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The p66Shc Protein Controls Redox Signaling and Oxidation-Dependent DNA Damage in Human Liver Cells.

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Key words: oxidative stress, p66Shc, hepatocyte, Akt, FoxO3a, alcoholic steatohepatitis, *Nrf2*.

Abstract

The p66^{Shc} protein mediates oxidative stress-related injury in multiple tissues. Steatohepatitis is characterized by enhanced oxidative stress-mediated cell damage. The role of p66^{Shc} in redox signaling was investigated in human liver cells and alcoholic steatohepatitis. HepG2 cells with overexpression of wild-type or mutant p66^{Shc}, with Ser^{36} replacement by Ala, were obtained through infection with recombinant adenoviruses. Reactive oxygen species and oxidation-dependent DNA damage were assessed by measuring dihydroethidium oxidation and 8-hydroxy-2'-deoxyguanosine accumulation into DNA, respectively. mRNA and protein levels of signaling intermediates were evaluated in HepG2 cells and liver biopsies from control and alcoholic steatohepatitis subjects. Exposure to H_2O_2 increased reactive oxygen species and phosphorylation of p66^{Shc} on Ser^{36} in HepG2 cells. Overexpression of p66^{Shc} promoted reactive oxygen species synthesis and oxidation-dependent DNA damage, which were further enhanced by H_2O_2. p66^{Shc} activation also resulted in increased Erk-1/2, Akt and FoxO3a phosphorylation. Blocking of Erk-1/2 activation inhibited p66^{Shc} phosphorylation on Ser^{36}. Increased p66^{Shc} expression was associated with reduced mRNA levels of anti-oxidant molecules, such as NF-E2-related factor 2 and its target genes. In contrast, overexpression of the phosphorylation defective p66^{Shc} Ala^{36} mutant inhibited p66^{Shc} signaling, enhanced anti-oxidant genes, and suppressed reactive oxygen species and oxidation-dependent DNA damage. Increased p66^{Shc} protein levels and Akt phosphorylation were observed in liver biopsies from alcoholic steatohepatitis compared to control subjects. Conclusions: in human alcoholic steatohepatitis, increased hepatocyte p66^{Shc} protein levels may enhance susceptibility to DNA damage by oxidative stress by promoting reactive oxygen species synthesis and repressing anti-oxidant pathways.

Introduction

Aberrant production of reactive oxygen species (ROS) has been recognized as a major determinant of DNA damage, leading to disruption of tissue homeostasis, organ dysfunction, and onset of chronic degenerative disorders (24, 27, 30). p66^{Shc} has recently emerged as a master regulator of ROS production and a critical intracellular switch conveying oxidative stress signals to DNA damage in multiple cells and tissues, including the vascular wall and heart (11), kidney (29), osteoblasts (1), lymphocytes (25), and hepatocytes (10).

Rodents with genetic deletion of *p66^{Shc}* demonstrate a prolonged life span due to significant resistance to oxidative stress (3, 21, 32) p66$^{Shc-/-}$ mice are also protected against experimental diabetic glomerulopathy (19), diabetic cardiomyopathy), and hyperglycemia-induced endothelial dysfunction and atherogenesis (36), confirming that p66^{Shc} mediates oxidative stress-dependent tissue damage. Furthermore, phosphorylation of p66^{Shc} on Ser^{36} has been identified as the key signaling event mediating p66^{Shc} activation and promotion of its downstream cellular effects (21). In the liver, the levels of total and Ser^{36}-phosphorylated p66^{Shc} protein were found to be significantly augmented in the mouse non-alcoholic steatohepatitis (NASH) model (31). Conversely, ethanol-induced oxidative stress was found to be attenuated in the liver of p66$^{Shc-/-}$ mice (12), suggesting that p66^{Shc} may be involved in the hepatocyte damage in response to metabolic injuries. In addition, ablation of *p66^{shc}* gene in mouse hepatocytes suppressed cellular apoptosis and ROS production after hypoxia/reoxygenation through up-regulation of Mn superoxide dismutase (*SOD*) and redox factor-1 (13).

Normally, cells adapt to increased ROS levels by up-regulating antioxidant genes (24, 30) and neutralizing ROS through the low-molecular weight antioxidant and phase II detoxifying enzymes (2, 35). The NF-E2-related factor 2 (*Nrf2*) is a master gene involved in the regulation of phase II and antioxidant enzymes (e.g., glutathione S-transferase alpha 5 [*GSTA5*], glutathione S-transferase

muscle 2 [*GSTM2*], and *MnSOD*) (2, 15). Reduced expression of cardiac *Nrf2* was indeed associated with significant increase in nitrosative DNA damage (5). In hepatocytes, *Nrf2* was shown to be required for cell survival during liver development, its deficiency resulting in enhanced oxidative stress both in the normal and injured liver (4). While the detoxifying and ROS-scavenging role of *Nrf2* has been recognized in multiple cytoprotective activities (4, 15, 35), the relationship between p66^{Shc} and *Nrf2* has not been explored.

In this study, we show that p66^{Shc} protein expression is increased in human alcoholic steatohepatitis (ASH) and that in human liver cells p66^{Shc} controls intracellular ROS levels, the anti-oxidant *Nrf2* and Forkhead box protein O3a (FoxO3a) pathways, and the extent of oxidative DNA damage.

Glossary: ROS, reactive oxygen species; NASH, non-alcoholic steatohepatitis; *SOD*, superoxide dismutase; *Nrf2*, NF-E2-related factor 2; *GSTA5*, glutathione S-transferase alpha 5; *GSTM2*, glutathione S-transferase muscle 2; FoxO3a, Forkhead box protein O3a; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; DAPI, 4',6-diamidino-2-phenylindole; ASH, alcoholic steatohepatitis; HepG2/p66^{Shc}, HepG2 cells overepressing p66^{Shc}; HepG2/mock, HepG2 cells expressing a control empty adenovirus; HepG2/p66^{Shc}-Ala^{36}, HepG2 cells overexpressing a mutant p66^{Shc} protein with replacement of Ser^{36} by Ala; ARE, anti-oxidant response element; *CYP*, cytochrome P450; NAFLD, non-alcoholic fatty liver disease.

Materials and Methods

Antibodies and reagents.

Antibodies and reagents.

Anti-Shc monoclonal antibody was from BD Transduction Laboratories (Lexington, KY). Anti-Shc/p66 (pSer^{36}) antibody was from Calbiochem (Darmstadt, Germany). Anti-MAP kinase (ERK-1/2) antibodies were obtained from Zymed Laboratories (San Francisco, CA). Anti-GAPDH antibody (FL-335) was from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Akt (Ser^{473}), total Akt, phospho-p42/p44 MAP kinase (Thr^{202}/Tyr^{204}), phospho-FoxO1a(Thr^{24})/FoxO3a(Thr^{32}), total FoxO3a, phosphorylated Thr^{183}/Tyr^{185}-SAPK/JNK, total SAPK/JNK, phosphorylated Thr^{180}/Tyr^{182}-p38 MAPK and total p38 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). MEK inhibitor U0126 was obtained from Calbiochem (La Jolla, CA, USA). Anti-8-oxoguanine monoclonal antibody was purchased from Millipore (MAB3560; Millipore, Billerica, MA, USA). Alexa Fluor^{546} anti-rabbit antibody and the fluorescent dye dihydroethidium (DHE) were obtained from Invitrogen (Invitrogen, Carlsbad, CA). H_2O_2 was from Sigma Aldrich (St. Louis, MO, USA).

Cell cultures.

HepG2 human hepatoma cells were from American Type Culture Collection (Rockville, MD, USA) and were cultured in MEM supplemented with 10% FCS (both from GIBCO, Invitrogen, Paisley, UK), 100 U/ml penicillin, 100 mg/ml streptomycin (LONZA, MD, Iquique, Chile) and non-essential amino acids (NEA) (GIBCO, Invitrogen, Paisley, UK).

Adenoviral transfection studies.

The recombinant adenoviruses were generated by cloning either the wild type p66^{Shc}-encoding cDNA or the Ala^{36} p66^{Shc} mutant into the shuttle vector pAdTrack-CMV, containing a green fluorescent

protein epitope. Adenovirus production and cells infection were performed as previously described (18, 23).

Immunoblotting analysis.

Cell lysate preparation and immunoblotting analysis were performed as previously described (22,23). Briefly, HepG2 cells mechanically detached in ice-cold lysis buffer, containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM EDTA, 1% Triton X-100, 10% glycerol, 50 mM NaF, and 10 mM NaPP, supplemented with 100 µM PMSF, 5 ng/ml leupeptin, 1 µg/ml aprotinin, and 2 mM Na_3VO_4. Cell lysates were cleared by centrifugation. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA), and equal protein samples (60 µg) were separated on 7%-10% SDS-PAGE gels, as appropriate, and electrotransferred onto Hybond-P polyvinylidene difluoride filters (Amersham Life Science, Arlington Heights, IL, USA). The filters were then probed with the specific primary antibodies, and the immuno-reactive bands were visualized with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (H+L) (Bio-Rad, Hercules, CA, USA), as appropriate, using an ECL Plus Immunoblotting Detection System (Amersham Life Science, Arlington Heights, IL, USA), and quantified by densitometric analysis using the Versadoc imaging system (Bio-Rad, Hercules, CA, USA).

Immunofluorescence analysis of FoxO3a.

To visualize FoxO3a translocation, immunofluorescence analysis was performed, as previously described (7). Briefly, HepG2 cells were grown on coverslips in complete medium for the indicated times, then fixed with 3.7% formaldehyde at room temperature for 45 min, and permeabilized at room temperature with 0.1% Triton X-100. Subsequently, coverslips were incubated with primary antibodies (1:250 dilution) in PBS containing 2% BSA (16 h at 4 C), followed by 1 h of incubation with secondary Alexa546 Fluor anti-mouse goat antibody (1:500; Molecular Probes, Eugene, OR) or Alexa488 Fluor anti-rabbit goat antibody (1:500; Molecular Probes). Coverslips were mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired on a

Leica DM IRE2 confocal microscope or on Leica fluorescence microscope DM RXA2 (Leica Microsystems, Heerbrugg, Switzerland), as appropriate.

Measurement of ROS.

Intracellular ROS production was assessed through the evaluation of dihydroethidium oxidation using a Jasco FP6200 spectrofluorimeter (Jasco, Easton, MD) (7). Cells were incubated with 20 mM dihydroethidium for 0.5 h at 37°C in a serum-free medium in the dark, then washed with PBS, collected and resuspended in assay buffer (100 mM potassium phosphate, pH 7.4, 2 mM $MgCl_2$), using an aliquot for protein determination. The fluorescence increase (480 nm excitation and 567 nm emission wavelengths) caused by the ROS dependent oxidation of dihydroethidium was expressed as arbitrary units normalized by cell protein content.

Gene expression analysis.

RNA was extracted using RNeasy minikit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, as described (18). After total RNA was isolated from HepG2 cells, genomic DNA contamination was eliminated by DNase digestion (Qiagen, Hilden, Germany), and cDNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany). Oligonucleotide primers used for qRT-PCR: Human beta-glucoronidase: forward CTCATTTGGAATTTTGCCGATT, reverse CCGAGTGAAGATCC; human rRNA 18s: forward CGAACGTCTGCCCTATCAACTT, reverse ACCCGTGGTCACCATGGTA; human *Nrf2*: forward AAACCAGTGGATCTGCCAAC, reverse GACCGGGAATATCAGGAACA; human *CYP1A1*: forward GCTGACTTCATCCCTATTCTTCG, reverse TTTTGTAGTGCTCCTTGACCATCT; human *GSTA5*: forward CATTCACCTGGTGGAACTTTTCTA, reverse CTGCCAGGCTGCAGAAACTT; human *GSTM2*: forward CCGATTTGAGGGCTTGGA, reverse CCATCTTTGTGAACACAGGTCTTG; human *SOD2*: forward GTTGGCTTGGTTTCAATAAGGAA, reverse TCCCCAGCAGTGGAATAAGG;

158 human catalase: forward TTCGATCTCACCAAGGTTTGG, reverse
159 TGGATTCCGGTTTAAGACCAGTT.
160
161 The PCR reactions were carried out in an ABI PRISM 7500 System (Applied Biosystems,
162 Weiterstadt, Germany). The PCR reactions were carried out under the following conditions: 50°C for
163 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 60°C for 1 min. Relative gene expression
164 levels were determined by analysing the changes in SYBR green fluorescence during qRT-PCR using
165 the ΔΔCt method. To confirm amplification of specific transcripts, melting curve profiles were
166 produced at the end of each reaction. The mRNA level of each gene was normalized using β-actin as
167 internal control.

168 **Assessment of oxidative DNA damage.**
169 Oxidative DNA damage in the HepG2 cells was estimated by measuring the levels of 8-oxo-7,8-
170 dihydro-2'-deoxyguanosine (8-oxodG) in DNA using the method of Polytarchou et al. (26). Briefly,
171 HepG2 cells were fixed in 4% formaldehyde, either before or 30 min after treatment with H_2O_2, and
172 then stained with an anti-8-hydroxyguanine antibody. Coverslips were mounted on glass slides, with
173 Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Labs).
174 Images were obtained using a Nikon Eclipse 80i microscope with a 10X objective and a Spot charge-
175 coupled-device camera. Images were quantified as red/blue ratios by using Adobe Photoshop (Adobe
176 Systems Inc.).

177 ***Ex-vivo* studies in liver biopsies.**
178 Liver biopsy specimens were obtained from patients admitted to the Liver Unit (Clinical Division "A.
179 Murri", Azienda Ospedaliero-Universitaria Policlinico, Bari, Italy) with clinical and analytic features
180 of alcoholic steatohepatitis (ASH), including alcohol intake >80 g/day, increased aminotransferase
181 and gamma-glutamyl transpeptidase levels, and no other identifiable cause of liver disease (9).
182 Histologic grading was as follows: (1) degree of hepatocellular damage/ ballooning (0, none; 1, mild;

2, severe) and presence of Mallory bodies, mega-mitochondria, and cholestasis (0, no; 1, yes); (2) degree of lymphocytic infiltration (0, none; 1, mild; 2, moderate; 3, severe); (3) degree of polymorphonuclear infiltration (0, none; 1, mild; 2, moderate; 3, severe); (4) degree of steatosis (G0: <10%; G1 10% - 33%; G2, 33% - 66%; G3 ≥66%); (5) degree of lobular fibrosis (0, none; 1, mild; 2, moderate; 3, severe); and (6) fibrosis stage (0, no fibrosis; 1, portal; 2, portal fibrosis and few septa; 3, septal fibrosis without cirrhosis; 4, cirrhosis) (9, 20). The protocol was approved by the institutional Ethics Committee, and all patients gave their written informed consent.

Statistical analysis.

Data are presented as mean ±SE. Normal distribution of data was assessed by the Kolmogorov–Smirnov test ($p>0.05$). Statistical analysis was performed by the Student's t test or the one-way ANOVA with Tukey's multiple comparison test, as appropriate, using Minitab® 15.1. Significance was assumed at a p value <0.05.

Results

p66^{Shc} is activated by oxidative stress and promotes ROS synthesis in HepG2 cells.

To understand the relationship between oxidative stress and p66^{Shc}, p66^{shc} phosphorylation was examined in HepG2 cells exposed to H_2O_2. Although the endogenous p66^{Shc} levels were relatively low in wild-type HepG2 cells, phosphorylation of p66^{Shc} on Ser^{36} could be detected in a dose-dependent manner upon exposure to H_2O_2 (Fig. 1A), and this was associated with increased intracellular ROS levels (Fig. 1D). To investigate the effects of increased p66^{Shc} protein levels in liver cells, HepG2 cells with selective overexpression of p66^{Shc} (HepG2/p66^{Shc}) were obtained by infection with a recombinant adenovirus encoding p66^{Shc} (Fig. 2). In the absence of H_2O_2, phosphorylation of p66^{Shc} on Ser^{36} was found to be increased several-fold in HepG2/p66^{Shc} as compared to control HepG2/mock cells ($p<0.0001$; Fig. 1, B and C), and it was further enhanced in a dose-dependent manner upon H_2O_2 exposure (Fig. 1B), peaking at 15 min ($p<0.001$ vs basal, Fig. 1C). ROS levels were increased ~3-fold in HepG2/p66^{Shc} compared to control cells under basal conditions ($p<0.001$ vs wild-type HepG2 and HepG2/mock; Fig. 1D). In addition, exposure of HepG2/p66^{Shc} cells to H_2O_2 led to further increase in ROS synthesis ($p<0.005$ vs wild-type HepG2 and HepG2/mock; Fig. 1D). Thus, p66^{Shc} conveys extracellular oxidative stress signals to increase ROS synthesis in liver cells.

p66^{Shc} activates the Erk and Akt/FoxO3a pathways in HepG2 cells.

The activation of Erk and of Akt/FoxO3a pathways, which have been shown to be involved in p66^{Shc} signaling and oxidative stress responses (5, 11), were assessed next. Phosphorylation levels of Erk-1/2 (Fig. 3A) and Akt (Fig. 3B) were found to be significantly increased in response to H_2O_2 treatment in both HepG2/p66^{Shc} and HepG2/mock cells ($p<0.001$ vs basal; Fig. 3, A and B). However, Erk-1/2 and Akt phosphorylation showed higher levels and Erk-1/2 also an earlier 15-min peak after H_2O_2 challenge in HepG2/p66^{Shc} than in control cells ($p<0.001$ vs HepG2/mock; Fig. 3, A

and B). Both JNK-1/2 and p38 MAPK were also found to be activated upon H_2O_2 treatment in both HepG2/p66^{Shc} and control cells; however, phosphorylation levels of these kinases were similar in HepG2/p66^{Shc} and control cells (data not shown).

Akt-mediated FoxO3a phosphorylation on Thr^{32} promotes both its inactivation and translocation from the nucleus to the cytoplasm (8). In both HepG2/mock and HepG2/p66^{Shc} cells, the levels of Thr^{32} phosphorylation of FoxO3a were increased following challenge with H_2O_2 ($p<0.005$ vs basal; Fig. 3C) and augmented in p66^{Shc} overexpressing vs control cells ($p<0.005$ in HepG2/p66^{Shc} vs HepG2/mock; Fig. 3C), paralleling Akt phosphorylation (Fig. 3B). The subcellular distribution of FoxO3a in the cytoplasmic and nuclear compartment was then investigated. In control HepG2/mock cells, endogenous FoxO3a could be detected almost exclusively in the nucleus in the basal state, whereas it was relocated predominantly in the cytoplasm following 15 min of H_2O_2 stimulation (Fig. 4, Panel A). By contrast, in HepG2/p66^{Shc} cells, FoxO3a showed reduced nuclear staining and increased cytoplasmic localization already in the basal state, with minimal changes induced by exposure to H_2O_2 (Fig. 4, Panel B). Thus, H_2O_2-mediated p66^{Shc} activation is followed by activation of the Erk-1/2 and Akt/FoxO3a pathways and translocation of FoxO3a from the nuclear to the cytoplasmic compartment. These responses are enhanced, and already under basal conditions, when p66^{Shc} is overexpressed.

Role of Erk in Ser^{36} phosphorylation of p66^{Shc} and Akt/FoxO3a phosphorylation.

The role of Erk-1/2 activation in p66^{Shc} phosphorylation was investigated next by using the MEK inhibitor U0126. As expected, pretreatment with U0126 completely abrogated Erk-1/2 phosphorylation in both HepG2/mock and HepG2/p66^{Shc} cells, both under basal conditions and after H_2O_2 stimulation ($p<0.0001$ vs cells not exposed to U0126; Fig. 5A and data not shown). This was associated with a significant decrease in p66^{Shc} phosphorylation on Ser^{36}, both in the absence and presence of H_2O_2 (Fig. 5B and data not shown). Similar results were obtained using PD098059,

another inhibitor of MEK (data not shown). Furthermore, inhibiting the Erk-1/2 pathway with U0126 also significantly reduced the phosphorylation of Akt and FoxO3a following H_2O_2 challenge ($p<0.05$ vs cells not exposed to the MEK inhibitor; Fig. 5C and 5D). Finally, treatment with U0126 markedly reduced H_2O_2-induced ROS production in control and HepG2/p66^{Shc} cells, respectively ($p<0.01$ vs cells not exposed to U0126; Fig. 5E). Altogether, these results suggest that activation of Erk-1/2 contributes to p66^{Shc} phosphorylation and its downstream signaling.

Role of p66^{Shc} Ser^{36} phosphorylation in Erk and Akt/FoxO3a signaling and ROS production.

To assess whether phosphorylation of p66^{Shc} on Ser^{36} is necessary for efficient signal propagation, a phosphorylation-defective p66^{Shc} protein, in which Ser^{36} was mutated to Ala, was overexpressed in HepG2 cells (HepG2/p66^{Shc}-Ala^{36}). No significant differences in p66^{Shc} protein levels were observed between HepG2/p66^{Shc} and HepG2/p66^{Shc}-Ala^{36} cells (Fig. 6A), while Ser^{36}-phosphorylation of p66^{Shc} was undetectable in HepG2/p66^{Shc}-Ala^{36} cells both under basal conditions and after H_2O_2 stimulation (Fig. 6A and B). Interestingly, Erk-1/2 activation was significantly decreased and Akt/FoxO3a phosphorylation was completely blunted in HepG2/p66^{Shc}-Ala^{36} as compared to HepG2/p66^{Shc} cells, both in the absence and presence of H_2O_2 stimulation (Fig. 7, A-C; $p<0.01$ vs HepG2/p66^{Shc}). In addition, H_2O_2 exposure failed to increase ROS production in HepG2/p66^{Shc}-Ala^{36}, differently than in control cells (Fig. 7D), indicating that Ser^{36} phosphorylation of p66^{Shc} is critical for ROS production in response to oxidative stress. Thus, Ser^{36}-phosphorylation of p66^{Shc} plays an important role in activation of the Erk and Akt/FoxO3a signaling pathways and ROS synthesis in HepG2 cells.

Nrf2 and Nrf2 target genes in HepG2 cells overexpressing p66^{Shc}.

In the light of the protective role of *Nrf2* against oxidative stress (17-19), whether *Nrf2* and the Nrf2-induced anti-oxidant response element (ARE) target genes would be affected by changes in p66^{Shc} protein levels in HepG2 cells was examined next. Interestingly, *Nrf2* mRNA levels were significantly

lower in HepG2/p66^{Shc} than in control HepG2/mock cells in the absence of H_2O_2 ($p<0.05$ vs basal control cells; Fig. 8), and remained significantly reduced by >50% at multiple times following induction of oxidative stress with H_2O_2 up to 240 min ($p<0.05$ vs control cells at 15-60 min; Fig. 8). Changes in *Nrf2* mRNA levels were paralleled by similar changes in gene expression of major Nrf2 target genes. Indeed, mRNA levels of cytochrome P450 (*CYP*)*1A1*, *GSTM2*, and *GSTA5* were significantly reduced in HepG2/p66^{Shc} as compared to HepG2/mock cells both under basal conditions and at multiple time-points following exposure to H_2O_2 ($p<0.05$ vs HepG2/mock; Fig. 8). Furthermore, *Nrf2* and Nrf2-induced ARE genes showed markedly reduced mRNA levels in HepG2/p66^{Shc} compared to HepG2/p66^{Shc}-Ala^{36} cells ($p<0.05$ vs HepG2/p66^{Shc}-Ala^{36}; Fig. 8). Finally, HepG2/p66^{Shc}-Ala^{36} cells exhibited higher mRNA levels of *CYP1A1, GSTM2, GSTA5* and *SOD2* than control HepG2/mock cells ($p<0.05$ vs HepG2/mock; Fig. 8), consistent with the mutant p66^{Shc} Ala^{36} variant acting as a dominant-negative protein.

Overexpression of p66^{Shc} promotes oxidative DNA damage.

8-OxodG is a sensitive marker of ROS-induced DNA damage (27). To investigate the possibility that forced expression/activation of p66^{Shc} may lead to DNA damage via increased ROS synthesis, the extent of 8-oxodG accumulation was assessed in individual cells infected with the distinct adenoviral constructs by immunofluorescence. Expression of green fluorescent protein by the adenovirus allowed identification of infected cells. With this method, 8-oxodG staining was found to be increased in HepG2/p66^{Shc} as compared to HepG2/mock and HepG2/p66^{Shc}-Ala^{36} cells in the absence of H_2O_2 (Fig. 9, A-C). Treatment of HepG2/p66^{Shc} with H_2O_2 for 60 min further increased 8-oxodG accumulation at 15 min (data not shown) and 30 min (Fig. 9B). By contrast, 8-oxodG staining was almost undetectable in H_2O_2-treated HepG2/p66^{Shc}-Ala^{36} as compared to both HepG2/p66^{Shc} and HepG2/mock cells (Fig. 9, A-C). Overall, these data demonstrate that p66^{Shc} promotes ROS-induced DNA damage in liver cells, and that this is inhibited by the phosphorylation-defective p66^{Shc} protein.

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292 *p66^{Shc} in human liver biopsies from subjects with ASH.*

293 Finally, protein expression and phosphorylation levels of p66^{Shc} were examined in liver biopsies of
294 subjects with ASH. The severity of liver injury was graded according to the presence and extent of
295 steatosis, fibrosis, and lymphocytic/polymorphonuclear infiltration (9, 20), ranging from G0/F0 to
296 G3/F4. The protein levels of p66^{Shc} were found to be increased in the human liver biopsies in parallel
297 with the severity of liver injury (Fig. 10A), in the absence of changes in p52^{Shc} and p46^{Shc} protein
298 abundance (Fig. 10A). Furthermore, p66^{Shc} expression was found to be significantly increased in the
299 group with higher extent of steatosis (>33%) and presence of fibrosis and
300 lymphocytic/polymorphonuclear infiltration ($p<0.05$, G2-3/F1-4 vs G0-1/F0; Fig. 10A). Furthermore,
301 increased p66^{Shc} protein levels were associated with significant augmentation of Akt
302 phosphorylation, in the absence of significant changes in Akt protein levels ($p<0.05$, G2-3/F1-4 vs
303 G0-1/F0; Fig. 10B). Other signaling reactions, including p66^{Shc} phosphorylation on Ser^{36} and FoxO3a
304 phosphorylation on Thr^{32}, could not be assessed since they were below the sensitivity of the
305 immunoblotting technique (data not shown).

Discussion

The adapter protein p66^{Shc} has been shown to mediate oxidative stress-related injury in multiple cell types and under a variety of pathophysiological conditions, including obesity, diabetes, and steatohepatitis (1, 10–13, 19, 21, 24, 25, 27–32, 36). Specifically, p66^{Shc} has been involved in hepatocyte lipid accumulation and cytotoxicity in both experimental ethanol intoxication and NASH (17, 31). The results from this study demonstrate for the first time that p66^{Shc} is both a target and an enhancer of oxidative stress in liver cells and that p66^{Shc} protein levels are increased in the liver of individuals with ASH. In addition, forced expression of p66^{Shc} promotes ROS synthesis and reduces expression of Nrf2 and Nrf2-induced ARE genes, increasing incorporation of 8-oxodG into cellular DNA, a marker of oxidative stress-related DNA damage. All of these effects require phosphorylation of p66^{Shc} on Ser^{36}.

In control HepG2 cells the levels of p66^{Shc} were relatively low, yet phosphorylation of the protein could be induced by exposure to H_2O_2. However, when p66^{Shc} was overexpressed by adenoviral-mediated gene transfer its phosphorylation on Ser^{36} was increased (Fig. 1C) and this was associated with increased phosphorylation of Erk-1/2 and Akt/FoxO3a (Fig. 3 A-C) and ROS synthesis (Fig. 1D).

While overexpression of p66^{Shc} was associated with increased Erk-1/2 phosphorylation, which was reduced in HepG2 overexpressing a defective p66^{Shc}-Ala^{36} (Fig. 7A), inhibition of the Erk pathway using various MEK inhibitors significantly reduced Ser^{36} phosphorylation of p66^{Shc} both in the absence and presence of H_2O_2 stimulation. Altogether, these results suggest that p66^{Shc} and Erk-1/2 are involved in a reciprocal “regulatory loop”: increased p66^{Shc} expression/signaling results in Erk-1/2 activation that in turn promotes Ser^{36}-phosphorylation of p66^{Shc} (Fig. 11). These data are in line with previous observations in breast and prostate cancer cells, in which increased p66^{Shc} protein levels were positively correlated with Erk-1/2 phosphorylation (16, 34), and p66^{Shc} knockdown led to

reduced Erk-1/2 activation (34), but not with other studies showing inhibition of Erk signaling by p66^{Shc} (32). Conversely, Erk-dependent phosphorylation of p66^{Shc} on Ser^{36}, which is then responsible for p66^{Shc}-dependent phosphorylation of FoxO3a, as observed in this study (Fig. 5), was previously shown in mouse embryo fibroblasts (32). However, the phosphorylation levels of p66^{Shc} on Ser^{36} were not completely abolished when Erk-1/2 was fully inhibited (Fig. 5), suggesting that additional protein kinases other than Erk-1/2 may be involved in Ser^{36}-phosphorylation of p66^{Shc}. Indeed, depending on the cell type and stimulus (e.g., H_2O_2, TGF-β, various cytokines), protein kinase C (PKC) β and δ, and β1Pix (Pak-interacting exchange factor) have also been shown to be involved in p66^{Shc} phosphorylation on Ser^{36} (6, 22, 31). Furthermore, we have recently reported that TNFα promotes p66^{Shc} phosphorylation on Ser^{36} via the stress-kinase JNK in human endothelial cells (23). However, both JNK-1/2 and p38 MAPK activities were not affected by p66^{Shc} overexpression in HepG2 cells (data not shown), suggesting that they are not part of the same regulatory loop as Erk-1/2.

The involvement of the *Nrf2* signaling pathway in eliciting cell survival and resistance to oxidative stress has been recently reported (2, 5, 35). *Nrf2* plays a central role in cytoprotection, by detoxifying and eliminating ROS, xenobiotics and electrophilic carcinogens, as well as by removing damaged proteins and organelles (35). When compared to control cells, *Nrf2* knockout cardiomyocytes showed significantly higher ROS levels under basal conditions, which were further enhanced upon exposure to high glucose concentrations (14). Similarly, in primary mouse hepatocytes *Nrf2* gene ablation resulted in enhanced oxidative stress, impaired activation of the MAPK pathway and reduced mRNA expression of ROS-detoxifying enzymes (4). In line with these findings, our results show for the first time a link between the redox protein p66^{Shc} and the *Nrf2* pathway. Liver cells overexpressing p66^{Shc} showed reduced mRNA levels of *Nrf2*, and of its downstream detoxifying target genes, such as *CYP1A1*, *GSTM2*, and *GSTA5*, in association with enhanced ROS synthesis and increased oxidative DNA damage. Conversely, cells overexpressing the phosphorylation defective p66^{Shc} mutant

displayed augmentation of gene expression of *Nrf2* and its downstream target genes, reduced ROS levels and minimal 8-oxodG accumulation. Thus, p66^{Shc} appears to foster cellular oxidative stress responses by suppressing *Nrf2* expression (Fig. 11). The Akt/FoxO3a signaling pathway is also involved in preventing accumulation of ROS and consequent cell damage by up-regulating anti-oxidant enzymes (33). Since forced activation of p66^{Shc} resulted in increased FoxO3a phosphorylation and nuclear exclusion (Fig. 3 and 4), and this was not observed following overexpression of p66^{Shc}Ala^{36} (Fig. 7), enhanced p66^{Shc} signaling may potentially promote oxidative DNA damage by both repressing *Nrf2* and inactivating FoxO3a (Fig. 11).

Significant elevations of *p66^{Shc}* mRNA and protein levels were recently reported in liver biopsies from individuals with non-alcoholic fatty liver disease (NAFLD) and NASH, in comparison with normal liver samples (31). In line with these findings, we found increased p66^{Shc} protein levels in liver biopsies of subjects with ASH, a well-characterized condition of oxidative stress-induced cellular damage (10,29). Moreover, p66^{Shc} protein abundance correlated with the degree of histological abnormalities and disease severity, being increased to a greater extent in subjects with higher degree of fibrosis and steatosis (Fig. 10), in line with the results in NAFLD/NASH (14). Increased protein expression of p66^{Shc} in liver biopsies with more severe grading was associated with augmented Akt phosphorylation, suggesting functional relevance of these findings (Fig. 10). Inhibition of hepatic p66^{Shc} signaling may thus represent an attractive strategy to counteract progression of hepatocyte damage in both alcoholic and non-alcoholic fatty liver disease.

In conclusion, overexpression of p66^{Shc} in human hepatocytes promotes ROS accumulation and increases susceptibility to H_2O_2-induced oxidative stress, leading to reduced levels of cytoprotective genes and consequently increased DNA damage. Modulation of the redox homeostasis by limiting p66^{Shc} expression and/or activity in human hepatocytes may open novel therapeutic approaches for oxidative stress-associated liver diseases.

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386 **References**

387 1. **Almeida M, Han L, Ambrogini E, Bartell SM, Manolagas SC**. Oxidative stress stimulates
388 apoptosis and activates NF-kappaB in osteoblastic cells via a PKCbeta/p66shc signaling
389 cascade: counter regulation by estrogens or androgens. [Online]. *Mol Endocrinol* 24: 2030–
390 2037, 2010. http://www.hubmed.org/display.cgi?uids=20685851.

391 2. **Bataille AM, Manautou JE**. Nrf2: a potential target for new therapeutics in liver disease.
392 [Online]. *Clin Pharmacol Ther* 92: 340–348, 2012.
393 http://www.hubmed.org/display.cgi?uids=22871994.

394 3. **Beltrami E, Ruggiero A, Busuttil R, Migliaccio E, Pelicci PG, Vijg J, Giorgio M**. Deletion
395 of p66Shc in mice increases the frequency of size-change mutations in the lacZ transgene.
396 *Aging Cell* 12: 177–83, 2013.

397 4. **Beyer TA, Xu W, Teupser D, auf dem Keller U, Bugnon P, Hildt E, Thiery J, Kan YW,**
398 **Werner S**. Impaired liver regeneration in Nrf2 knockout mice: role of ROS-mediated
399 insulin/IGF-1 resistance. [Online]. *EMBO J* 27: 212–223, 2008.
400 http://www.hubmed.org/display.cgi?uids=18059474.

401 5. **Cai C, Teng L, Vu D, He J-Q, Guo Y, Li Q, Tang X-L, Rokosh G, Bhatnagar A, Bolli R**.
402 The heme oxygenase 1 inducer (CoPP) protects human cardiac stem cells against apoptosis
403 through activation of the extracellular signal-regulated kinase (ERK)/NRF2 signaling pathway
404 and cytokine release. [Online]. *J Biol Chem* 287: 33720–33732, 2012.
405 http://www.hubmed.org/display.cgi?uids=22879597.

406 6. **Chahdi A, Sorokin A**. Endothelin-1 induces p66Shc activation through EGF receptor
407 transactivation: Role of beta(1)Pix/Galpha(i3) interaction. [Online]. *Cell Signal* 22: 325–329,
408 2010. http://www.hubmed.org/display.cgi?uids=19804820.

409 7. **Cignarelli A, Melchiorre M, Peschechera A, Conserva A, Renna LA, Miccoli S,**
410 **Natalicchio A, Perrini S, Laviola L, Giorgino F**. Role of UBC9 in the regulation of the
411 adipogenic program in 3T3-L1 adipocytes. *Endocrinology* 151: 5255–5266, 2010.

412 8. **Clavel S, Siffroi-Fernandez S, Coldefy AS, Boulukos K, Pisani DF, Derijard B**. Regulation
413 of the intracellular localization of Foxo3a by stress-activated protein kinase signaling
414 pathways in skeletal muscle cells. [Online]. *Mol Cell Biol* 30: 470–480, 2010.
415 http://www.hubmed.org/display.cgi?uids=19917721.

416 9. **Colmenero J, Bataller R, Sancho-Bru P, Bellot P, Miquel R, Moreno M, Jares P, Bosch J,**
417 **Arroyo V, Caballeria J, Gines P**. Hepatic expression of candidate genes in patients with
418 alcoholic hepatitis: correlation with disease severity. [Online]. *Gastroenterology* 132: 687–
419 697, 2007. http://www.hubmed.org/display.cgi?uids=17258719.

420 10. **Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi**
421 **L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, Pelicci PG**.
422 Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that
423 trigger mitochondrial apoptosis. [Online]. *Cell* 122: 221–233, 2005.
424 http://www.hubmed.org/display.cgi?uids=16051147.

11. **Guo J, Gertsberg Z, Ozgen N, Steinberg SF**. p66Shc links alpha1-adrenergic receptors to a reactive oxygen species-dependent AKT-FOXO3A phosphorylation pathway in cardiomyocytes. [Online]. *Circ Res* 104: 660–669, 2009. http://www.hubmed.org/display.cgi?uids=19168439.

12. **Haga S, Morita N, Irani K, Fujiyoshi M, Ogino T, Ozawa T, Ozaki M**. p66(Shc) has a pivotal function in impaired liver regeneration in aged mice by a redox-dependent mechanism. [Online]. *Lab Invest* 90: 1718–1726, 2010. http://www.hubmed.org/display.cgi?uids=20567235.

13. **Haga S, Terui K, Fukai M, Oikawa Y, Irani K, Furukawa H, Todo S, Ozaki M**. Preventing hypoxia/reoxygenation damage to hepatocytes by p66(shc) ablation: up-regulation of anti-oxidant and anti-apoptotic proteins. [Online]. *J Hepatol* 48: 422–432, 2008. http://www.hubmed.org/display.cgi?uids=18191273.

14. **He X, Kan H, Cai L, Ma Q**. Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. [Online]. *J Mol Cell Cardiol* 46: 47–58, 2009. http://www.hubmed.org/display.cgi?uids=19007787.

15. **Huang X-S, Chen H-P, Yu H-H, Yan Y-F, Liao Z-P, Huang Q-R**. Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for hypoxic preconditioning-mediated delayed cardioprotection. [Online]. *Mol Cell Biochem* 385: 33–41, 2014. http://www.hubmed.org/display.cgi?uids=24048861.

16. **Jackson JG, Yoneda T, Clark GM, Yee D**. Elevated levels of p66 Shc are found in breast cancer cell lines and primary tumors with high metastatic potential. [Online]. *Clin Cancer Res* 6: 1135–1139, 2000. http://www.hubmed.org/display.cgi?uids=10741744.

17. **Koch OR, Fusco S, Ranieri SC, Maulucci G, Palozza P, Larocca LM, Cravero AAM, Farre' SM, De Spirito M, Galeotti T, Pani G**. Role of the life span determinant P66(shcA) in ethanol-induced liver damage. [Online]. *Lab Invest* 88: 750–760, 2008. http://www.hubmed.org/display.cgi?uids=18490896.

18. **Laviola L, Orlando MR, Incalza MA, Caccioppoli C, Melchiorre M, Leonardini A, Cignarelli A, Tortosa F, Labarbuta R, Martemucci S, Pacelli C, Cocco T, Perrini S, Natalicchio A, Giorgino F**. TNFα signals via p66(Shc) to induce E-Selectin, promote leukocyte transmigration and enhance permeability in human endothelial cells. [Online]. *PLoS One* 8, 2013. http://www.hubmed.org/display.cgi?uids=24349153.

19. **Menini S, Amadio L, Oddi G, Ricci C, Pesce C, Pugliese F, Giorgio M, Migliaccio E, Pelicci P, Iacobini C, Pugliese G**. Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress. [Online]. *Diabetes* 55: 1642–1650, 2006. http://www.hubmed.org/display.cgi?uids=16731826.

20. **Michalak S, Rousselet M-C, Bedossa P, Pilette C, Chappard D, Oberti F, Gallois Y, Cales P**. Respective roles of porto-septal fibrosis and centrilobular fibrosis in alcoholic liver disease. [Online]. *J Pathol* 201: 55–62, 2003. http://www.hubmed.org/display.cgi?uids=12950017.

21. **Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG**. The p66shc adaptor protein controls oxidative stress response and life span in

mammals. [Online]. *Nature* 402: 309–313, 1999.
http://www.hubmed.org/display.cgi?uids=10580504.

22. **Morita M, Matsuzaki H, Yamamoto T, Fukami Y, Kikkawa U**. Epidermal growth factor receptor phosphorylates protein kinase C {delta} at Tyr332 to form a trimeric complex with p66Shc in the H2O2-stimulated cells. [Online]. *J Biochem* 143: 31–38, 2008.
http://www.hubmed.org/display.cgi?uids=17956904.

23. **Natalicchio A, De Stefano F, Perrini S, Laviola L, Cignarelli A, Caccioppoli C, Quagliara A, Melchiorre M, Leonardini A, Conserva A, Giorgino F**. Involvement of the p66Shc protein in glucose transport regulation in skeletal muscle myoblasts. *Am. J. Physiol. Endocrinol. Metab.* 296: E228–37, 2009.

24. **Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA**. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. [Online]. *Gastroenterology* 122: 366–375, 2002.
http://www.hubmed.org/display.cgi?uids=11832451.

25. **Pagnin E, Fadini G, de Toni R, Tiengo A, Calo L, Avogaro A**. Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: relationship to oxidative stress. [Online]. *J Clin Endocrinol Metab* 90: 1130–1136, 2005.
http://www.hubmed.org/display.cgi?uids=15562031.

26. **Polytarchou C, Pfau R, Hatziapostolou M, Tsichlis PN**. The JmjC domain histone demethylase Ndy1 regulates redox homeostasis and protects cells from oxidative stress. [Online]. *Mol Cell Biol* 28: 7451–7464, 2008.
http://www.hubmed.org/display.cgi?uids=18838535.

27. **Reid AE**. Nonalcoholic steatohepatitis. [Online]. *Gastroenterology* 121: 710–723, 2001.
http://www.hubmed.org/display.cgi?uids=11522755.

28. **Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME, Esposito G, Vitale S, Urbanek K, Casarsa C, Giorgio M, Luscher TF, Pelicci PG, Anversa P, Leri A, Kajstura J**. Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. [Online]. *Circ Res* 99: 42–52, 2006.
http://www.hubmed.org/display.cgi?uids=16763167.

29. **Sun L, Xiao L, Nie J, Liu F-Y, Ling G-H, Zhu X-J, Tang W-B, Chen W-C, Xia Y-C, Zhan M, Ma M-M, Peng Y-M, Liu H, Liu Y-H, Kanwar YS**. p66Shc mediates high-glucose and angiotensin II-induced oxidative stress renal tubular injury via mitochondrial-dependent apoptotic pathway. [Online]. *Am J Physiol Ren. Physiol* 299: 1014–1025, 2010.
http://www.hubmed.org/display.cgi?uids=20739391.

30. **Thannickal VJ, Fanburg BL**. Reactive oxygen species in cell signaling. [Online]. *Am J Physiol Lung Cell Mol Physiol* 279: 1005–1028, 2000.
http://www.hubmed.org/display.cgi?uids=11076791.

31. **Tomita K, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T, Okada Y, Kurihara C, Ebinuma H, Saito H, Shimizu I, Yoshida Y, Hokari R, Sugiyama K, Hatsuse K, Yamamoto J, Kanai T, Miura S, Hibi**

T. p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. [Online]. *J Hepatol* 57: 837–843, 2012. http://www.hubmed.org/display.cgi?uids=22641095.

32. **Trinei M, Giorgio M, Cicalese A, Barozzi S, Ventura A, Migliaccio E, Milia E, Padura IM, Raker VA, Maccarana M, Petronilli V, Minucci S, Bernardi P, Lanfrancone L, Pelicci PG**. A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. [Online]. *Oncogene* 21: 3872–3878, 2002. http://www.hubmed.org/display.cgi?uids=12032825.

33. **Veal EA, Day AM, Morgan BA**. Hydrogen peroxide sensing and signaling. [Online]. *Mol Cell* 26: 1–14, 2007. http://www.hubmed.org/display.cgi?uids=17434122.

34. **Veeramani S, Igawa T, Yuan T-C, Lin F-F, Lee M-S, Lin JS, Johansson SL, Lin M-F**. Expression of p66(Shc) protein correlates with proliferation of human prostate cancer cells. [Online]. *Oncogene* 24: 7203–7212, 2005. http://www.hubmed.org/display.cgi?uids=16170380.

35. **Wang Z, Dou X, Li S, Zhang X, Sun X, Zhou Z, Song Z**. Nuclear factor (erythroid-derived 2)-like 2 activation-induced hepatic very-low-density lipoprotein receptor overexpression in response to oxidative stress contributes to alcoholic liver disease in mice. [Online]. *Hepatology* 59: 1381–1392, 2014. http://www.hubmed.org/display.cgi?uids=24170703.

36. **Zhou S, Chen H-Z, Wan Y-Z, Zhang Q-J, Wei Y-S, Huang S, Liu J-J, Lu Y-B, Zhang Z-Q, Yang R-F, Zhang R, Cai H, Liu D-P, Liang C-C**. Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction. [Online]. *Circ Res* 109: 639–648, 2011. http://www.hubmed.org/display.cgi?uids=21778425.

Figure Legends

Figure 1. p66^{Shc} phosphorylation on Ser^{36} and ROS synthesis in HepG2 cells. (**A**) Dose-response of p66^{Shc} phosphorylation on Ser^{36} in wild-type HepG2 cells exposed to H_2O_2 for 15 min. Representative immunoblots of p66^{Shc} phosphorylation (*top*) and protein content (*bottom*) are shown. (**B**) Dose-response of p66^{Shc} phosphorylation on Ser^{36} in HepG2/p66^{Shc} and HepG2/mock cells exposed to H_2O_2 for 15 min. (**C**) Time-course of p66^{Shc} phosphorylation in HepG2/p66^{Shc} and HepG2/mock cells exposed to 0.5 mM H_2O_2. Representative immunoblots of p66^{Shc} phosphorylation on Ser^{36} (*top*) and Shc protein content (*bottom*; all three Shc protein isoforms are shown). The quantitation of phosphorylated p66^{Shc} on Ser^{36} in multiple experiments is also shown (HepG2/p66^{Shc} *black diamonds*; HepG2/mock, *black squares*). (**D**) Quantification of ROS levels in wild-type HepG2, HepG2/p66^{Shc} and HepG2/mock cells stimulated with H_2O_2 for 15 minutes (*black bars*) or left untreated (*white bars*). Results in A-D represent the mean ± SE of at least n = 5 independent experiments. *p<0.05 vs basal; #p<0.05 vs wild-type HepG2 and HepG2/mock. A.U., arbitrary units.

Figure 2. p66^{Shc} overexpression in HepG2 cells. HepG2 cells were transduced with different doses of Ad/p66^{Shc}, Ad/mock and Ad/p66^{Shc}Ala^{36} adenoviral constructs, expressing a green fluorescence protein, at 90% confluence, as described under Material and Methods. (**A**) Morphology of confluent wild-type HepG2, HepG2/p66^{Shc}, HepG2/mock and Ad/p66^{Shc}Ala^{36} cells incubated with the indicated doses of adenovirus under light microscopy (*top*) and corresponding fluorescent signal assessed under a fluorescent microscope (*bottom*). Magnification: X10. (**B**) Representative immunoblot of all three Shc isoforms in HepG2/p66^{Shc}, HepG2/mock and Ad/p66^{Shc}Ala^{36} cells incubated with the indicated doses of adenovirus or left untreated. (**C**) Quantitation of p66^{Shc} in HepG2/p66^{Shc} incubated with the indicated doses of adenovirus or left untreated. *p<0.05 vs control. A.U., arbitrary units.

Figure 3. Activation of Erk-1/2 and Akt/FoxO3a in HepG2/p66^{Shc} and HepG2/mock cells. HepG2/p66^{Shc} and HepG2/mock cells were incubated with 0.5 mM H_2O_2 for the indicated times or

left untreated. (**A**), (**B**), and (**C**) show respectively representative immunoblots of Erk-1/2, Akt, and FoxO3a total protein content and phosphorylation (*left*), and the quantitation of results from multiple experiments (*right*; HepG2/p66^{Shc}, *black diamonds*; HepG2/mock, *black squares*). Results represent the mean ± SE of at least n = 5 independent experiments. *p<0.05 vs basal; #p<0.05 vs HepG2/mock. A.U., arbitrary units.

Figure 4. FoxO3a localization in HepG2/mock and HepG2/p66^{Shc} cells. HepG2/mock (**A**) and HepG2/p66^{Shc} (**B**) cells were incubated with 0.5 mM H_2O_2 for 15 min or left untreated. The magnified images show FoxO3a localization in representative HepG2/mock and HepG2/p66^{Shc} cells. Adenovirus-infected cells are shown in green according to green fluorescence protein expression (*green*, top images). FoxO3a was visualized with a rabbit polyclonal antibody followed by the addition of ALEX488 (*red*) labeled anti-rabbit antisera. TOPRO (*blue*) was used to visualize the nuclei. In control HepG2/mock cells, endogenous FoxO3a could be detected almost exclusively in the cell nucleus in the absence of H_2O_2 (Panel A, white arrows in images on the left), whereas it was relocated predominantly in the cytoplasm after exiting the nucleus following H_2O_2 stimulation for 15 min (Panel A, white arrows in images on the right). In HepG2/p66^{Shc} cells, FoxO3a showed predominant cytoplasmic localization and reduced nuclear staining already in the absence of H_2O_2 (Panel B, white arrows in images on the left), and this was not significantly modified by exposure to H_2O_2 (Panel B, white arrows in images on the right). Images are representative of four independent experiments.

Figure 5. Effects of the MEK inhibitor on p66^{Shc} phosphorylation, Akt/FoxO3a signaling, and ROS production. Representative immunoblots (*left*) assessing total protein content and phosphorylation of Erk-1/2 (**A**), p66^{Shc} (**B**), Akt (**C**), and FoxO3a (**D**) in HepG2/p66^{Shc} cells. The quantitation of results from multiple experiments is also shown (*right*). HepG2/p66^{Shc} were pretreated with 20 μM U0126 for 2 h or left untreated before exposure to 0.5 mM H_2O_2 for 15 min. (**E**) Quantitation of ROS levels in HepG2/mock and HepG2/p66^{Shc} cells stimulated with 0.5 mM H_2O_2

for 15 minutes (*black bars*) or left untreated (*white bars*). Results represent the mean ± SE of at least n = 5 independent experiments. *p<0.05 vs basal; #p<0.05 vs H_2O_2-stimulated cells treated with U0126; §p<0.05 vs unstimulated HepG2/mock treated with U0126; †p<0.05 vs HepG2/mock. A.U., arbitrary units.

Figure 6. p66^{Shc} phosphorylation on Ser^{36} in HepG2 cells overexpressing the mutant p66^{Shc}Ala^{36} protein. (**A**) Representative immunoblots of Shc protein content (*top*) and p66^{Shc} phosphorylation on Ser^{36} (*bottom*). (B) Densitometric analysis of 5 independent experiments (*white bars*: untreated cells; *black bars*: H_2O_2-stimulated cells). Wild-type HepG2, HepG2/p66^{Shc}, HepG2/p66^{Shc}-Ala^{36} and HepG2/mock cells were incubated with 0.5 mM H_2O_2 for 15 min or left untreated. *p<0.05 vs basal; #p<0.05 vs wild-type HepG2, HepG2/mock and HepG2/p66^{Shc}-Ala^{36}. Data represent the quantitation of at least n = 5 independent experiments. A.U., arbitrary units.

Figure 7. Activation of Erk and Akt/FoxO3a pathways and ROS synthesis in HepG2 cells overexpressing the phosphorylation defective p66^{Shc} Ala^{36} mutant. HepG2/p66^{Shc} and HepG2/p66^{Shc}-Ala^{36} cells were incubated with 0.5 mM H_2O_2 for the indicated times or left untreated. Representative immunoblots (*left*) of Erk-1/2 (**A**), Akt (**B**), and FoxO3a (**C**) total protein content and phosphorylation, respectively. The quantitation of results from multiple experiments is also shown (*right*; HepG2/p66^{Shc}, *black diamonds*; HepG2/p66^{Shc}-Ala^{36}, *black squares*). Results represent the mean ± SE of at least n = 5 independent experiments. *p<0.05 vs basal; #p<0.05 vs HepG2/p66^{Shc}-Ala^{36}. A.U., arbitrary units. (**D**) Quantification of ROS levels in wild-type HepG2, HepG2/p66^{Shc}, HepG2/p66^{Shc}-Ala^{36} and HepG2/mock cells stimulated with 0.5 mM H_2O_2 for 15 minutes (*black bars*) or left untreated (*white bars*). Data represent the quantitation of at least n = 5 independent experiments. *p<0.05 vs basal; #p<0.05 vs wild-type HepG2, HepG2/mock and HepG2/p66^{Shc}-Ala^{36}.

Figure 8. mRNA expression levels of *Nrf2* and Nrf2-target genes in HepG2/mock, HepG2/p66^{Shc} and HepG2/p66^{Shc}-Ala^{36} cells. *Nrf2*, *CYP1A1*, *GSTM2*, *GSTA5*, *SOD2* mRNA expression levels

were measured by qRT-PCR in HepG2/mock (*grey squares*), HepG2/p66^{Shc} (*black squares*) and HepG2/p66^{Shc}-Ala^{36} (*white triangles*) following exposure to 0.5 mM H_2O_2 for the indicated times. Data represent the mean ± SE of at least n = 5 independent experiments. *p<0.05 HepG2/p66^{Shc} vs HepG2/mock; #p<0.05 HepG2/p66^{Shc} vs HepG2/p66^{Shc}-Ala^{36}; §p<0.05 HepG2/p66^{Shc}-Ala^{36} vs HepG2/mock.

Figure 9. 8-oxodG accumulation following p66^{Shc} overexpression in HepG2 cells. (**A**) HepG2/mock, (**B**) HepG2/p66^{Shc} and (**C**) HepG2/p66^{Shc}-Ala^{36} cells were treated with 0.5 mM H_2O_2 or left untreated and then analyzed 30 min later by immunofluorescence, evaluating accumulation of 8-oxodG, a sensitive marker of DNA damage. For each condition, from left to right, the first column shows DAPI nuclei staining, the second column shows the green fluorescence protein in infected cells, the third column shows 8-oxodG staining, and the fourth column displays the merged staining (A-C). Bar, 50 μm. Results are representative of n = 4 independent experiments.

Figure 10. Protein levels of p66^{Shc} in liver from ASH subjects. (**A**) Protein levels of Shc isoforms in human liver biopsies. Representative immunoblots of Shc protein isoforms in liver biopsies from individual subjects with various degrees of steatosis, fibrosis and lymphocytic/polymorphonuclear infiltration are shown (*top*). GAPDH protein content was used as loading control. The quantitation of p66^{Shc} vs p52-p46 ^{Shc} ratio in liver samples considered individually (*bottom left*) and grouped as G0-1/F0 (steatosis <33% and absence of fibrosis and lymphocytic/polymorphonuclear infiltration) and as G2-3/F1-4 (steatosis >33% and presence of fibrosis and lymphocytic/polymorphonuclear infiltration) (*bottom right*) is also shown. (**B**) Akt in human liver biopsies. Representative immunoblots of Akt phosphorylation and total protein content in liver biopsies from individual subjects with various degrees of steatosis, fibrosis and lymphocytic/polymorphonuclear infiltration are shown (*top*). The quantitation of phospho-Akt vs total Akt liver biopsies considered individually (*bottom left*) and

624 grouped as G0-1/F0 and G2-3/F1-4 (*bottom right*) is also shown. *p<0.05 vs G0-1/F0. A.U., arbitrary
625 units.

626 **Figure 11. Hypothetical model of p66^{Shc}-dependent redox signaling in human liver cells.**