings, the requirement for hepatoma cell lines in the cultivation and study of both  
76 sub-genomic HCV replicons and infectious viral variants of HCV (e.g. the genotype  
77 2a JFH1 strain) represents a major barrier to the study of liver tumorigenesis in the  
78 context of HCV infection.

79           The growth arrest and DNA damage (Gadd45) gene family encodes three  
80 highly conserved nuclear proteins that contribute to cellular homeostasis in response  
81 to a number of stressors (16). Several lines of evidence suggest that these proteins  
82 fulfill similar functions in cellular survival, cell cycle control, apoptosis and repair of  
83 DNA damage (17). Gadd45 $\beta$  has been demonstrated to interact with several key  
84 cellular regulators including cyclin B1, p21, proliferating cell nuclear antigen (PCNA)  
85 and mitogen-activated protein kinase 7 (MKK7) (16, 18-21). The cellular function of  
86 Gadd45 $\beta$  is dependent upon its interacting partner. Notably, Gadd45 $\beta$  is able to  
87 suppress G2/M progression in response to genotypic stress via its ability to interact  
88 with, and suppress the kinase activities, of the cyclinB1/cell division cycle 2 (cdc2)  
89 complex (20). Accordingly, RNA silencing of Gadd45 $\beta$  expression impairs G2/M  
90 checkpoint activity. It remains to be determined whether interactions between  
91 Gadd45 $\beta$  and p21 play a role in G1 arrest. Gadd45 $\beta$  has also been suggested to  
92 function in DNA excision repair through its interaction with PCNA (20, 22). This  
93 interaction may contribute towards the proposed role for Gadd45 $\beta$  in epigenetic gene  
94 activation (23, 24). Furthermore, several lines of evidence implicate Gadd45 $\beta$  in  
95 apoptosis, particularly in response to transforming growth factor  $\beta$  (TGF- $\beta$ ) (25, 26).

96           Such observations have implicated Gadd45 proteins, including Gadd45 $\beta$ , as  
97 crucial sensors of genotoxic stress. In line with this proposal, Gadd45 $\beta$ -/- mice  
98 display increased susceptibility to ionizing radiation and chemical carcinogenesis,  
99 and accelerated melanoma growth compared to wild-type (wt) littermates (27, 28).  
100 Accordingly, in human hepatocellular tumors, and in several hepatoma cell lines,  
101 Gadd45 $\beta$  expression is diminished (29). This diminution has been attributed to  
102 hypermethylation of the Gadd45 $\beta$  promoter, as treatment with DNA  
103 methyltransferase inhibitors restores Gadd45 $\beta$  expression to non-tumoral levels (30).

104 Moreover, down-regulation of Gadd45 $\beta$  is closely associated with the degree of  
105 malignancy in tumoral samples. Further studies demonstrated that treatment of  
106 tumoral cell lines with the hepatoprotector S-adenosylmethione could stimulate  
107 nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF- $\kappa$ B)-mediated  
108 Gadd45 $\beta$  transcription (31). Therefore, Gadd45 $\beta$  downregulation may play an  
109 important role in hepatocarcinogenesis.

110 Transgenic mice of the FL-N/35 lineage expressing the entire open reading  
111 frame (ORF) of a genotype 1b isolate of HCV have previously been shown to  
112 develop hepatic steatosis and hepatocellular carcinomas in the absence of cirrhosis  
113 (32, 33). These animals also exhibit impaired viral clearance and decreased  
114 expression of bid, a pro-apoptotic B-cell leukemia/lymphoma 2 (Bcl2)-homology  
115 domain 3 (BH3)-only protein which has also been implicated in the response to DNA  
116 damage (34, 35). Unlike other experimental systems for HCV infection, the entire  
117 complement of viral proteins is expressed at low levels, resembling those found in  
118 the livers of chronically-infected patients (32) and does not trigger non-specific ER  
119 stress (33). Moreover, viral protein expression in this model does not elicit an  
120 immune response, allowing the role of HCV proteins in tumorigenesis to be studied in  
121 the absence of the chronic inflammation usually observed during HCV infection.

122 In this context, we asked whether Gadd45 $\beta$  expression was specifically  
123 affected by HCV protein expression. To that end, we characterized Gadd45 $\beta$   
124 expression in human tissue samples, *in vitro* systems of HCV replication and  
125 infection in hepatoma cell lines, and the FL-N/35 murine model of HCV protein  
126 expression.

127

128

## 129 **MATERIALS AND METHODS**

130

### 131 **Human liver tissues**

132 Tumoral and adjacent non-tumoral liver tissues from 7 patients with chronic  
133 HCV infection, including 4 with cirrhosis, were examined. Similar tissues from 5  
134 uninfected patients and 5 patients infected with hepatitis B virus (HBV) were used as  
135 controls. The patients' characteristics are detailed in Table 1.

136

### 137 **Cell culture models of HCV replication and infection**

138 Huh7.5 cells, and Huh7.5 cells harboring the genotype 1b bicistronic HCV  
139 subgenomic replicon I389-neo/NS3-3'/5.1, kindly provided by Dr. Ralf Bartenschlager  
140 (University of Heidelberg, Heidelberg, Germany), were maintained as previously  
141 described (36). Uninfected HuAP cells, and cells infected with the HCV infectious  
142 strain JFH-1, were a kind donation from Dr Czeslaw Wychowski (Institut de Biologie  
143 de Lille, CNRS-UMR 8161, Lille, France).

144

### 145 **HCV transgenic mice**

146 Wild-type C57/Bl6 mice and mice transgenic for the entire HCV open reading  
147 frame (FL-N/35 lineage (32)), were bred and maintained as previously described  
148 (34). Eighteen-month-old male littermates were used for transcriptional and  
149 proteomic studies. Ten-month-old females were used for primary hepatocyte  
150 cultures. For benzo[a]pyrene treatment, mice received a single intraperitoneal  
151 injection of 55 µg benzo[a]pyrene (Sigma-Aldrich, Saint Louis, Missouri) per gram of  
152 body weight, diluted in corn oil. Treated mice were euthanized 36 hours post  
153 treatment, the livers extracted and RNA isolated as described below.

154

**155 Primary cell culture**

156 Murine hepatocytes from both HCV transgenic and non-transgenic animals  
157 were isolated by portal vein perfusion of collagenase. Freshly isolated hepatocytes  
158 were cultured in DMEM supplemented with 10 % fetal calf serum, 10 U/mL penicillin,  
159 10 µg/mL streptomycin, 10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL sodium  
160 selenite. Four hours post perfusion, media was removed and fresh media,  
161 supplemented with 0.1 µM dexamethasone (Sigma-Aldrich) and 50 ng/mL epidermal  
162 growth factor, was added.

163

**164 Plasmids and antibodies**

165 Plasmid pGL2, containing firefly luciferase under the control of the SV40  
166 promoter, was from Promega (Madison, Wisconsin), whilst pmaxGFP was from  
167 Lonza (Basel, Switzerland). A plasmid encoding the human Gadd45β promoter (-  
168 1604 to +141; pGL2-Gadd45β) upstream of firefly luciferase was kindly provided by  
169 Dr. Mary Goldring (Hospital for Special Surgery, Laboratory for Cartilage Research,  
170 New York) (37). The antibodies used in this study are outlined in Supplementary  
171 Materials.

172

**173 RNA isolation and quantitative real-time PCR analysis**

174 Total RNA was purified using the PARIS purification kit (Ambion, Austin,  
175 Texas). RNA integrity and quantity were determined using an Agilent Bioanalyser,  
176 with samples displaying an RNA integrity number of 6.8-8.1. Complementary DNA  
177 was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied  
178 Biosystems, Foster City, California). Quantitative PCR were performed with an



179 Applied Biosystems 7300 Thermal Cycler using Taqman reagents. Primer  
180 information is supplied in Supplementary Materials. Statistical significance of the  
181 results was determined using a Mann-Whitney nonparametric test.

182

### 183 **Methylation-specific PCR**

184 Genomic DNA was isolated from murine livers by phenol chloroform  
185 extraction, and bisulphite treated (38). Amplification of the Gadd45 $\beta$  promoter in  
186 methylated and unmethylated states was performed by methylation-specific PCR  
187 using the primers described in Supplemental Materials.

188

### 189 **Cell fractionation, tumor samples and western blotting**

190 Nuclear fractions of murine livers were prepared using the Pierce NE-PER  
191 Nuclear Cytoplasmic extraction kit (ThermoFisher Scientific, Waltham,  
192 Massachusetts). Crude extracts of human liver samples and of cultured hepatocytes  
193 were prepared by homogenization in PBS containing 10 mM EDTA and protease  
194 inhibitors. In all cases, proteins were quantified using the BCA assay (ThermoFisher  
195 Scientific). For protein analysis, polypeptides from nuclear fractions or crude extracts  
196 were separated on 4-12% SDS-polyacrylamide gels, transferred to polyvinylidene  
197 fluoride membranes (GE Healthcare, Chalfont St. Giles, UK), and proteins were  
198 detected by immunoblotting. Where appropriate, relative protein expression was  
199 quantified using National Institutes of Health ImageJ software.

200

### 201 **Luciferase reporter gene assays**

202 Primary murine hepatocytes were co-transfected with 1  $\mu$ g pmaxGFP and  
203 either 2  $\mu$ g pGL2-Gadd45 $\beta$  or 2  $\mu$ g pGL2, using Lipofectamine LTX (Invitrogen,

204 Carlsbad, California). Four hours post transfection, monolayers were either mock  
205 treated or exposed to 10 J/m<sup>2</sup> UV-C using a UV-C Crosslinker (GE Healthcare).  
206 Luciferase activity was assayed 40 hours post treatment using a Mithras LB 940  
207 plate reader (Berthold Technologies, Bad Wilbad, Germany), and normalized to the  
208 expression of green fluorescent protein (GFP). Data are presented as the  
209 mean±standard error (SEM) of three experiments.

210

### 211 **Mitotic index analysis**

212 Freshly isolated murine hepatocytes plated on Permanox slides  
213 (ThermoFisher Scientific) were either mock treated or exposed to 5 J/m<sup>2</sup> UV-C 4  
214 hours post plating. Six hours post treatment, cells were fixed and processed for  
215 immunofluorescent detection of phospho-histone H3 as previously described (39).  
216 Images were captured using a Zeiss Axioskop 40 microscope in conjunction with a  
217 Zeiss MRc5 AxioCam and Axiovision LE software. The percent of phospho-histone  
218 H3 positive (mitotic) nuclei was calculated for each field of view at 40X magnification.  
219 Data represent the mean±SEM mitotic index of at least 300 cells from two  
220 independent cultures. Statistical significance was determined using a Mann-Whitney  
221 nonparametric test.

222

### 223 **RNA silencing**

224 Gadd45 $\beta$  expression in primary murine hepatocytes was suppressed by  
225 transfection with siRNA (Silencer Gadd45 $\beta$  siRNA s70735 and nonsilencing control  
226 4398043, Applied Biosystems) by electroporation using a Mouse Hepatocyte  
227 Nucleofector kit (Lonza) prior to plating. Twenty hours after transfection, cells were

228 left untreated, or treated with 5 J/m<sup>2</sup> UV-C, and processed for mitotic index analysis  
229 or quantitative transcript analysis as appropriate.

230

### 231 **5-azacytidine treatment**

232 Attached primary hepatocytes were treated with 50 μM 5-azacytidine (Sigma-  
233 Aldrich) for 12 hours, before treatment with 5 J/m<sup>2</sup> UV. Cells were harvested for  
234 quantitative transcript analysis or were examined for the presence of phospho-  
235 histone H3 by immunofluorescence.

236

### 237 **Plasmid host cell reactivation assays**

238 The ability of primary hepatocytes cultures to repair a UV-C damaged reporter  
239 was assayed using a host cell reactivation assay (40, 41). Briefly, cultures of  
240 attached primary hepatocytes were cotransfected with UV-C-damaged (15 KJ/m<sup>2</sup>)  
241 pGL2, together with pGFP to control for transfection efficiency. Luciferase activity  
242 was assayed 48 hours post-transfection, and normalized to GFP expression. Relative  
243 DNA repair efficiency was calculated, and is presented as the mean±SEM of three  
244 experiments.

245

## 246 **RESULTS**

247

### 248 **Gadd45β expression is inhibited in tumoral and non-tumoral liver tissues** 249 **from HCV-infected patients**

250 We measured Gadd45β expression in tumoral and adjacent non-tumoral liver  
251 tissues from patients with HCC and chronic HCV infection with or without cirrhosis.  
252 Patients infected with HBV only and non-infected patients were used as controls

253 (Table 1). Immunoblotting revealed that Gadd45 $\beta$  was equivalently expressed in non-  
254 tumoral tissues from both uninfected and HBV-infected patients. In contrast,  
255 Gadd45 $\beta$  was undetectable in non-tumoral tissues from both cirrhotic and non-  
256 cirrhotic HCV-infected patients (Fig. 1A and 1B). In tumoral tissues, Gadd45 $\beta$   
257 expression was nearly undetectable in uninfected and HBV-infected patients, and  
258 remained undetectable in HCV-infected patients (Fig. 1A and 1B).

259 Gadd45 $\beta$  transcripts were quantified by quantitative RT-PCR in the same  
260 samples. As shown in Fig. 1C, Gadd45 $\beta$  was similarly expressed in non-tumoral  
261 tissues from both uninfected and HBV-infected patients. It was reduced at least two-  
262 fold on average ( $p=0.013$ ) in tumors from these individuals when compared to  
263 adjacent non-tumoral tissues. In HCV-infected patients, Gadd45 $\beta$  mRNA levels were  
264 significantly lower in non-tumoral tissues than in uninfected and HBV-infected  
265 controls ( $p=0.02$ ). Gadd45 $\beta$  mRNA levels were also lower in tumors from HCV-  
266 infected patients than in the controls ( $p=0.02$ ; Fig. 1C). In contrast, the levels of  
267 neither Gadd45 $\alpha$  nor Gadd45 $\gamma$  were significantly affected by the presence of HCV in  
268 either tumoral or non-tumoral tissues (Fig. 1D). Decreased Gadd45 $\beta$  expression was  
269 also observed in HCV-infected patients with no history of HCC (data not shown).

270 Correlations between Gadd45 $\beta$  protein expression and patients'  
271 clinicopathological features (Table 1) were examined using Spearman's non-  
272 parametrical test. Such analyses verified that reduced Gadd45 $\beta$  expression strongly  
273 correlated ( $p=0.007$ ) with HCV infection, and confirmed previous observations (29)  
274 that Gadd45 $\beta$  expression was associated with differentiation ( $p=0.02$ ). No  
275 relationship was observed between Gadd45 $\beta$  expression and either tumor size or  
276 frequency. Together, these data indicate that Gadd45 $\beta$  expression is down-regulated  
277 in liver tissues from HCV-infected individuals.

278

279 **Gadd45 $\beta$  expression is inhibited in cultured hepatocytes harboring a**  
280 **sub-genomic replicon or the infectious HCV strain JFH1**

281 The expression of the Gadd45 $\beta$  protein was next examined in cultured  
282 hepatoma cell lines harboring a replicating genotype 1b subgenomic replicon or the  
283 genotype 2a infectious HCV strain JFH1. Immunoblotting analysis revealed that  
284 Gadd45 $\beta$  expression was reduced in both models compared to uninfected cells (Fig.  
285 2A and 2B, respectively).

286

287 **Expression of the entire HCV ORF inhibits Gadd45 $\beta$  expression in**  
288 **transgenic mice**

289 In order to assess the direct role of HCV protein expression in Gadd45 $\beta$  down-  
290 regulation in HCV-infected patients, we set out to confirm our previous observations  
291 in the absence of HCV replication and local inflammation in the FL-N/35 mouse  
292 lineage, which expresses the entire HCV ORF in a liver-specific fashion. Analysis of  
293 liver-specific Gadd45 $\beta$  protein and mRNA expression revealed a two-fold decrease in  
294 Gadd45 $\beta$  expression in FL-N/35 mice compared to their wt littermates ( $p=0.016$ ; Fig.  
295 3A and 3B). Gadd45 $\beta$  expression reduction was liver-specific (data not shown).

296

297 Both wt and FL-N/35 mice were then treated with a single intraperitoneal  
298 injection of benzo[a]pyrene, which induces mutagenic DNA lesions repaired by  
299 nucleotide excision repair (42). Gadd45 $\beta$  mRNA levels increased after  
300 benzo[a]pyrene treatment in both wt and FL-N/35 mice, but to a markedly lesser  
301 extent in FL-N/35 mice (3.5-fold vs 5.5-fold in the wt mice) (Fig. 3B). Expression of  
302 other Gadd family members in this model was not affected by the expression of the  
HCV transgene, even in the presence of benzo[a]pyrene (data not shown).

303 Collectively, these findings demonstrate that HCV protein expression is responsible  
304 for Gadd45 $\beta$  expression inhibition *in vivo*.

305

306 **Gadd45 $\beta$  promoter activity is decreased in primary hepatocytes from FL-**  
307 **N/35 mice**

308 In order to understand the mechanisms underlying inhibition of Gadd45 $\beta$   
309 expression by the HCV proteins, we sought to determine whether Gadd45 $\beta$  promoter  
310 activity was affected in this context. Isolated hepatocytes from FL-N/35 mice or their  
311 wt littermates were transfected with a reporter plasmid expressing luciferase under  
312 the control of either the SV40 promoter (pGL2) or the human Gadd45 $\beta$  promoter  
313 (pGL2-Gadd45 $\beta$ -luc). As shown in Fig. 3C, basal activity of the Gadd45 $\beta$  promoter  
314 was significantly decreased in hepatocytes isolated from FL-N/35 mice compared to  
315 wt controls. Moreover, although acute UV-C treatment stimulated promoter activity in  
316 both wt and FL-N/35 hepatocytes, such activity in UV-treated FL-N/35 cells remained  
317 significantly reduced compared to UV-treated wt controls. Expression of the HCV  
318 proteins had no effect on basal activity of the SV40 promoter of pGL2, indicating that  
319 the HCV proteins do not induce global promoter repression. These data imply that  
320 the observed reduction in Gadd45 $\beta$  expression is a consequence of impaired  
321 promoter activity in the presence of HCV.

322

323 **The Gadd45 $\beta$  promoter is hypermethylated in HCV transgenic mice**

324 Decreased Gadd45 $\beta$  gene expression in tumoral cell lines has previously  
325 been attributed to hypermethylation of the Gadd45 $\beta$  promoter. This hypermethylation  
326 can be alleviated by *in vitro* treatment with the DNA methyltransferase inhibitor 5-  
327 azacytidine (30). In order to investigate whether decreased Gadd45 $\beta$  promoter

328 activity in FL-N/35 hepatocytes could be due to its hypermethylation, isolated  
329 hepatocytes from wt and FL-N/35 animals were treated with 5-azacytidine for 12  
330 hours, and then either mock-irradiated or treated with UV-C. Cells were harvested six  
331 hours post UV-treatment and analyzed for expression of Gadd45 $\beta$  mRNA (Fig. 4A).  
332 Isolated hepatocytes from FL-N/35 mice displayed reduced Gadd45 $\beta$  levels in both  
333 the presence and absence of acute UV-C treatment. However, 5-azacytidine  
334 treatment restored Gadd45 $\beta$  mRNA expression to wt levels in both mock irradiated  
335 and UV-irradiated FL-N/35 cells. To confirm these data, methylation-specific PCR  
336 was performed on hepatocyte DNA from wt or FL-N/35 mice. Methylation of the  
337 Gadd45 $\beta$  promoter was observed solely in FL-N/35 mice (Fig. 4B). Collectively, these  
338 results demonstrate that inhibition of Gadd45 $\beta$  promoter activity by HCV is due to  
339 promoter hypermethylation.

340

341 **Down-regulation of Gadd45 $\beta$  expression by HCV proteins leads to**  
342 **defective cell cycle arrest**

343 Gadd45 $\beta$  is involved in the induction of G2/M arrest and increased Gadd45 $\beta$   
344 expression is associated with a decreased mitotic index (20, 43). Thus, we assessed  
345 whether reduced Gadd45 $\beta$  expression in hepatocytes isolated from HCV transgenic  
346 FL-N/35 mice was associated with defective cell cycle arrest and increased G2/M  
347 progression in the presence of DNA damage. To this end, isolated hepatocytes from  
348 FL-N/35 or wt animals were mock-treated or exposed to 5 J/m<sup>2</sup> UV-C to induce  
349 transient cell cycle arrest. Six hours post treatment, cells undergoing mitosis were  
350 identified by immunofluorescence using an antibody to phosphorylated histone H3  
351 (Ser-10) (Fig. 5A), and the mitotic index was calculated.

352 Mitotic index analyses revealed no significant difference between basal mitotic  
353 indices in hepatocytes from wt and FL-N/35 mice (Fig. 5B). Upon UV-C treatment,  
354 the percentage of mitotic wt cells decreased significantly ( $p=0.001$ ), denoting cell  
355 cycle arrest to allow repair of DNA damage. Inhibition of Gadd45 $\beta$  expression by  
356 RNA silencing completely abrogated UV-induced cell cycle arrest, with cells  
357 displaying mitotic indices close to those observed in untreated cells (not significant  
358 compared to untreated cultures; Fig. 5B). In FL-N/35 mice hepatocytes, the  
359 percentage of mitotic cells also decreased upon UV-C treatment, although to a lesser  
360 extent than in wt cells. Overall, a significantly greater percentage of mitotic FL-N/35  
361 hepatocytes were observed after exposure to UV-C compared to wt cultures  
362 ( $p=0.001$ ; Fig. 5B), suggesting that cell cycle arrest in response to DNA damage in  
363 HCV transgenic cells was defective. RNA silencing of Gadd45 $\beta$  did not significantly  
364 increase mitotic indexes in UV-C treated FL-N/35 mice (Fig. 5B), suggesting that the  
365 previously observed decrease in Gadd45 $\beta$  expression was sufficient to perturb  
366 temporary cell cycle arrest in response to UV-C.

367 In order to affirm the role of Gadd45 $\beta$  promoter hypermethylation in altering  
368 cell cycle arrest in HCV transgenic cells, UV-C exposed wt and FL-N/35 cells were  
369 treated with 5-azacytidine. Reactivation of Gadd45 $\beta$  expression by 5-azacytidine in  
370 FL-N/35 cells was able to restore UV-C-induced cell cycle arrest to wt levels  
371 ( $p=0.001$ ; Fig. 5B). Cells in which Gadd45 $\beta$  expression was inhibited by RNA  
372 silencing prior to 5-azacytidine treatment displayed abrogated cell cycle arrest, with  
373 mitotic indices close to mock irradiated controls, suggesting that the observed  
374 restorative effect of 5-azacytidine was specifically due to increased Gadd45 $\beta$   
375 expression (Fig 5B). These experiments demonstrate that inhibited hepatic Gadd45 $\beta$   
376 expression in the context of HCV protein expression is due to hypermethylation of the



377 Gadd45 $\beta$  promoter, and is associated with impaired cell cycle arrest in response to  
378 DNA damage.

379

380 **Down-regulation of Gadd45 $\beta$  expression by HCV proteins adversely**  
381 **affects repair of damaged DNA**

382 As Gadd45 $\beta$  is important for efficient nucleotide excision repair of damaged  
383 DNA, we examined whether decreased Gadd45 $\beta$  expression in FL-N/35 hepatocytes  
384 was associated with reduced DNA excision repair using a host cell reactivation  
385 assay. This assay permits the indirect analysis of the ability of cells to perform  
386 nucleotide excision repair of UV-C-induced TT or TC dimers in a reporter plasmid by  
387 measuring transcription of the corresponding reporter gene (40, 41).

388 Hepatocytes isolated from wt and FL-N/35 mice were transfected with UV-  
389 irradiated pGL2, and relative DNA repair calculated for three independent  
390 experiments from the resulting luciferase activity. As demonstrated in Fig. 5C,  
391 relative levels of luciferase activity, and thus DNA repair, were consistently and  
392 significantly ( $p=0.02$ ) lower in cells isolated from FL-N/35 mice compared to wt  
393 controls. These data suggest that, in addition to impaired cell cycle arrest, HCV-  
394 mediated hypermethylation of the Gadd45 $\beta$  promoter also has a deleterious impact  
395 on repair of damaged DNA.

396

397 **DISCUSSION**

398

399 Despite advances in our knowledge of HCV-host cell interactions, the basis for  
400 HCV-associated hepatocarcinogenesis remains unclear. In infected humans,  
401 cirrhosis appears to play a major role as very few non-cirrhotic HCV-infected patients

402 develop primary liver tumors. However, it is not clear whether the carcinogenetic  
403 process is triggered by the expression of HCV viral proteins. Gadd45 family members  
404 play a central role in cell cycle regulation and DNA repair, and have been linked with  
405 hepatocarcinogenesis (27, 29, 30, 44). In the present study, we demonstrate a  
406 previously unknown link between HCV infection and diminished Gadd45 $\beta$   
407 expression. Firstly, we show, in liver biopsies from HCV-infected patients,  
408 significantly lessened Gadd45 $\beta$  mRNA and protein expression in both tumoral and  
409 non-tumoral tissues, in the presence and absence of cirrhosis. Gadd45 $\beta$  expression  
410 was also diminished in hepatoma cell lines harboring either an HCV subgenomic  
411 replicon or the infectious JFH1 strain. However, hepatoma-based cell culture models  
412 are not ideal for the study of HCV-related HCC development, as they are tumor-  
413 derived, and conflicting results have been reported (45). Importantly, similar  
414 reductions in Gadd45 $\beta$  expression were apparent in non-tumoral tissues from a  
415 transgenic mouse model expressing the entire HCV open reading frame, a model  
416 that develops hepatocellular carcinogenesis in the absence of chronic inflammation  
417 (32), suggesting that our observations were a direct consequence of HCV protein  
418 expression. Although Gadd45 $\gamma$  mRNA expression was reduced in tumoral human  
419 tissues compared to adjacent controls, we observed no effect of HCV on the  
420 expression of either Gadd45 $\alpha$  or Gadd45 $\gamma$  in non-tumoral tissues. Thus, we provide  
421 the first evidence that Gadd45 $\beta$  expression is specifically disrupted by HCV protein  
422 expression.

423 Mechanistically, the Gadd45 $\beta$  promoter was shown to be hypermethylated in  
424 the presence of HCV. Treatment with the DNA methyltransferase suicide inhibitor 5-  
425 azacytidine restored both Gadd45 $\beta$  expression and cell cycle arrest. The effects of  
426 such treatment could be completely abrogated by RNA silencing of Gadd45 $\beta$

427 expression, confirming that the effect of 5-azacytidine in this context was confined to  
428 Gadd45 $\beta$ . Methylation-specific PCR confirmed hypermethylation of the Gadd45 $\beta$   
429 promoter in HCV transgenic mice. The mechanisms by which HCV alters gene  
430 methylation during infection remain to be explored. In addition, our data suggests that  
431 alternative mechanisms are responsible for hypermethylation of the Gadd45 $\beta$  and  
432 Gadd45 $\gamma$  promoters during tumorigenesis in the presence or absence of HCV.

433         Previously, it was demonstrated that inhibition of Gadd45 $\beta$  expression by RNA  
434 interference perturbed G2/M checkpoint activity (20). Accordingly, we demonstrated  
435 that Gadd45 $\beta$  silencing abrogated UV-induced cell cycle arrest in murine primary  
436 hepatocytes. Consistent with these data, hepatocytes isolated from HCV transgenic  
437 mice exhibited not only diminished Gadd45 $\beta$  expression, but also decreased  
438 Gadd45 $\beta$  promoter activity, defective cell cycle arrest and DNA repair. Aberrant cell  
439 cycle arrest contributes to the development of somatic mutations, and therefore may  
440 be a contributing factor in the onset and progression of hepatocellular carcinoma.  
441 Moreover, deregulation of Gadd45 $\beta$  expression by HCV may directly provoke further  
442 changes in methylation patterns of several genes, since Gadd45 $\beta$  has itself been  
443 implicated in epigenetic regulation (23). Since epigenetic inactivation of tumor  
444 suppressors and cycle inhibitors has been implicated in the development of several  
445 human cancers including HCCs (46-48), our data suggest that similar mechanisms  
446 might play an important role in the onset and progression of HCV-associated HCC. It  
447 should be emphasized that Gadd45 $\beta$  downregulation in our transgenic mouse model  
448 occurred in the absence of chronic inflammation or cirrhosis, suggesting that  
449 expression of the viral proteins contribute directly to the development of HCC.

450         In conclusion, our data indicate that HCV proteins have a direct effect on liver  
451 tumorigenesis by promoting the hypermethylation of the Gadd45 $\beta$  promoter in the

452 absence of chronic inflammation, impacting DNA repair, epigenetic gene control and  
453 cell cycle regulation. Further study is underway to determine the effect of the HCV  
454 proteins on the promoter methylation of other genes implicated in carcinogenesis.

455

456

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458

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610

611

612

613 **FIGURE LEGENDS**

614

615 **Fig. 1. Gadd45 $\beta$  expression in tumoral and non-tumoral tissues from HCV-**

616 **infected patients.** (A) Gadd45 $\beta$  protein expression in tumoral (T) and adjacent non-  
617 tumoral (NT) liver samples from uninfected (HBV-/HCV-), HBV-infected (HBV+) or  
618 HCV-infected (HCV+) patients. Tissue samples were analyzed by western blotting,  
619 and bands corresponding to 18 kDa Gadd45 $\beta$  or 45 kDa actin are indicated.

620 (B) Densitometric quantification of multiple western blot analyses of tumoral and non-  
621 tumoral tissues from HBV-/HCV- (n=5), HBV+ (n=5) and HCV+ (n=7) patients.  
622 Gadd45 $\beta$  protein levels were normalized to the expression of actin, and mean $\pm$ SEM  
623 densities are displayed in relation to non-tumoral non-infected (HBV-/HCV-) tissues.

624 (C) Gadd45 $\beta$  mRNA expression in non-tumoral and tumoral liver tissues from HBV-  
625 /HCV- (n=5), HBV+ (n=5) and HCV+ (n=7) patients. Expression levels of Gadd45 $\beta$   
626 and SFRS4 transcripts were determined by real-time PCR. Data were normalized to  
627 expression of SFRS4, and expressed as the mean $\pm$ SEM mRNA expression.

628 (D) Gadd45 $\alpha$  and Gadd45 $\gamma$  mRNA expression in non-tumoral and tumoral liver  
629 tissues from HBV-/HCV- (n=5), HBV+ (n=5) and HCV+ (n=7) patients. Gadd45 $\alpha$ ,  
630 Gadd45 $\gamma$  and SFRS4 transcripts were quantified by real-time PCR. Data were  
631 normalized to SFRS4 expression, and expressed as the mean $\pm$ SEM mRNA  
632 expression for each gene. None of the comparisons showed significant differences.

633

634 **Fig. 2. Gadd45 $\beta$  expression in hepatocyte cell lines harboring an HCV genotype**  
635 **1b replicon or infected with the HCV genotype 2a JFH1 strain.**

636 (A) Immunoblotting analysis of Gadd45 $\beta$  expression in Huh7.5 cells in the absence  
637 and presence of an HCV genotype 1b subgenomic replicon. (B) Immunoblotting

638 analysis of Gadd45 $\beta$  expression in mock-infected HuAP cells and cells infected with  
639 the full length HCV infectious variant JFH1. In each case, whole cell lysates of  
640 cultured cells were analyzed by western blotting, with bands corresponding to 18 kDa  
641 Gadd45 $\beta$  or 45 kDa actin indicated.

642

643 **Fig. 3. Gadd45 $\beta$  expression in FL-N/35 mice transgenic for the entire HCV open**  
644 **reading frame.** (A) Western blot analysis of Gadd45 $\beta$  expression in FL-N/35 and wt  
645 mice using representative samples from each genotype. Nuclear extracts of isolated  
646 livers were analyzed for Gadd45 $\beta$  and lamin A/C expression, and bands  
647 corresponding to these proteins are indicated. (B) Liver-specific Gadd45 $\beta$  mRNA  
648 expression in untreated and benzo[a]pyrene-treated wt and FL-N/35 mice. The  
649 expression levels of Gadd45 $\beta$ , GusB, GAPDH and HPRT1 were determined by  
650 quantitative real-time PCR. The results were normalized to expression of GAPDH,  
651 GusB and HPRT1, and the mean $\pm$ SEM mRNA quantities are expressed in relation to  
652 untreated wt controls. Statistical significance was analyzed using a Mann-Whitney  
653 nonparametric test. (C) Gadd45 $\beta$  promoter activity in FL-N/35 hepatocytes. Isolated  
654 hepatocytes from wt or FL-N/35 animals were cotransfected with pmaxGFP and a  
655 plasmid expressing firefly luciferase under the control of either the human Gadd45 $\beta$   
656 promoter (pGL2-Gadd45 $\beta$ -luc) or the SV40 promoter (pGL2), and either mock  
657 irradiated or treated with UV-C. The mean $\pm$ SEM luciferase levels (normalized to GFP  
658 expression) from three independent experiments are shown relative to those  
659 obtained in untreated wt hepatocytes. The statistical significances of the data were  
660 analyzed with a Mann-Whitney nonparametric test.

661

662 **Fig. 4. Gadd45 $\beta$  promoter hypermethylation in FL-N/35 mice.** (A) Expression of  
663 the Gadd45 $\beta$  transcript in mock irradiated or UV-irradiated primary hepatocytes from  
664 wt or FL-N/35 animals in the presence and absence of 5-azacytidine. Gadd45 $\beta$  and  
665 actin mRNA expression levels were determined by quantitative RT-PCR. Data from  
666 three independent experiments were normalized to the expression of  $\beta$ -actin, and are  
667 expressed relative to untreated wt controls. The statistical significance of differences  
668 was analyzed with a Mann–Whitney nonparametric test. (B) Analysis of Gadd45 $\beta$   
669 promoter methylation in hepatocyte DNA from wt or FL-N/35 animals by methylation-  
670 specific PCR. U indicates unmethylated PCR products, M denotes hypermethylated  
671 PCR products.

672

673 **Fig. 5. Mitotic index and DNA repair analyses in primary wt or FL-N/35**  
674 **hepatocytes.** (A) Immunofluorescent detection of phosphorylated histone-H3. Cells  
675 were analyzed for the presence of phosphorylated histone-H3 (left panel), and nuclei  
676 were counterstained with DAPI (right panel). A representative image (40X  
677 magnification) of phosphorylated histone-H3 and DAPI signals is shown. Scale bar =  
678 10  $\mu$ m. (B) Quantitative analysis of mitotic indices. Isolated cells receiving the  
679 indicated siRNA or a non-silencing control were subsequently either mock treated or  
680 treated with 5-azacytidine for 12 hours. Monolayers were then mock irradiated or  
681 treated with UV-C, and the presence of phosphorylated histone-H3 was analyzed six  
682 hours after irradiation. For each field of view (40X magnification), phosphorylated  
683 histone-H3-positive and DAPI-positive cells were quantified. Data are expressed as  
684 the percentage of phospho-histone H3 positive cells per field of view, and represent  
685 the analysis of at least 300 cells from two independent experiments. The statistical  
686 significance of differences was analyzed with a Mann-Whitney nonparametric test

687 (denoted in text). (C) DNA excision repair ability of primary wt and FL-N/35  
688 hepatocytes. Primary hepatocytes isolated from wt or FL-N/35 animals were  
689 cotransfected with pmaxGFP and UV-C-irradiated pGL2. Luciferase levels were  
690 assayed 48 hours post transfection, and normalized to GFP expression. DNA repair  
691 efficiency was calculated relative to wt control cultures, and mean $\pm$ SEM repair  
692 efficiency from three independent experiments is shown. Statistical significance was  
693 analyzed with a Mann-Whitney nonparametric test.

694

695 **Table 1.** Clinical and pathological features of patients with HCCs.

Patient number	Sex	Age (years)	Etiology	HCV genotype	Activity/Fibrosis*	Number of tumors	Size of largest tumor (cm)	Differentiation <sup>‡</sup>	Gadd45 $\beta$ protein expression <sup>Δ</sup>
1	M	50	HCV	ND	A2/F4	2	6	Pd	0
2	M	48	HCV	3a	F4	5	3	Md/Pd	0
3	M	67	HCV	ND	F4	7	2	Pd	0
4	M	51	HCV	3a	F4	2	6	Pd	0
5	M	75	HCV+alcohol	1b	A2/F3	1	3	Md	+
6	F	71	HCV	1b	A1/F1	1	12	Wd	+
7	M	69	HCV+alcohol	2a	A1/F2	1	3	Pd	0
8	M	61	HBV	NA	A1/F1	1	4	Md	+++
9	M	57	HBV	NA	A1/F2	2	3	Pd	++
10	M	45	HBV	NA	A2/F2	1	13	Md	++
11	M	60	HBV	NA	A1/F3	1	3	Pd	++
12	M	50	HBV	NA	F3/F4	2	3	Md	+++
13	M	78	ND	NA	A0/F3	10	6	Md	+++
14	M	73	alcohol	NA	A1/F2	6	13	Pd	+++
15	M	71	alcohol	NA	F1	1	7	Pd	+
16	M	67	alcohol	NA	F1	1	10	Md	+++
17	M	33	ND	NA	ND	1	10	Wd	+++

696 HCV: hepatitis C virus; HBV: hepatitis B virus; ND: not determined; NA: not applicable

697 \*According to Metavir scoring: A = activity: absent (A0); mild (A1); moderate (A2); severe (A3); F = fibrosis: absent (F0); mild (F1), moderate (F2), severe (F3); cirrhosis (F4)

699 ‡According to Edmonson grade: Wd (well-differentiated); Md (moderately differentiated); Pd (poorly differentiated)

700 <sup>Δ</sup>Gadd45 $\beta$  protein expression in tumoral tissues as determined by densitometric analysis of immunoblots. Scores: 0 = no expression; + = weak expression; ++ = moderate expression; +++ = strong expression.

701