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

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ABSTRACT

Members of the Gadd45 family play central roles in the cellular response to genotoxic stress, and have been implicated in several human cancers including hepatocellular carcinomas. Chronic infection by hepatitis C virus (HCV) is a major risk factor for the onset and development of primary hepatocellular tumors, although the underlying mechanisms are unclear. Here, we demonstrate a novel link between diminished Gadd45β expression and HCV infection. Inhibited Gadd45β expression was observed in both non-tumoral and tumoral tissues from infected individuals, and in cell lines harboring an HCV replicon and the infectious HCV strain JFH1. Decreased Gadd45β expression was confirmed *in vivo* in a transgenic murine model expressing the entire HCV open reading frame. Mechanistically, hypermethylation of the Gadd45β promoter in the presence of HCV is responsible for this defect. Diminished Gadd45β expression leads to aberrant cell cycle arrest and diminished DNA excision repair. Together, these results provide a novel insight into the mechanisms involved in HCV-associated hepatocellular carcinomas, showing that reduced Gadd45β expression may play a contributory role to this process, and providing evidence that HCV may interfere with epigenetic gene expression by altering promoter methylation.

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). Gadd45β 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 Gadd45β is dependent upon its interacting partner. Notably, Gadd45β 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 Gadd45β expression impairs G2/M
90 checkpoint activity. It remains to be determined whether interactions between
91 Gadd45β and p21 play a role in G1 arrest. Gadd45β 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 Gadd45β in epigenetic gene
94 activation (23, 24). Furthermore, several lines of evidence implicate Gadd45β in
95 apoptosis, particularly in response to transforming growth factor β (TGF-β) (25, 26).

96 Such observations have implicated Gadd45 proteins, including Gadd45β, as
97 crucial sensors of genotoxic stress. In line with this proposal, Gadd45β-/- 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 Gadd45β expression is diminished (29). This diminution has been attributed to
102 hypermethylation of the Gadd45β promoter, as treatment with DNA
103 methyltransferase inhibitors restores Gadd45β expression to non-tumoral levels (30).

104 Moreover, down-regulation of Gadd45β 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 κ-light-chain-enhancer of activated B cells (NF-κB)-mediated
108 Gadd45β transcription (31). Therefore, Gadd45β 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 Gadd45β expression was specifically
123 affected by HCV protein expression. To that end, we characterized Gadd45β
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

MATERIALS AND METHODS

Human liver tissues

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

Cell culture models of HCV replication and infection

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

HCV transgenic mice

Wild-type C57/Bl6 mice and mice transgenic for the entire HCV open reading frame (FL-N/35 lineage (32)), were bred and maintained as previously described (34). Eighteen-month-old male littermates were used for transcriptional and proteomic studies. Ten-month-old females were used for primary hepatocyte cultures. For benzo[a]pyrene treatment, mice received a single intraperitoneal injection of 55 μg benzo[a]pyrene (Sigma-Aldrich, Saint Louis, Missouri) per gram of body weight, diluted in corn oil. Treated mice were euthanized 36 hours post 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 Gadd45β 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 μg pmaxGFP and
203 either 2 μg pGL2-Gadd45β or 2 μg pGL2, using Lipofectamine LTX (Invitrogen,

204 Carlsbad, California). Four hours post transfection, monolayers were either mock
205 treated or exposed to 10 J/m² 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/m² 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 Gadd45β expression in primary murine hepatocytes was suppressed by
225 transfection with siRNA (Silencer Gadd45β 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/m² 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/m² 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/m²)
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 Gadd45β was equivalently expressed in non-
254 tumoral tissues from both uninfected and HBV-infected patients. In contrast,
255 Gadd45β was undetectable in non-tumoral tissues from both cirrhotic and non-
256 cirrhotic HCV-infected patients (Fig. 1A and 1B). In tumoral tissues, Gadd45β
257 expression was nearly undetectable in uninfected and HBV-infected patients, and
258 remained undetectable in HCV-infected patients (Fig. 1A and 1B).

259 Gadd45β transcripts were quantified by quantitative RT-PCR in the same
260 samples. As shown in Fig. 1C, Gadd45β 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, Gadd45β mRNA levels were
264 significantly lower in non-tumoral tissues than in uninfected and HBV-infected
265 controls (p=0.02). Gadd45β 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 Gadd45α nor Gadd45γ were significantly affected by the presence of HCV in
268 either tumoral or non-tumoral tissues (Fig. 1D). Decreased Gadd45β expression was
269 also observed in HCV-infected patients with no history of HCC (data not shown).

270 Correlations between Gadd45β protein expression and patients'
271 clinicopathological features (Table 1) were examined using Spearman's non-
272 parametrical test. Such analyses verified that reduced Gadd45β expression strongly
273 correlated (p=0.007) with HCV infection, and confirmed previous observations (29)
274 that Gadd45β expression was associated with differentiation (p=0.02). No
275 relationship was observed between Gadd45β expression and either tumor size or
276 frequency. Together, these data indicate that Gadd45β expression is down-regulated
277 in liver tissues from HCV-infected individuals.

278

279 **Gadd45β expression is inhibited in cultured hepatocytes harboring a**
280 **sub-genomic replicon or the infectious HCV strain JFH1**

281 The expression of the Gadd45β 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 Gadd45β 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 Gadd45β expression in**
288 **transgenic mice**

289 In order to assess the direct role of HCV protein expression in Gadd45β 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 Gadd45β protein and mRNA expression revealed a two-fold decrease in
294 Gadd45β expression in FL-N/35 mice compared to their wt littermates ($p=0.016$; Fig.
295 3A and 3B). Gadd45β expression reduction was liver-specific (data not shown).

296 Both wt and FL-N/35 mice were then treated with a single intraperitoneal
297 injection of benzo[a]pyrene, which induces mutagenic DNA lesions repaired by
298 nucleotide excision repair (42). Gadd45β mRNA levels increased after
299 benzo[a]pyrene treatment in both wt and FL-N/35 mice, but to a markedly lesser
300 extent in FL-N/35 mice (3.5-fold *vs* 5.5-fold in the wt mice) (Fig. 3B). Expression of
301 other Gadd family members in this model was not affected by the expression of the
302 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 Gadd45β expression inhibition *in vivo*.
305

306 **Gadd45β promoter activity is decreased in primary hepatocytes from FL-**
307 **N/35 mice**

308 In order to understand the mechanisms underlying inhibition of Gadd45β
309 expression by the HCV proteins, we sought to determine whether Gadd45β 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 Gadd45β promoter
313 (pGL2-Gadd45β-luc). As shown in Fig. 3C, basal activity of the Gadd45β 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 Gadd45β expression is a consequence of impaired
321 promoter activity in the presence of HCV.
322

323 **The Gadd45β promoter is hypermethylated in HCV transgenic mice**

324 Decreased Gadd45β gene expression in tumoral cell lines has previously
325 been attributed to hypermethylation of the Gadd45β 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 Gadd45β 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 Gadd45β mRNA (Fig. 4A).
332 Isolated hepatocytes from FL-N/35 mice displayed reduced Gadd45β levels in both
333 the presence and absence of acute UV-C treatment. However, 5-azacytidine
334 treatment restored Gadd45β 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 Gadd45β promoter was observed solely in FL-N/35 mice (Fig. 4B). Collectively, these
338 results demonstrate that inhibition of Gadd45β promoter activity by HCV is due to
339 promoter hypermethylation.

340

341 **Down-regulation of Gadd45β expression by HCV proteins leads to**
342 **defective cell cycle arrest**

343 Gadd45β is involved in the induction of G2/M arrest and increased Gadd45β
344 expression is associated with a decreased mitotic index (20, 43). Thus, we assessed
345 whether reduced Gadd45β 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/m² 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 Gadd45β 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 Gadd45β 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 Gadd45β expression was sufficient to perturb
366 temporary cell cycle arrest in response to UV-C.

367 In order to affirm the role of Gadd45β 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 Gadd45β 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 Gadd45β 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 Gadd45β
375 expression (Fig 5B). These experiments demonstrate that inhibited hepatic Gadd45β
376 expression in the context of HCV protein expression is due to hypermethylation of the

377 Gadd45β promoter, and is associated with impaired cell cycle arrest in response to
378 DNA damage.
379

380 **Down-regulation of Gadd45β expression by HCV proteins adversely**
381 **affects repair of damaged DNA**

382 As Gadd45β is important for efficient nucleotide excision repair of damaged
383 DNA, we examined whether decreased Gadd45β 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 Gadd45β 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 Gadd45β
407 expression. Firstly, we show, in liver biopsies from HCV-infected patients,
408 significantly lessened Gadd45β mRNA and protein expression in both tumoral and
409 non-tumoral tissues, in the presence and absence of cirrhosis. Gadd45β 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 Gadd45β 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 Gadd45γ 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 Gadd45α or Gadd45γ in non-tumoral tissues. Thus, we provide
421 the first evidence that Gadd45β expression is specifically disrupted by HCV protein
422 expression.

423 Mechanistically, the Gadd45β promoter was shown to be hypermethylated in
424 the presence of HCV. Treatment with the DNA methyltransferase suicide inhibitor 5-
425 azacytidine restored both Gadd45β expression and cell cycle arrest. The effects of
426 such treatment could be completely abrogated by RNA silencing of Gadd45β

427 expression, confirming that the effect of 5-azacytidine in this context was confined to
428 Gadd45β. Methylation-specific PCR confirmed hypermethylation of the Gadd45β
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 Gadd45β and
432 Gadd45γ promoters during tumorigenesis in the presence or absence of HCV.

433 Previously, it was demonstrated that inhibition of Gadd45β expression by RNA
434 interference perturbed G2/M checkpoint activity (20). Accordingly, we demonstrated
435 that Gadd45β 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 Gadd45β expression, but also decreased
438 Gadd45β 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 Gadd45β expression by HCV may directly provoke further
442 changes in methylation patterns of several genes, since Gadd45β 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 Gadd45β 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 Gadd45β 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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463 established in our laboratory by Abdelhakim Ahmed-Belkacem.

464

465

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611

612

613 **FIGURE LEGENDS**

614

615 **Fig. 1. Gadd45β expression in tumoral and non-tumoral tissues from HCV-**
616 **infected patients.** (A) Gadd45β 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 Gadd45β 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 Gadd45β protein levels were normalized to the expression of actin, and mean±SEM
623 densities are displayed in relation to non-tumoral non-infected (HBV-/HCV-) tissues.
624 (C) Gadd45β 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 Gadd45β
626 and SFRS4 transcripts were determined by real-time PCR. Data were normalized to
627 expression of SFRS4, and expressed as the mean±SEM mRNA expression.
628 (D) Gadd45α and Gadd45γ mRNA expression in non-tumoral and tumoral liver
629 tissues from HBV-/HCV- (n=5), HBV+ (n=5) and HCV+ (n=7) patients. Gadd45α,
630 Gadd45γ and SFRS4 transcripts were quantified by real-time PCR. Data were
631 normalized to SFRS4 expression, and expressed as the mean±SEM mRNA
632 expression for each gene. None of the comparisons showed significant differences.

633

634 **Fig. 2. Gadd45β 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 Gadd45β expression in Huh7.5 cells in the absence
637 and presence of an HCV genotype 1b subgenomic replicon. (B) Immunoblotting

638 analysis of Gadd45β 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 Gadd45β or 45 kDa actin indicated.

642

643 **Fig. 3. Gadd45β expression in FL-N/35 mice transgenic for the entire HCV open**
644 **reading frame.** (A) Western blot analysis of Gadd45β expression in FL-N/35 and wt
645 mice using representative samples from each genotype. Nuclear extracts of isolated
646 livers were analyzed for Gadd45β and lamin A/C expression, and bands
647 corresponding to these proteins are indicated. (B) Liver-specific Gadd45β mRNA
648 expression in untreated and benzo[a]pyrene-treated wt and FL-N/35 mice. The
649 expression levels of Gadd45β, 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±SEM mRNA quantities are expressed in relation to
652 untreated wt controls. Statistical significance was analyzed using a Mann-Whitney
653 nonparametric test. (C) Gadd45β 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 Gadd45β
656 promoter (pGL2-Gadd45β-luc) or the SV40 promoter (pGL2), and either mock
657 irradiated or treated with UV-C. The mean±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. Gadd45β promoter hypermethylation in FL-N/35 mice.** (A) Expression of
663 the Gadd45β 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. Gadd45β and
665 actin mRNA expression levels were determined by quantitative RT-PCR. Data from
666 three independent experiments were normalized to the expression of β-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 Gadd45β
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 μm. (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±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‡	Gadd45β protein expression$^{\Delta}$
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
698 (F2), severe (F3); cirrhosis (F4)
699 ‡According to Edmonson grade: Wd (well-differentiated); Md (moderately differentiated); Pd (poorly differentiated)
700 $^{\Delta}$Gadd45β protein expression in tumoral tissues as determined by densitometric analysis of immunoblots. Scores: 0 = no expression; + = weak
701 expression; ++ = moderate expression; +++ = strong expression.