Original Research

p53 antagonizes the unfolded protein response and inhibits ground glass hepatocyte development during endoplasmic reticulum stress

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Abstract

The unfolded protein response (UPR) is triggered during stress of the endoplasmic reticulum (ER) and facilitates tissue homeostasis. Considering the role of p53 tumor suppressor gene in the interpretation of stress-inducing stimuli, in this study, we explored whether p53 modulates UPR. We found that p53 ablation resulted in a profound sensitivity to tunicamycin that was associated with liver dysfunction, ground glass hepatocyte (GGH) development and nuclear atypia/dysplasia. Binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78) chaperone was readily detected in the cytoplasm of GGHs, confirming ER expansion. Tunicamycin administration induced BiP/GRP78 and GRP94 expression more potently in the p53-deficient mice than in controls and elevated phosphatidylcholine, the major lipid of ER, by a p53-dependent mechanism. Furthermore, alternative splicing of XBP1, the transcription factor that executes the UPR, was more efficient in cells which do not express p53. The cytoprotective effects of p53 were confirmed by cell viability studies, indicating that p53 deficiency conferred sensitivity against tunicamycin. Our findings show that p53 protects from the hepatotoxic effects of chronic ER stress. Stimulation of p53 activity when intense UPR is undesirable may possess therapeutic implications.

Keywords: ER stress, ground glass hepatocyte, endoplasmic reticulum biogenesis, phosphatidylcholine

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Introduction

Accumulation of misfolded proteins in the endoplasmic reticulum (ER) induces stress in the ER.[1] A diverse array of factors that disturb the balance between protein synthesis and folding may cause ER stress. These include, but are not limited to, viral infections,[2] inhibition of protein glycosylation and calcium homeostasis,[3] and aberrant lipid or glucose metabolism.[4–6] In order to preserve tissue homeostasis, cells have evolved an adaptive response, designated as the unfolded protein response (UPR) that aims to relieve ER from the overload of the excessive misfolded proteins. UPR involves the reversible blockade of protein synthesis by phosphorylation of eIF2, and the specific production of various chaperone proteins in order to increase the efficiency of the cell's folding machinery.[1] At the same time, terminally misfolded proteins are cleared by a process termed ER-associated degradation.[1,7] If prolonged or intense ER stress prevents the establishment of homeostasis, then apoptosis is initiated.[8,9] The differential regulation of p21 by the transcription factor CHOP has been proposed to play a role in this transition between the prosurvival and the proapoptotic states of ER stress.[10] According to the most widely accepted model, UPR is initiated by the activation of three ER resident proteins, activating transcription factor-6 (ATF6), inositol requiring protein-1 (IRE1) and protein kinase RNA-like ER kinase (PERK).[1] The activation of these three ER-transmembrane transducers follows their dissociation from binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78) that senses the presence of unfolded and misfolded proteins.[1] Activation of IRE1, ATF6 and PERK initiates a complex cascade of biochemical events that collectively constitute the UPR. Activation of IRE1 in particular by autophosphorylation activates its endonuclease activity, inducing the unconventional cytoplasmic splicing of the transcription factor X-box binding protein-1 (XBP1).[11,12] In turn, spliced XBP1 (XBP1s) triggers the transcription of various genes encoding for chaperone proteins. XBP1s is also involved in the regulation of ER expansion by stimulating the biosynthesis of phosphatidylcholine (PhCh), the major lipid component of ER.[13]

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ER-stress related mechanisms have been identified in several pathologies such as diabetes, obesity, neurodegenerative diseases and inflammation and cancer.[11,14,15] Various liver diseases, including non-alcoholic steatohepatitis, alcoholic liver disease and alcohol-induced liver injury, viral hepatitis, as well as cholestatic models of liver disease, have also been associated with ER stress responses. All these conditions, which are related to steatosis, implicate ER stress in altered lipid metabolism and hepatocyte physiology.[16,17] In viral hepatitis, stimulation of ER stress is related to the overproduction of viral antigens in the hepatocytes.[17] During chronic hepatitis B infection in particular, pre-S viral mutants are capable of inducing ER stress and are contained in characteristic ground glass hepatocytes (GGHs).[18–20] These GGHs are typical for chronic hepatitis B and have been classified predominantly as type I or type II depending on their morphology and distribution within the infected liver: type I GGHs are usually scattered sporadically in liver lobules and contain pre-S1 mutants as opposed to type II GGHs that are distributed in large clusters and contain pre-S2 mutants.[21–23]

p53 is a tumor suppressor gene that plays a major role in the regulation of the cells' response during genotoxic stress and is frequently inactivated in various human cancers.[24] Depending on the cellular context and the intensity of the stress-inducing signals, p53 activation may induce either cell cycle arrest or apoptosis, thus regulating the cell's life and death decisions.[24,25] During anticancer therapy, the status of p53 has been recognized as a major determinant of therapeutic efficacy, with p53 deficiency contributing to radio- and chemoresistance of tumors.[26] During ER stress, both p53 levels and activity have been shown to decrease by cytoplasmic localization and degradation of p53 in a glycogen synthase kinase-3β-dependent manner[27,28] or to increase by a mechanism coupling ribosomal stress with the inhibition of Hdm2-mediated ubiquitination of p53.[29]

In order to determine whether p53 plays an antagonistic or synergistic role in the execution of the UPR *in vivo*, we evaluated the effects of tunicamycin (Tun), an antibiotic inducing ER stress by inhibiting protein glycosylation, in mice deficient for p53.[30] We found that genetic ablation of p53 sensitizes mice to ER stress, causing characteristic liver pathology involving the generation GGHs. Our results are consistent with a protective role for p53 in response to ER stress, by reducing the levels of UPR.

Materials and methods

Animal housing and treatment

p53-deficient (p53KO) mice[31] and wild type (wt) controls were originally obtained from Jackson Laboratory (Bar Harbor, ME, USA), and maintained in our laboratory in a mixed C3H/C57BL6 genetic background. For all experiments, isogenic littermates have been used. Animals were kept on a 12:12 light:dark cycle and provided *ad libitum* access to water and food. Conditions within rooms were maintained at $21 \pm 1^{\circ}C$ with $50\% \pm 20\%$ relative humidity and ventilated at a minimum of 15 high-efficiency particulate-filtered air changes per hour. In order to induce chronic ER stress, mice were injected intraperitoneally with a 0.2 mL volume containing a dose of 2.5 μg of Tun (Sigma, St Louis, MO, USA) in phosphate-buffered saline (0.1 mg/kg of body weight) twice weekly. Control groups were injected with vehicle alone. A dose of 100×100 μg of Tun (4 mg/kg) was used to induce acute ER stress and tissues and tissues were harvested eight hours later. The experimental protocol was reviewed and approved by an Institutional Committee.

Cell culture treatments

Mouse embryo fibroblasts (MEFs) from wt and p53KO mice were isolated using standard protocols. All cell lines used were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics/antimycotics. Tissue-cultured reagents were obtained from Gibco (Life Technologies, Grand Island, NY, USA). Cells were plated at 60–70% confluence, and were cultured for 24 h before treatment with 5 μg/mL Tun and 25 μmol/L Nutlin-3 (Sigma) for 24 h. Experiments with MEFs involved cells of early passage, usually of less than passage 8.

Cell proliferation and migration assays

The rate of cell proliferation was evaluated by cell counting, using the Trypan blue exclusion assay under an inverted microscope. Cells were seeded at a density of 5×10^5 per well, in six-well plates, and were treated for 24 h with Tun at the indicated concentration.

RNA isolation, cDNA formation and analysis of XBP1 splicing

RNA was isolated using a Trizol RNA isolation protocol (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions. cDNA was obtained after a two-step reaction, using AMV reverse transcriptase (Promega, Madison, WI, USA). Cells were approximately 90% confluent when RNA was isolated. XBP1 splicing was detected using primers as described previously[32] and polymerase chain reactions were performed using Go-Taq polymerase (Promega), according to the manufacturer's instruction.

Western blotting

Proteins were extracted from livers of p53 wt and KO mice after homogenization. The samples were lysed at 4°C for 30 min using RIPA lysis buffer (Invitrogen) supplemented with proteinase inhibitors. Whole tissue extracts were subsequently centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants were collected. Protein content in the supernatants was quantified by the Bradford assay. Electrophoresis was performed on a 10% sodium dodecyl sulfate polyacrylamide gel under reducing conditions using 100 μg of the protein sample. The separated proteins were subsequently transferred to nitrocellulose membranes and exposed overnight to primary antibodies, followed by one hour of incubation with secondary antibodies. Antibodies were obtained from Santa Cruz Biotechnology

(Santa Cruz, CA, USA). The antigen–antibody complexes were detected with the ECL chemiluminescence detection system (Thermo Scientific, Waltham, MA, USA).

Immunohistochemical studies

Immunostaining of mouse liver for Bip/GRP78 (Santa Cruz Biotechnology) was performed by using the Superpicture Polymer (Dab) Kit (Novocastra, Newcastle Upon Tyne, UK), following the manufacturer's instructions. Before observation, tissue sections were weakly counterstained with hematoxylin.

PhCh concentrations

PhCh concentrations were measured in liver tissue, after homogenization, according to the manufacturer's instructions (Phosphatidylcholine Assay Kit; Biovision, Milpitas, CA, USA). Concentrations were normalized to DNA concentration.

Statistical analysis

Histological results were analyzed by the chi-squared test while the statistical analysis of PhCh concentrations and cell proliferation were performed using the Student's *t*-test (two-tailed, two-parameter). Mouse survival analysis generated a Kaplan–Meier plot (CDC-Epi Info; Centers for Disease Control and Prevention, Atlanta, GA, USA), analyzed by the logrank test.

Results

p53 deficiency sensitizes mice to chronic ER stress

In order to evaluate the consequences of p53 gene ablation on ER stress *in vivo*, p53KO ($n = 20$) and wt ($n = 14$) mice

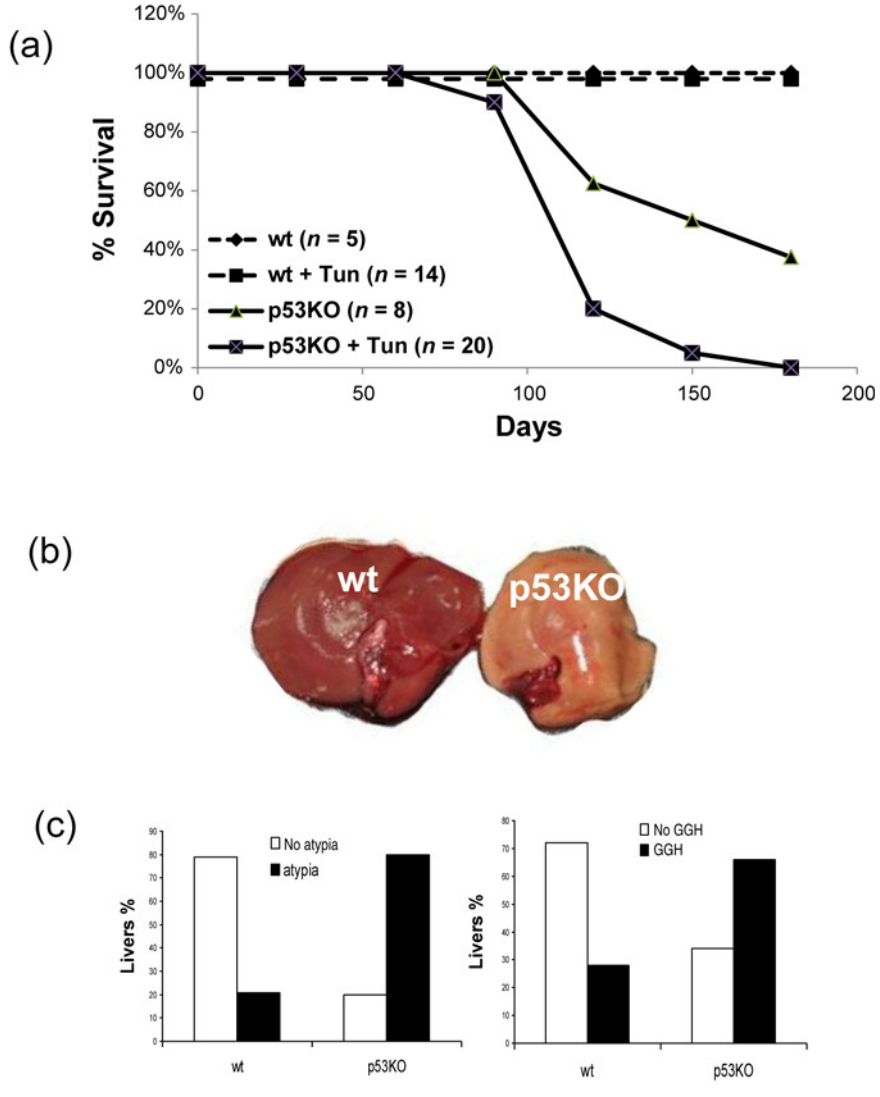

Figure 1 p53 deficiency confers sensitivity to chronic endoplasmic reticulum stress in mice. (a) Survival of wt and p53KO mice receiving chronically tunicamycin or solvent. Experiment was terminated and mice of all experimental groups were sacrificed by day 180 when all p53KO animals receiving tunicamycin died or were sacrificed due to sickness and distress. The number of animals in each experimental group is shown in the graph (Kaplan–Meier, $P < 0.0005$ logrank test). (b) Macroscopic appearance of livers from wt and p53KO mice receiving tunicamycin. (c) Graphical presentation of atypic nuclei (left panel) and ground glass hepatocytes (right panel) detected in wt and p53KO mice receiving tunicamycin. The percentage of animals whose livers showed the corresponding pathology is represented in the graph. (A color version of this figure is available in the online journal)

were administered, intraperitoneally, 0.1 mg/kg Tun twice per week. p53wt and p53KO animals not receiving Tun were also included as controls. By day 150, approximately 95% of p53KO mice that were receiving Tun either died ($n = 5$) or were sacrificed due to exhibiting signs of distress ($n = 15$, $P < 0.0005$, logrank test, Figure 1a). In contrast to the p53KO animals, the wt controls receiving a similar course of Tun administration for 150 d showed no signs of sickness. During the 150-day study, about 50% of the p53KO mice without Tun treatment also died or were sacrificed due to sickness. Autopsy, performed in all animals, identified the presence of spontaneous tumors, predominantly lymphomas, in the untreated p53KO mice. This was consistent with the previously shown increased susceptibility to carcinogenesis in these animals.[31] However, in the p53KO mice receiving Tun, with the exception of two animals, there was no evidence of malignancy, probably because these animals were sacrificed before tumors could have developed. These animals appeared to suffer from hepatic disease and the color of their livers ranged from pale to whitish. Additionally, whitish nodules and gray-brown friable areas, most probably necrotic tissue,

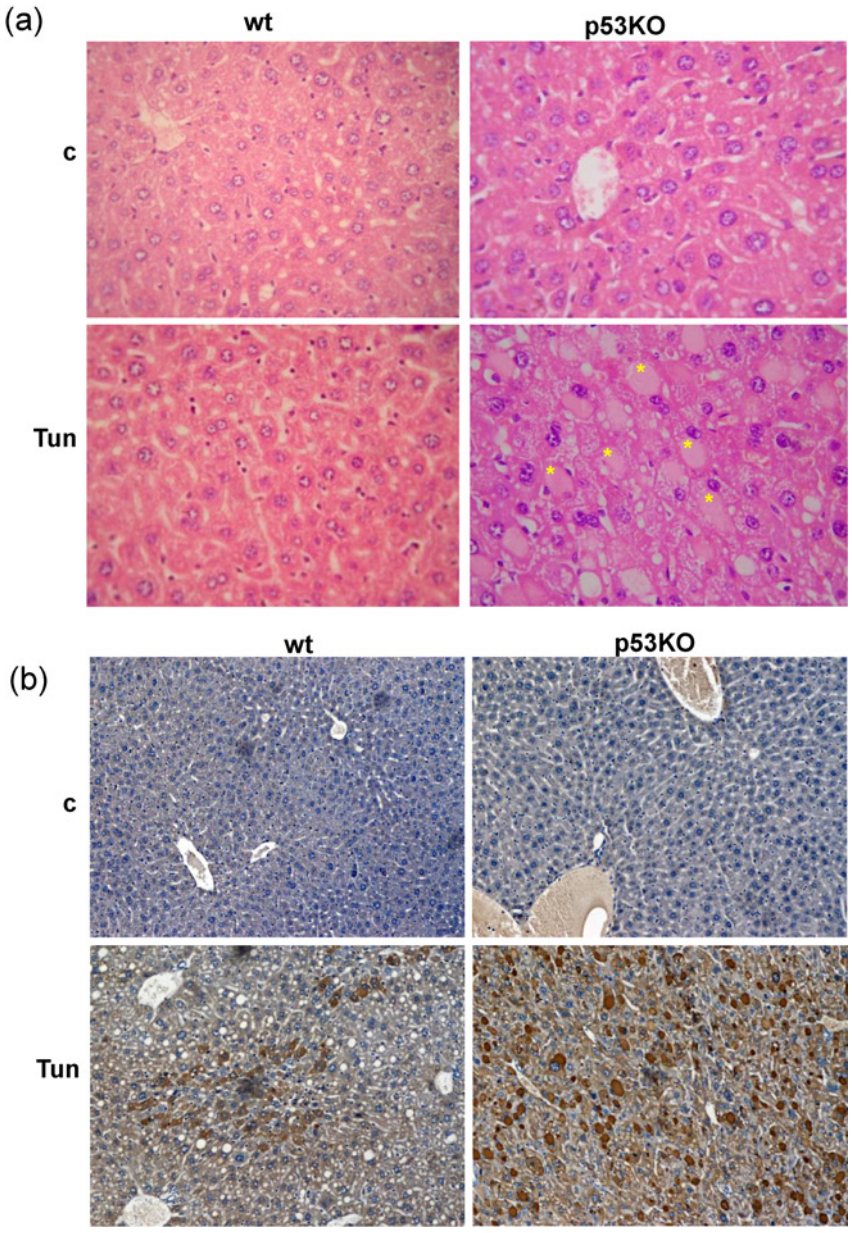

Figure 2 Ground glass hepatocyte (GGH) development and unfolded protein response induction in the livers of p53KO mice treated with tunicamycin. (a) Hematoxylin and eosin-stained sections of livers from wt and p53KO mice receiving chronically tunicamycin. The GGHs in the p53KO mice are recognized by their eccentric nuclei and the granular eosinophilic cytoplasm with a glassy appearance and are indicated by yellow asterisks. (b) Immunohistochemistry for binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78) in the livers of wt and p53KO mice receiving tunicamycin. Anti-BiP immunoreactivity was nearly absent from the animals that did not receive tunicamycin, but was detectable in the wt mice and strongly present in the p53KO mice receiving tunicamycin. Immunoreactivity is manifested by the brown cytoplasmic staining (Supplementary Figure 1; please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.012140/-/DC1). (A color version of this figure is available in the online journal)

were noted occasionally in these animals (Figure 1b). This characteristic gross appearance was absent in the liver of any of the wt mice that were receiving Tun or the p53KO mice that were not receiving treatment. Thus, genetic ablation of p53 sensitizes mice to the chronic induction of ER stress, elicited pharmacologically by Tun-induced damage in liver tissue.

Detection of GGHs in the livers of p53KO mice receiving Tun

In order to understand the cause of liver disease that was triggered by Tun in the absence of p53, hepatic tissue from these animals was subjected to histopathological examination. A characteristic liver pathology, consisting of the presence of abundant GGHs, was readily observed in 10 of the 15 (66%) p53KO livers evaluated (Figure 2a). These characteristic cells, which represent the hallmark of viral hepatitis B infection, consist of hepatocytes with eccentric nuclei and eosinophilic granular, glassy cytoplasms that have been associated with the activation of ER stress response. Their sporadic presence resembles the type I GGHs seen during viral hepatitis.[22] GGHs have also been detected in the livers of patients treated with drugs such as cyanamide, which is used for treating alcohol abuse and the overdose of diazepam and barbiturates.[33] GGHs were also identified in the livers of wt mice receiving Tun, but only in 28% (4 out of 14) of the cases as compared with 66% ($P < 0.05$, chi-square test) in the p53KO mice (Figure 1c). In all cases examined, the number of GGHs ranged 5–20 per high power optic field, while in three cases from the p53KO mice more than 30 GGHs were recorded. Besides GGHs, nuclear atypia/dysplasia could also be recognized in the Tun-treated animals. This was more common in the p53KO group, indicating the presence of dysplasia caused by chronic ER stress (Figures 1c, 2b and Supplementary Figure 1; please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.012140/-/DC1). Dysplastic cells with oversized and pleomorphic nuclei could be seen in only three of 14 (21%) wt mice livers, as compared with 12 of 15 p53KO mice (80%, $P < 0.05$, chi-square test). Interestingly, the occurrence of GGHs was associated with the presence of cellular atypia/dysplasia in both wt and p53KO mice that were receiving Tun. In wt mice, all three specimens with cellular atypia/dysplasia had GGHs, as well as in the p53KO group in which all 10 GGH-positive specimens also exhibited evidence of cellular atypia/dysplasia. This is consistent with a pro-oncogenic role for chronic ER stress that is intensified in the absence of p53.

Immunohistochemistry for BiP/GRP78 was performed in tissue sections from mice livers in order to confirm that under our experimental conditions, Tun induced ER stress (Figure 2b and Supplementary Figure 1b). In agreement with the above histological assessment, anti-BiP immunoreactivity was more pronounced in the p53KO mice, with GGHs exhibiting strong anti-BiP immunopositivity (Figure 2b and Supplementary Figure 1b).

Induction of PhCh by Tun is more pronounced in the livers of p53KO mice

ER stress stimulates ER biogenesis, inducing the biosynthesis of PhCh by mechanisms that may involve XBP1 activation.[13] The fact that GGHs possess an expanded ER in association with the observation that UPR appears to be more pronounced in the absence of p53 prompted us to address whether p53 modulates PhCh production *in vivo* and *in vitro*. The concentrations of PhCh were assessed in the livers of wt and p53KO mice that received either an acute (4 mg/kg once intraperitoneally) or chronic (0.1 mg/kg, twice per week, intraperitoneally, for 2 months) injections of Tun. Consistent with previous findings on the stimulation of ER biogenesis by UPR, we noticed an increase in PhCh concentrations in both experimental groups following prolonged administration of Tun. However, following the single administration of Tun, increased PhCh concentrations were detected only in the p53-deficient mice, implying that p53 restricts PhCh production and, thus, ER biosynthesis (Figure 3). This differential response elicited by the acute versus chronic administration of Tun is possibly because of the prolonged induction of ER stress bypassing the

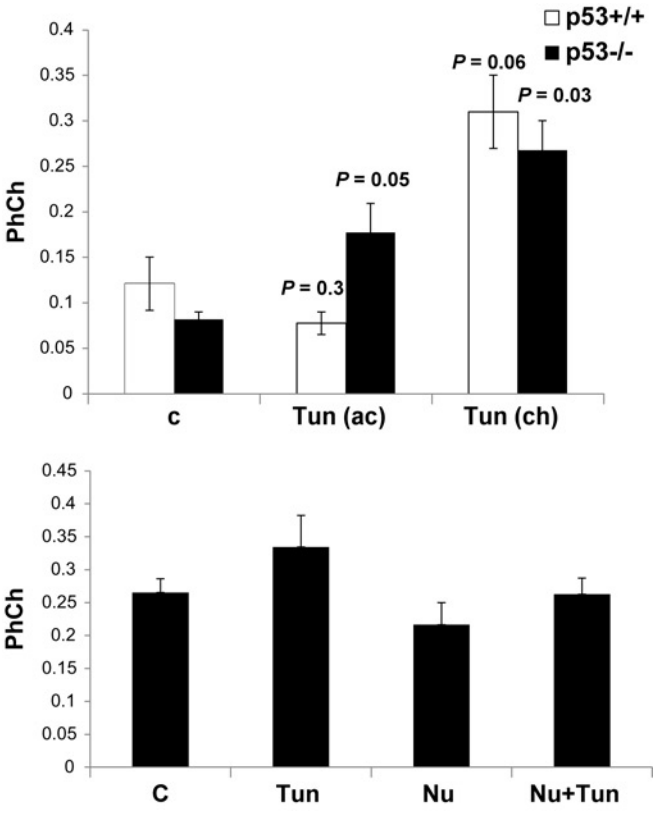

Figure 3 p53 modulates phosphatidylcholine (PhCh) biosynthesis *in vivo* and *in vitro*, in response to tunicamycin (Tun). In the upper panel, the relative concentrations of PhCh are shown in livers from mice that have received acute (ac) or chronic (ch) administration of Tun ($n = 2$ mice per group). *P* values (Student's *t*-test) versus untreated mice of the same genotype are indicated. In the bottom panel, the concentrations of PhCh in A549 human lung adenocarcinoma cells cultured *in vitro* in the presence of Tun and Nutlin (Nu), alone or combined, are shown. The concentrations of PhCh (*y*-axis) are expressed in arbitrary units following normalization with the content of the tissue in DNA

inhibition conferred by p53 in the wt p53-bearing animals. These results were also in agreement with the concentrations of PhCh in a different experimental system such as A549 human lung adenocarcinoma cells cultured *in vitro*. We found that exposure of A549 cells in culture to Tun for 24 h stimulated PhCh concentrations, and that this effect was abolished in the presence of Nutlin, which stabilizes p53, thus stimulating its activity.[34]

p53-deficiency potentiates the UPR and increases the cytotoxicity of Tun in cells *in vitro*

In order to confirm and extend the role of p53 ablation in the sensitization against ER stress, we evaluated total BiP and GRP94 chaperone levels in livers of mice that received Tun. BiP levels were consistently higher in the Tun-treated mice and this effect was more pronounced in the p53-deficient animals (Figure 4a). GRP94 was detected in three out of four p53KO mice that received Tun and in one of two untreated p53KO mice examined, but in none of the wt mice, regardless of Tun treatment. The absence of GRP94 expression in wt mice in association with the variable response of p53KO mice to Tun as regards GRP94 expression probably indicates an ER stress-independent regulation of GRP94 by p53 and/or higher sensitivity of this chaperone to local ER stress-inducing stimuli. Thus, p53 deficiency sensitizes cells to ER stress with BiP exhibiting a more robust and uniform stimulation than GRP94, during the execution of UPR.

Subsequently, we tested *in vitro* the splicing of XBP1 in MEFs isolated from the wt and p53KO mice, which had been cultured in the presence of Tun. Higher levels of spliced and thus active[12] XBP1 were detected in the p53-deficient MEFs (Figure 4b). In an analogous experiment involving A549 cells cultured *in vitro*, Nutlin inhibited the Tun-induced splicing of XBP1 (Figure 4b). Thus, modulation of p53 activity, in two different experimental systems, either by genetic ablation or pharmacological stabilization, is sufficient to modulate the efficiency of XBP1 splicing during ER stress.

These studies suggest that Tun-induced cytotoxicity is potentiated by the absence of p53. In order to test this hypothesis, p53-deficient and wt MEFs, as well as human hepatocellular carcinoma cells that differ in their status of p53, were exposed to Tun and cell viability was evaluated. In these studies, HepG2 cells represented p53 wt hepatocytes, and Hep3B cells represented p53 mutant hepatocytes.[35] As predicted, in both p53-deficient MEFs and Hep3B hepatocytes, the absence of functional p53 increased the sensitivity to Tun (Figure 4c).

Discussion

Intact p53 activity is typically associated with sensitivity against agents inducing genotoxic stress, such as chemotherapy and radiotherapy. Depending on the applied experimental system, ER stress was shown to either facilitate the reduction[27,28] or the increase[29] in p53 levels and activity. Thus, p53 reduction during ER stress may imply an

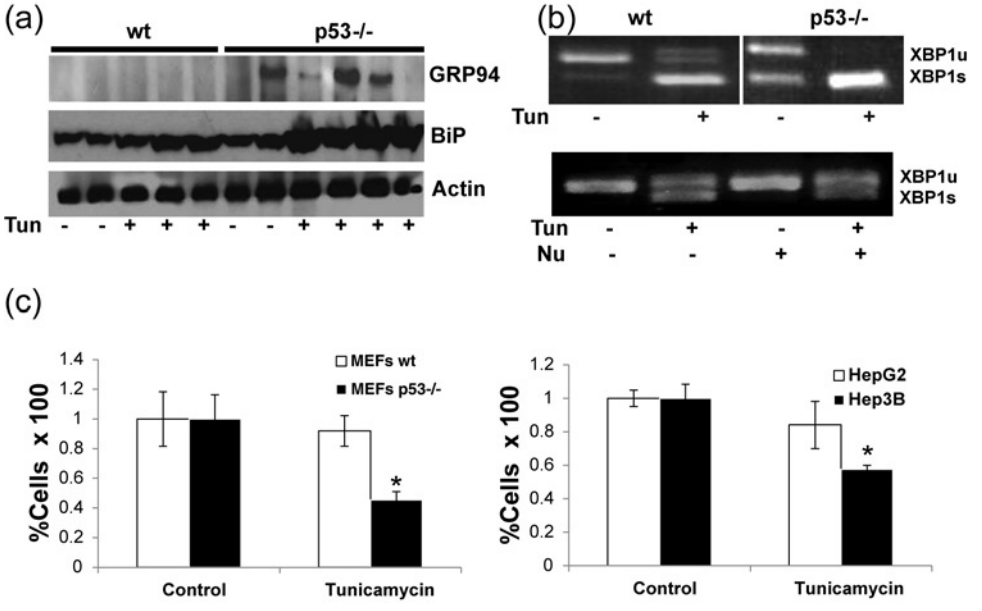

Figure 4 Potentiation of the UPR in the absence of p53. (a) Binding immunoglobulin protein (BiP) and glucose-regulated protein 94 (GRP94) protein concentrations in the livers of wt and p53KO mice. Beta-actin levels are shown as a loading control. Single bands at a size of about 45 kD (beta-actin), 78 kDa (BiP) and 100 kDa (GRP94) were obtained. BiP consistently and GRP94 with some variability were stimulated in the absence of p53 following tunicamycin treatment. (b) Splicing of XBP1 in wt and p53KO mouse embryo fibroblasts (MEFs) (upper panel) or A549 human lung cancer cells (lower panel) in the presence of tunicamycin. In A549 cells, the activity of p53 was also stimulated by Nutlin. p53 inhibits the splicing of XBP1 that is induced by tunicamycin. (c) Tunicamycin-induced cytotoxicity in wt and p53-deficient MEFs (left panel) and human hepatocellular carcinoma HepG2 (p53 wt) and Hep3B (deleted p53). Cells were exposed to tunicamycin at 5 μg/mL tunicamycin and 25 μmol/L Nutlin-3 for 24 h and cell number was evaluated by trypan blue exclusion assay. Percent cell number versus controls is shown in the graphs. $^*P < 0.05$

antagonistic, while its increase a synergistic, action of p53 during UPR. Recently, we showed that p53 activity was also required for the stimulation of chaperone ERp29 during adriamycin-induced cytotoxic stress; this in turn regulates PERK levels and activity.[36] In the present study, in order to assess directly the role of p53 during ER stress *in vivo*, we explored the consequences of chronic induction of ER stress in a genetic background that is deficient for p53. We showed that in the absence of p53, UPR is potentiated, an observation that is consistent with an antagonistic action for p53 in ER stress. Tun-mediated toxicity in the experimental animals was associated with a characteristic liver pathology that involved the development of GGHs, a histological characteristic of viral hepatitis. This is a direct evidence that this pathology is due to ER stress induction and involves p53-related mechanisms. Furthermore, we showed that this activity of p53 is likely mediated, at least in part, by interference with the efficiency of XBP1 splicing during UPR, PhCh production and ER biogenesis. It is conceivable that the ability of p53 to inhibit the ER stress-associated biogenesis of ER is also related to its anti-oncogenic activity. Consistently with the tumor suppressive role of p53, inactivation of p53 in cancer cells facilitates ER expansion in response to elevated demands for protein synthesis. Finally, our findings suggest that activation of p53 activity in pathological conditions in which intense UPR is undesirable and cytotoxic may reduce the consequent tissue damage. ER stress-mediated sensitization of cancer cells deficient in p53 may also be considered as a strategy for enhancing the efficacy of anticancer therapy.

Author contributions: ND and IC performed the experiments, analyzed the results and wrote the paper; EF performed the experiments and analyzed the results; and AGP and HK designed the experiments, analyzed the results and wrote the paper.

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