Oncogene (2016) **35,** 4200–4211

www.nature.com/onc

ORIGINAL ARTICLE

Suppressor of cytokine signaling 1-dependent regulation of the expression and oncogenic functions of $p21^{CIP1/WAF1}$ in the liver

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The *SOCS1* gene coding for suppressor of cytokine signaling 1 is frequently repressed in hepatocellular carcinoma (HCC), and hence SOCS1 is considered a tumor suppressor in the liver. However, the tumor-suppressor mechanisms of SOCS1 are not yet well understood. SOCS1 is known to inhibit pro-inflammatory cytokine production and signaling and to promote activation of the p53 tumor suppressor. However, we observed that SOCS1-deficient mice developed numerous and large liver tumor nodules following treatment with the hepatocarcinogen diethylnitrosamine (DEN) without showing increased interleukin-6 production or activation of p53. On the other hand, the livers of DEN-treated *Socs1*-null mice showed elevated levels of $p21^{CIP1/WAF1}$ protein (p21). Even though p21 generally functions as a tumor suppressor, paradoxically many cancers, including HCC, are known to express elevated levels of p21 that correlate with poor prognosis. We observed elevated p21 expression also in the regenerating livers of SOCS1-deficient mice and in cisplatin-treated *Socs1*-null hepatocytes, wherein the p21 protein showed increased stability. We show that SOCS1 interacts with p21 and promotes its ubiquitination and proteasomal degradation. Besides, the DEN-treated livers of *Socs1*-null mice showed increased nuclear and cytosolic p21 staining, and the latter was associated with growth factor-induced, phosphatidylinositol 3-kinase-dependent phosphorylation of p21 in SOCS1-deficient hepatocytes. Cytosolic p21 is often associated with malignancy and chemo-resistance in many cancers. Accordingly, SOCS1-deficient hepatocytes showed increased resistance to apoptosis that was reversed by shRNA-mediated p21 knockdown. In the regenerating livers of *Socs1*-null mice, increased p21 expression coincided with elevated cyclinD levels. Correspondingly, SOCS1-deficient hepatocytes showed increased proliferation to growth factor stimulation that was reversed by p21 knockdown. Overall, our findings indicate that the tumor-suppressor functions of SOCS1 in the liver could be mediated, at least partly, via regulation of the expression, stability and subcellular distribution of p21 and its paradoxical oncogenic functions, namely, resistance to apoptosis and increased proliferation.

Oncogene (2016) **35,** 4200–4211; doi:10.1038/onc.2015.485; published online 4 January 2016

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common and the third most lethal cancer.[1] The high mortality associated with HCC results from insidious onset, delayed diagnosis, lack of efficient therapy and rapid disease progression. Development of efficient therapies for HCC requires a better understanding of its molecular pathogenic mechanisms.[2] HCC evolves as a long-term consequence of chronic inflammation caused by persistent liver injury that leads to compensatory hepatocyte proliferation and formation of dysplastic nodules.[3] Within these nodules, proliferating hepatocytes accumulate genetic lesions that could promote neoplastic transformation.

Analysis of aberrant DNA patterns in human HCC revealed frequent epigenetic repression of the gene coding for suppressor of cytokine signaling 1 (SOCS1).[4] Subsequent studies showed increased susceptibility of $Socs1^{+/-}$ mice to the hepatocarcinogen diethylnitrosamine (DEN).[5] Although these studies strongly suggested a tumor-suppressor role for SOCS1 in the liver, the underlying molecular mechanisms are not yet clear.

Members of the SOCS family regulate cytokine and growth factor signaling pathways.[6,7] SOCS1 is an indispensable regulator of interferon-γ (IFNγ) signaling. *Socs1*-null mice die within 3 weeks of birth that can be prevented by ablating the *Ifng* gene.[8] SOCS1 also attenuates tumor necrosis factor-α (TNFα) and interleukin (IL)-6 signaling, which are critical for hepatocyte priming during liver regeneration and hepatocarcinogenesis.[9,10] Similarly, SOCS1 controls hepatocyte growth factor signaling, which is essential for liver regeneration and is implicated in HCC progression.[11] SOCS1 also promotes p53-dependent cellular senescence, an important tumor-suppression mechanism.[12]

To investigate whether SOCS1 deficiency increases susceptibility to HCC via promoting inflammatory cytokine signaling and by compromising p53-mediated tumor suppression, we studied DEN-induced HCC and associated molecular events in SOCS1-deficient mice and hepatocytes. Our results show that SOCS1 deficiency does not affect IL-6 signaling or compromise p53-dependent gene expression in the liver but deregulates the expression of the cyclin-dependent kinase (CDK) inhibitor 1A

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Received 25 May 2015; revised 2 November 2015; accepted 17 November 2015; published online 4 January 2016

(CDKN1A; p21$^{CIP1/WAF1}$; p21). Although p21 generally functions as a tumor suppressor, paradoxically it can also promote oncogenesis in many cancers, including HCC.[13–17] Our findings show that SOCS1 is an important regulator of p21 expression and its potential oncogenic functions.

RESULTS

Loss of SOCS1 promotes DEN-induced HCC independently of IFNγ

SOCS1 is an indispensable regulator of IFNγ signaling, which is known to exert pro-oncogenic role in DEN-induced HCC.[8,18] In order to determine whether the increased susceptibility of *Socs1*-haploinsufficient mice to experimental HCC[5] arises from increased IFNγ signaling, we evaluated DEN-induced HCC in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice. *Ifng*$^{-/-}$ and C57Bl/6 mice served as controls. Compared with C57Bl/6 controls, *Ifng*$^{-/-}$ mice developed fewer hepatic tumor nodules (Figure 1a), confirming the requirement of IFNγ in DEN-induced HCC. However, SOCS1-deficient mice developed numerous and large liver nodules even in the absence of IFNγ (Figure 1a), indicating that SOCS1 may also suppress IFNγ-independent hepatocarcinogenesis pathways. Hepatocyte-specific SOCS1-deficient (*Socs1*$^{fl/fl}$*Alb*^{Cre}) mice with intact *Ifng* gene also showed increased susceptibility to DEN-induced HCC (Figure 1b).

DEN-treated SOCS1-deficient mice showed abundant proliferating cell nuclear antigen-positive cells in the liver (Figure 1c),

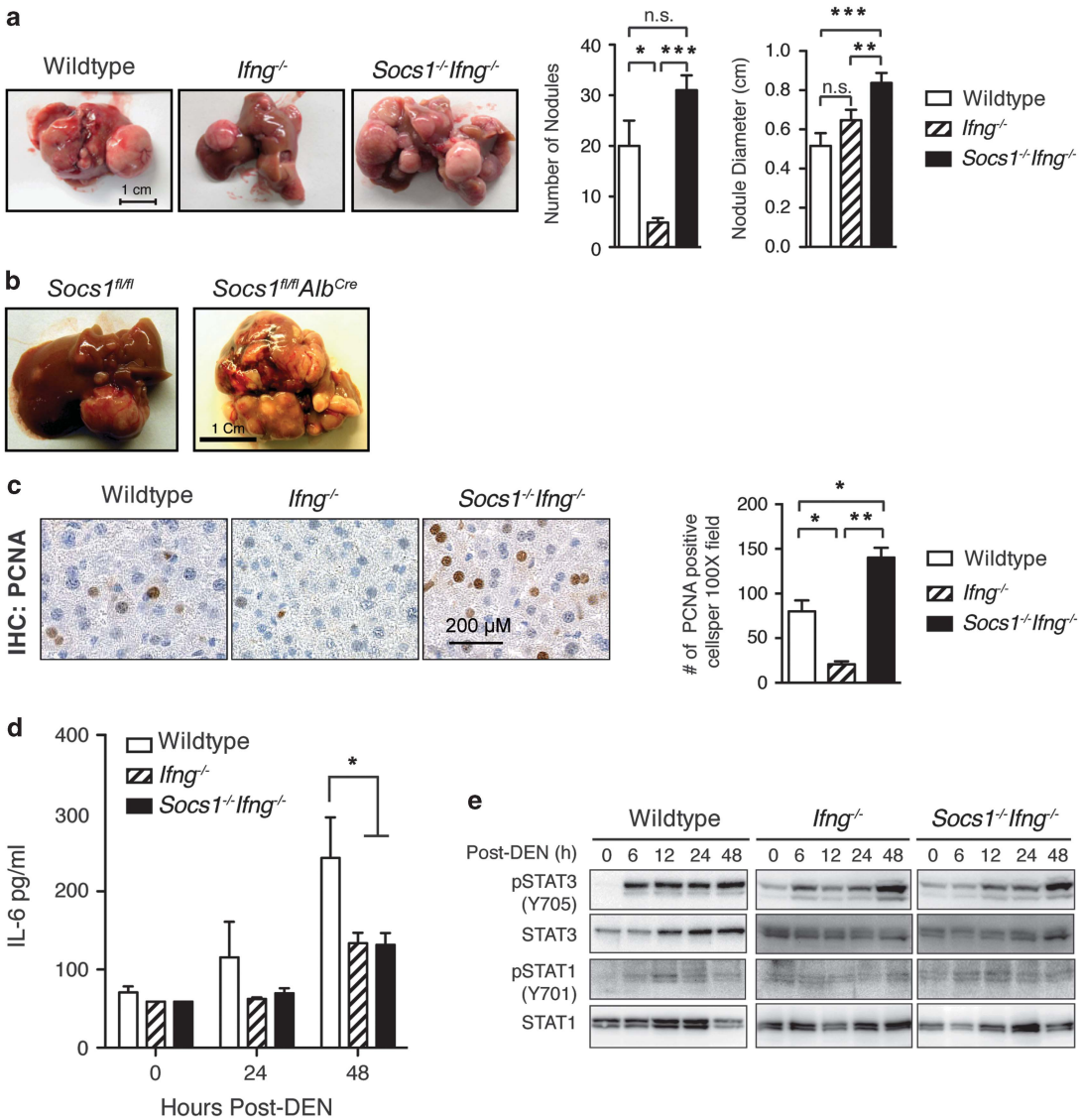

Figure 1. SOCS1 deficiency enhances DEN-induced HCC independently of IFNγ and without augmenting IL-6 production or signaling. (**a**) Two-week-old male mice were injected with DEN (25 mg/Kg; i.p.). Hepatic tumor nodules were evaluated 10 months later. Left: Representative images. Right: The number and diameter of tumor nodules. Data pooled from 7 to 8 mice/group from three experiments. (**b**) Two-week-old male hepatocyte-specific SOCS1 knockout mice were administered DEN and HCC development was evaluated 10 months later. Representative data from two mice for each group are shown. (**c**) Six-to-eight-week-old male mice were injected with high-dose DEN (100 mg/Kg; i.p.). Hepatocyte proliferation was evaluated 48 h later by immunohistochemistry for proliferating cell nuclear antigen (PCNA). Left: Representative PCNA staining. Right: Quantification from five mice/group from two experiments. (**d**) Serum IL-6 levels in high-dose DEN-treated mice ($n=6$ mice/group from two independent experiments). (**e**) Phosphorylation of STAT3 and STAT1 in the livers of high-dose DEN-treated mice. Data represent two independent experiments. Mean ± s.e.m. (**a**) Mann–Whitney, (**c**, **d**) analysis of variance with Tukey's test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; NS, $P>0.05$.

reflecting increased hepatocyte proliferation. IL-6 is essential for priming hepatocyte proliferation during liver regeneration and is also critical for experimental hepatocarcinogenesis induced by DEN or high-fat diet.[10,19] *Ifng*$^{-/-}$ mice, which developed fewer liver tumor nodules, showed reduced serum IL-6 levels than wild-type mice after DEN treatment (Figure 1d), suggesting that IFNγ acts upstream of IL-6 in DEN-induced HCC. However, *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice, which developed more numerous and larger tumor nodules, also showed reduced circulating IL-6 following DEN treatment, suggesting that SOCS1 deficiency may circumvent the need for IFNγ and IL-6 to induce HCC. In agreement, phosphorylation of STAT3 in the liver, mainly induced by IL-6 signaling in experimental HCC models,[10,19] was not significantly altered in *Ifng*$^{-/-}$ or *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice compared with wild-type controls (Figure 1e). Likewise, STAT1 phosphorylation (Y701), implicated in DEN-induced HCC in *Socs1*$^{+/-}$ mice,[5] was comparable between *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice (Figure 1e). These data indicated that the increased susceptibility of SOCS1-deficient mice to HCC is unlikely to result from deregulated IL-6 production or signaling.

Several other pro-inflammatory cytokines such as IL-1β, IL-12, TNFα and transforming growth factor-β (TGFβ) are also reported to be elevated in human HCC, which are invariably associated with hepatitis virus infections.[20] We examined how SOCS1 deficiency modulates inflammatory cytokine gene expression in the livers of DEN-treated mice. We observed significantly elevated *Il6* and *Tgfb* expression in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice, which also showed slightly increased *Tnfa* and *Il1b* expression compared with wild-type but not *Ifng*$^{-/-}$ mice (Supplementary Figure S1). Intriguingly, despite elevated *Il6* mRNA levels, circulating IL-6 levels were not elevated in SOCS1-deficient mice (Figure 1d), suggesting that SOCS1 may control hepatic IL-6 expression at the posttranscriptional level and/or its secretion.

SOCS1 deficiency increases the expression of p21$^{CIP1/WAF1}$ in hepatocytes

Previously, we have shown that SOCS1 promotes phosphorylation and transcriptional activation of p53 during oncogene-induced senescence.[12] This raised the possibility that increased DEN-induced HCC in SOCS1-deficient mice might result from impaired p53 activation. To examine whether SOCS1 promoted p53 activation in hepatocytes, we treated HepG2 human hepatoma cells stably expressing SOCS1 (HepG2-SOCS1) or control vector (HepG2-vector) with cisplatin and evaluated p53 phosphorylation. Phospho-p53 was detectable in control cells within 3 h after cisplatin withdrawal and lasted for up to 18 h, accompanied by increased p53 expression (Figure 2a). Stable SOCS1 expression only marginally augmented p53 phosphorylation. In HepG2-vector cells, cisplatin elicited rapid and persistent induction of p21 protein (Figure 2a), an important mediator of p53 functions. Surprisingly, p21 induction in HepG2-SOCS1 cells was initially normal but profoundly diminished by 18 h after cisplatin treatment (Figure 2a; right panel), at which time point the expression of *CDKN1A* gene coding for p21 was also diminished (Figure 2b).

Next we evaluated cisplatin-induced p53 phosphorylation, p21 expression and transcription of candidate p53 target genes in hepatocytes from *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice. Cisplatin strongly induced *Socs1* gene expression in murine hepatocytes (Supplementary Figure S2). Cisplatin-treated *Socs1*$^{-/-}$*Ifng*$^{-/-}$ hepatocytes showed phosphorylation and stabilization of p53 for up to 18 h, similarly to control hepatocytes (Figure 2c, left panels). However, SOCS1-deficient hepatocytes showed increased p21 protein levels, consistent with its decreased expression in HepG2-SOCS1 cells (Figure 2a). Similar results were obtained with hepatocyte-specific *Socs1*-null mice (Figure 2c, right panels), indicating that elevated p21 expression in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ hepatocytes was not related to IFNγ deficiency. Increased p21 expression without substantial difference in p53 phosphorylation also occurred in irradiated SOCS1-deficient hepatocytes (Supplementary Figure S3). Following cisplatin treatment, *Socs1*-null cells showed increased levels of *Cdkn1a* mRNA while other p53 target genes remained unchanged (Figure 2d). The increased *Cdkn1a* mRNA expression in SOCS1-deficient hepatocytes was dependent on p53, as it was abrogated following siRNA-mediated silencing of p53 (Supplementary Figure S4).

Next we treated SOCS1-deficient mice with high-dose DEN and evaluated p21 expression *in vivo*. The livers of both *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Socs1*$^{fl/fl}$*:Alb*^{Cre} mice showed increased levels of p21 protein that was more pronounced at 48 h post-DEN compared with control mice (Figures 3a and b). Even though p53 phosphorylation was not altered in the SOCS1-deficient livers at 24 h post-DEN, a discernible increase was evident at 48 h (Figures 3a and b). The latter finding stands in contrast to the ability of SOCS1 in enhancing p53 phosphorylation in a cellular senescence model.[12] The livers of DEN-treated SOCS1-deficient mice also showed increased *Cdkn1a* mRNA levels, whereas the expression of other p53 target genes was not significantly altered (Figures 3c and d). These observations were confirmed using a PCR array containing a large number of p53 target genes (Supplementary Figure S5), which also showed upregulation of *Tfnrsf10b* (TNF receptor superfamily member 10b) but not other p53 target genes in the SOCS1-deficient livers. Collectively, the above findings indicate that SOCS1 is dispensable for p53 activation in hepatocytes and that SOCS1 deficiency increases *Cdkn1a* expression without significantly affecting most other p53 target genes.

Loss of SOCS1 promotes p21 protein stability

Although SOCS1-deficient primary hepatocytes showed elevated steady-state levels of p21 protein (Figure 2c, Supplementary Figure S3), this was not the case in the steady-state livers of *Socs1*-null mice (Figures 3a and b). This suggested that elevated p21 expression in SOCS1-deficient hepatocytes might result from the loss of contact with extracellular matrix and exit from quiescence. To investigate this, we cultured primary hepatocytes in serum-deprived medium to induce G0 arrest, when p21 expression is low.[21] We observed that *Socs1*-null hepatocytes showed increased p21 expression upon starvation, which was reversed upon restoration of SOCS1 (Figures 4a and b). SOCS1 also reversed radiation-induced p21 upregulation in *Socs1*-null hepatocytes (Figure 4b). To determine whether SOCS1 affected the stability of p21 protein, we irradiated SOCS1-deficient and control hepatocytes to induce p21 and inhibited protein synthesis 24 h later with cycloheximide (CHX). Evaluation of p21 at different time points revealed that SOCS1 deficiency prolonged the half-life of p21 by more than two-fold (23 ± 7 versus 53 ± 7.5 min; Figure 4c). These findings indicated that SOCS1 regulated p21 protein stability.

SOCS1 is known to function as a substrate adaptor for the CRL5^{SOCS1} E3 Ub-ligase, comprised of elongin BC, cullin5 and RBX1.[22] To address whether SOCS1 targeted p21 for ubiquitination and proteasomal degradation, we transfected COS-7 cells with p21 along with different SOCS1 constructs (Supplementary Figure S6). SOCS1 caused a dose-dependent reduction in p21 (Supplementary Figure S7). Despite such reduced expression in the presence of SOCS1, p21 co-immunoprecipitated SOCS1 (Figure 5a), indicating an interaction between SOCS1 and p21. Using the Hepa1-6 mouse hepatoma cells stably expressing SOCS1, which also showed reduced expression of endogenous p21, we observed interaction between these two proteins (Figure 5b). We confirmed SOCS1–p21 interaction by glutathione *S*-transferase (GST) pull-down assay using human and mouse p21 (Figure 5c; Supplementary Figure S8). Mutant SOCS1-GST constructs revealed that the loss of N-terminal or C-terminal segments or the whole SH2 domain of SOCS1 profoundly decreased its binding to p21 (Figure 5c).

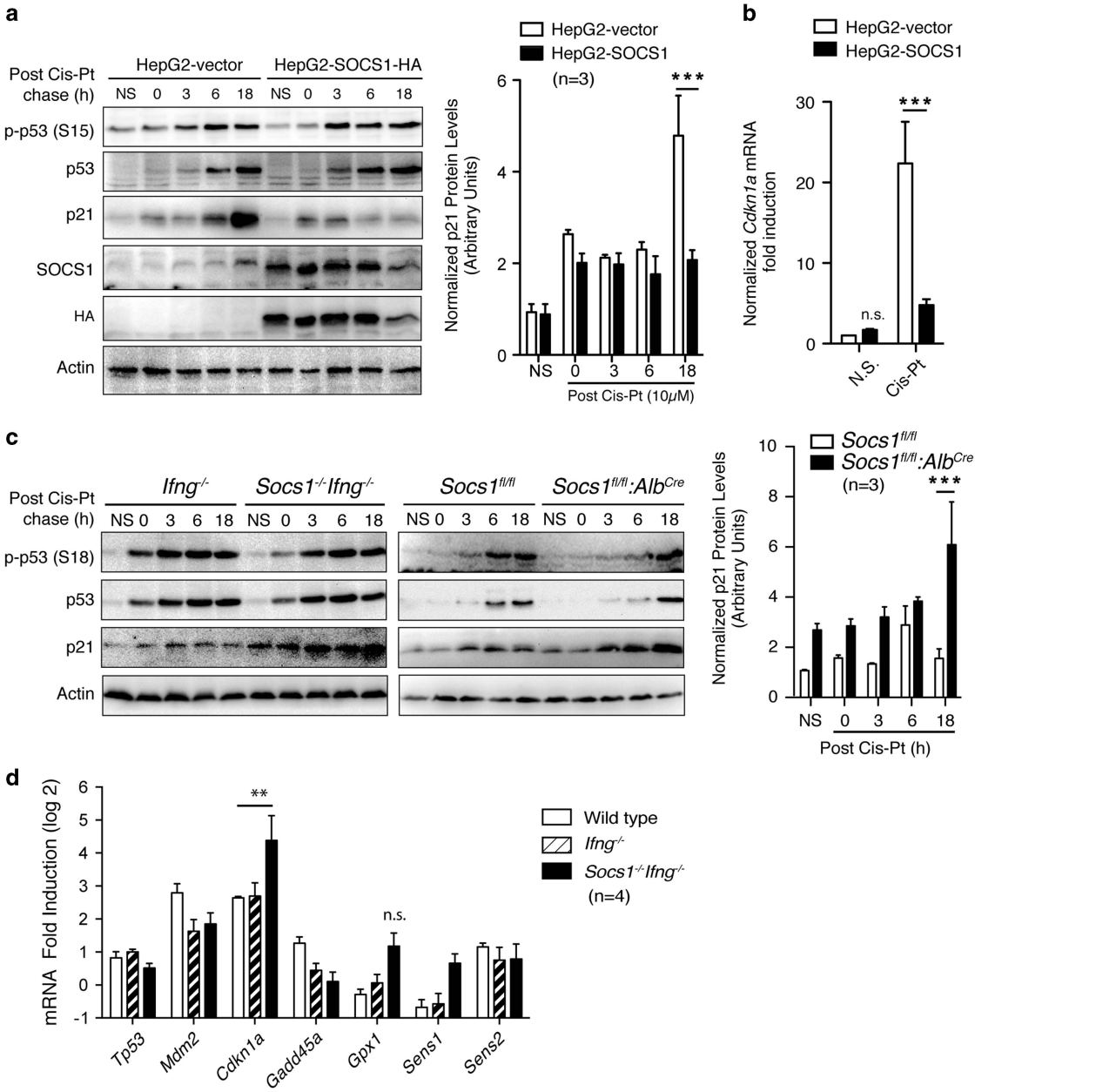

Figure 2. SOCS1 negatively regulates p21 expression in hepatocytes exposed to genotoxic stress. (**a**) HepG2-SOCS1 and HepG2-vector control cells were treated with cisplatin (Cis-Pt; 10 μM) for 3 h and then replenished with fresh medium. At the indicated time points, cells were lysed and analyzed for phosphorylation and induction of p53, and p21. Left panel: Representative data from three similar experiments. Right panel: normalized intensity of p21 bands relative to actin. (**b**) Quantitative reverse transcriptase–PCR evaluation of *CDKN1A* gene transcript 18 h after Cis-Pt treatment from three independent experiments. (**c**, **d**) Primary hepatocytes from *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Socs1*$^{fl/fl}$*Alb*^{Cre} mice and corresponding control mice were exposed to Cis-Pt for 3 h, washed and cultured for up to 18 h. At the indicated time points, phosphorylation of p53 and protein expression were analyzed. (**c**) Representative data for three similar experiments is shown. (**d**) Induction of p53 target genes was evaluated 18 h after cisplatin treatment (10 μM), and the fold-induction was calculated based on the basal expression levels in the corresponding genotype. Cumulative data from there independent experiments are shown. All graphs represent mean ± s.e.m. Analysis of variance with Bonferroni test: $^{**}P < 0.01$; $^{***}P < 0.001$; NS, $P > 0.05$.

The reduced expression of p21 in the presence of SOCS1 (Figure 5a, Supplementary Figure S7) raised the possibility that SOCS1 might destabilize p21 via promoting its ubiquitination. To test this idea, COS-7 cells were transfected with p21, HA-tagged Ub and wild-type SOCS1 or the ΔCT-SOCS1 lacking the SOCS-box, which is critical for assembling the CRL5^{SOCS1} Ub-ligase.[22] In this assay, wild-type SOCS1 but not ΔCT-SOCS1 decreased p21 expression (Figure 5d, left panels). The proteasome inhibitor MG-132 stabilized polyubiquitinated p21 in cells expressing wild-type SOCS1 (Figure 5d, right panels). The Ub moiety contains several internal Lys residues that are differentially utilized to generate distinct Ub chains, which influence the fate of target proteins.[23] Although K63-mediated poly-ubiquitination promotes endocytosis of cell surface receptors, proteins modified by K48-linked Ub chains are degraded by proteasomes. Using HA-Ub constructs expressing only K48 or K63, or none (K0) of the internal

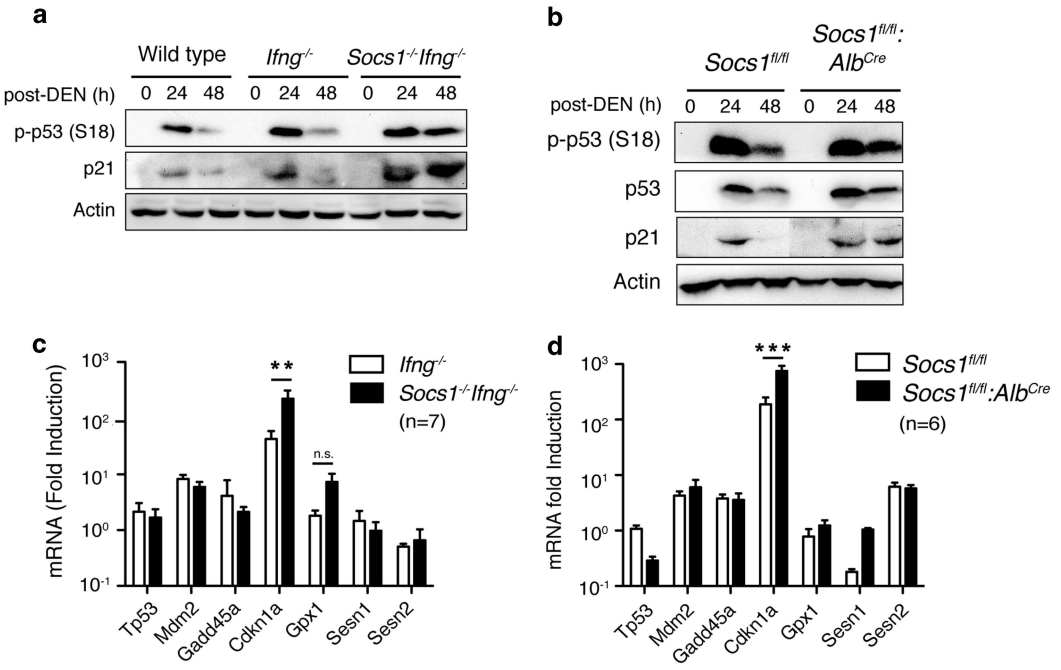

Figure 3. The livers of SOCS1-deficient mice show increased expression of p21 following DEN treatment. (**a**, **b**) *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Socs1*$^{fl/fl}$*Alb*^{Cre} mice and corresponding control groups were treated with high-dose DEN (100 mg/Kg). At the indicated time points, liver tissues were collected and analyzed by western blotting. Data represent three independent experiments. (**c**, **d**) Induction of p53 target genes at 24 h post-DEN in the liver. Cumulative data from more than two experiments are shown. Mean ± s.e.m. Analysis of variance with Bonferroni test: $^{**}P < 0.01$; $^{***}P < 0.001$; NS, $P > 0.05$.

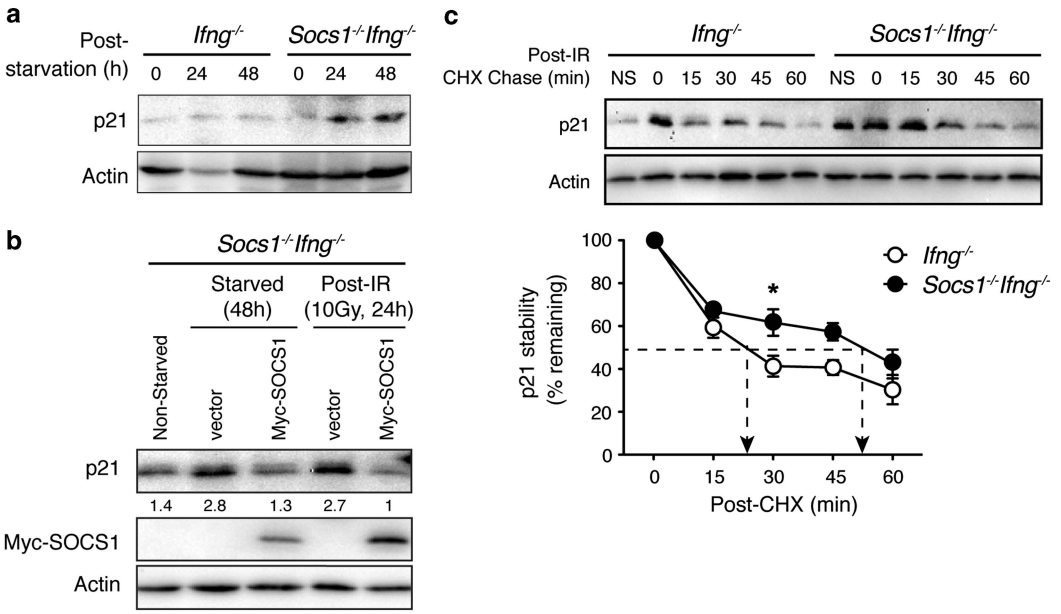

Figure 4. SOCS1 reduces the stability of p21 protein. (**a**) Primary hepatocytes from *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice, plated at 90% confluence, were serum-starved (0.25% FBS) and examined for p21 protein expression 24 and 48 h later. Data represent two independent experiments. (**b**) *Socs1*$^{-/-}$*Ifng*$^{-/-}$ hepatocytes, transfected with SOCS1 or control vector, were either starved for 48 h or exposed to gamma radiation (10 Gy) and cultured for 24 h and evaluated for p21 protein expression. The numbers represent p21 expression normalized to actin and relative to the lowest expression in irradiated SOCS1-expressing cells (last lane). Representative data from two experiments are shown. (**c**) Irradiated (10 Gy) primary hepatocytes were treated 24 h later with CHX (100 μM) and p21 protein levels were evaluated at the indicated times points. Representative data from three experiments are shown. Normalized p21 levels from three experiments were plotted to determine protein stability (mean ± s.e.m.). Analysis of variance with Bonferroni test: $^{*}P < 0.05$.

Lys residues, we observed that SOCS1 promoted K48-dependent poly-ubiquitination of p21 (Figure 5e). These findings show that SOCS1 interacts with p21 and promotes its polyubiquitination and subsequent proteasomal degradation.

Loss of SOCS1 enhances phosphatidylinositol 3-kinase (PI3K)-dependent cytosolic localization of p21

Although p21 generally functions as a tumor suppressor by inhibiting cell cycle progression, several cancers show increased

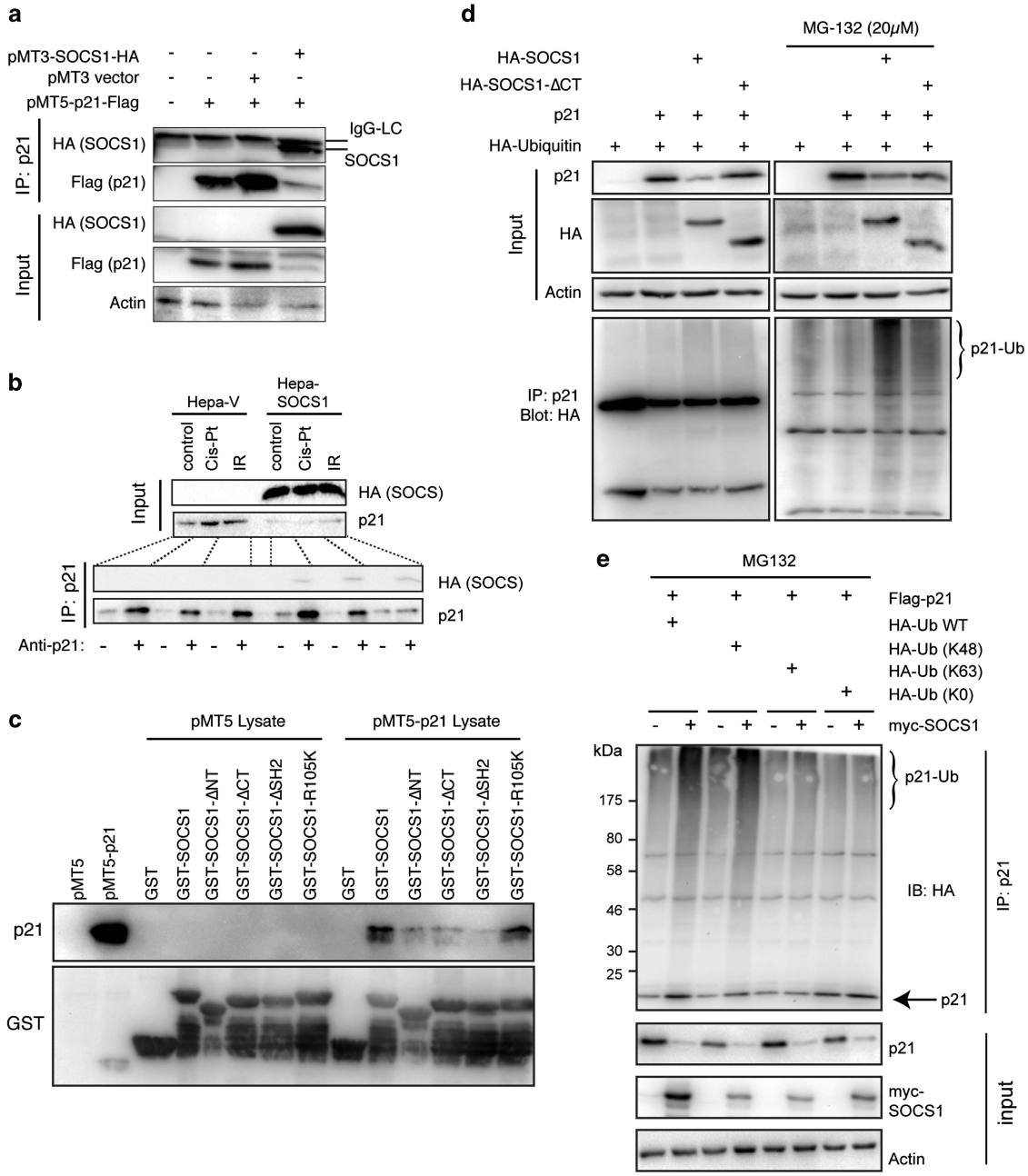

Figure 5. SOCS1 interacts with p21 and promotes its ubiquitination and proteasomal degradation. (**a**) COS-7 cells were co-transfected with Flag-tagged p21 and HA-tagged SOCS1. p21 was immunoprecipitated (IP) and blotted for HA. Data represent two independent experiments. (**b**) Hepa1-6 murine hepatoma cells stably expressing SOCS1 (Hepa-SOCS1) or control vector (Hepa-V) were exposed to Cis-Pt or subjected to irradiation, lysed and p21 immunoprecipitates were evaluated for SOCS1. (**c**) Equal amounts of wild-type or the indicated mutant (Supplementary Figure S6) SOCS1-GST fusion proteins bound to glutathione-Sepharose beads were incubated with lysates of COS-7 cells transfected with Flag-p21 or empty vector. Data represent two independent experiments. (**d**) Wild-type or SOCS-Box-deleted (ΔCT) SOCS1 were co-transfected with HA-tagged ubiquitin (Ub) and Flag-p21 into COS-7 cells. After 24 h, cells were treated or not with MG-132 (20 μM) for 4 h before cell lysis. p21 was immunoprecipitated and probed with an anti-HA antibody to detect polyubiquitination of p21. Data represent three separate experiments. (**e**) COS-7 cells were co-transfected with Ub constructs expressing wild-type Ub, K48-Ub, K63-Ub or K0-Ub. After 24 h, p21 was immunoprecipitated and its ubiquitination was examined by anti-HA blot. Data represent two independent experiments.

p21 expression that correlates with malignancy, poor prognosis and drug resistance.[14–16] Genetic studies have also shown pro-oncogenic potential of p21.[24] The paradoxical oncogenic function of p21 is associated with phosphorylation of Thr145 and Ser146 (Thr140, Ser141 in mouse p21) by AKT downstream of growth factor receptors, leading to its cytoplasmic accumulation and increased stability.[16,25] To examine the sub-cellular localization of p21 in SOCS1-deficient hepatocytes, we evaluated hepatic p21 expression in DEN-treated mice. Immunohistochemical staining showed increased p21 expression in the SOCS1-deficient livers that was detectable in both nucleus and cytoplasm (Figure 6a). Immunoblot analysis of nuclear and cytoplasmic fractions confirmed the increased cytoplasmic distribution of p21 in the SOCS1-deficient livers (Figure 6b). Moreover, SOCS1-deficient hepatocytes showed increased cytosolic p21 at steady state and after stimulation with TGFα (Figure 6c, lanes 1 and 3 versus 5 and 7),

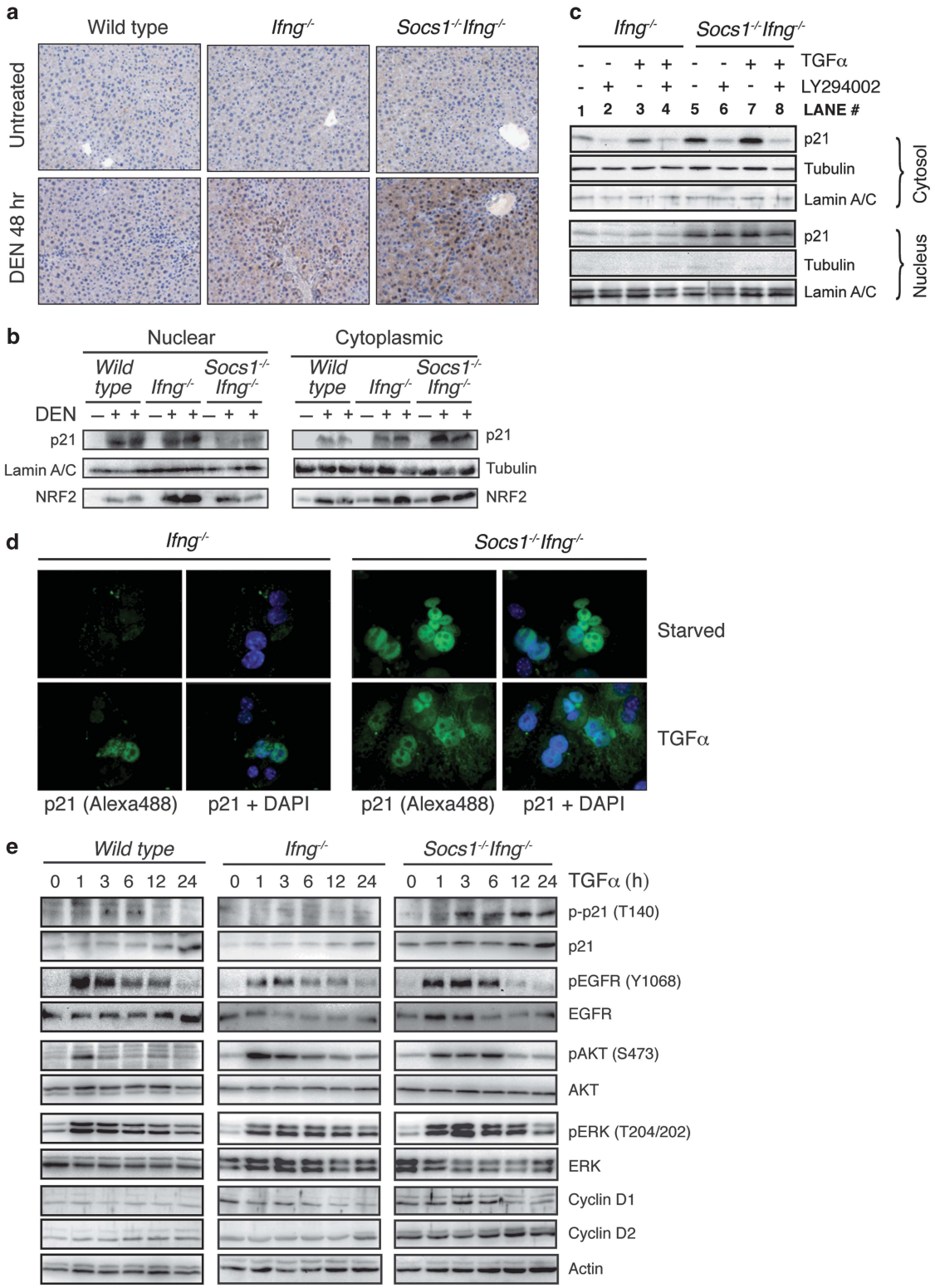

Figure 6. Loss of SOCS1 induces cytoplasmic p21 accumulation in hepatocytes. (**a**) Immunohistochemical staining of p21 in the livers of wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice 48 h after high-dose DEN treatment. Representative data from five mice per group from two experiments are shown. (**b**) Nuclear and cytosolic fractions of the liver tissues from control and DEN-treated wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice were evaluated for the distribution of p21, NRF2 and the indicated subcellular markers by western blotting. (**c**) *Socs1*-null and control primary hepatocytes were stimulated with TGFα (10 ng/ml, 12 h) in the presence or absence of LY249002 (30 μM). Nuclear and cytoplasmic fractions were evaluated for p21. LaminA/C and tubulin served as respective subcellular markers. Data represent two similar experiments. (**d**) *Socs1*-null and control primary hepatocytes treated with TGFα (10 ng/ml, 12 h) were fixed and stained for p21 and examined by immunofluorescence microscopy. Data represent two independent experiments. (**e**) Primary hepatocytes from *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice were starved overnight in serum-free medium, treated with TGFα (10ng/ml) and evaluated for the indicated proteins. Data represent two independent experiments.

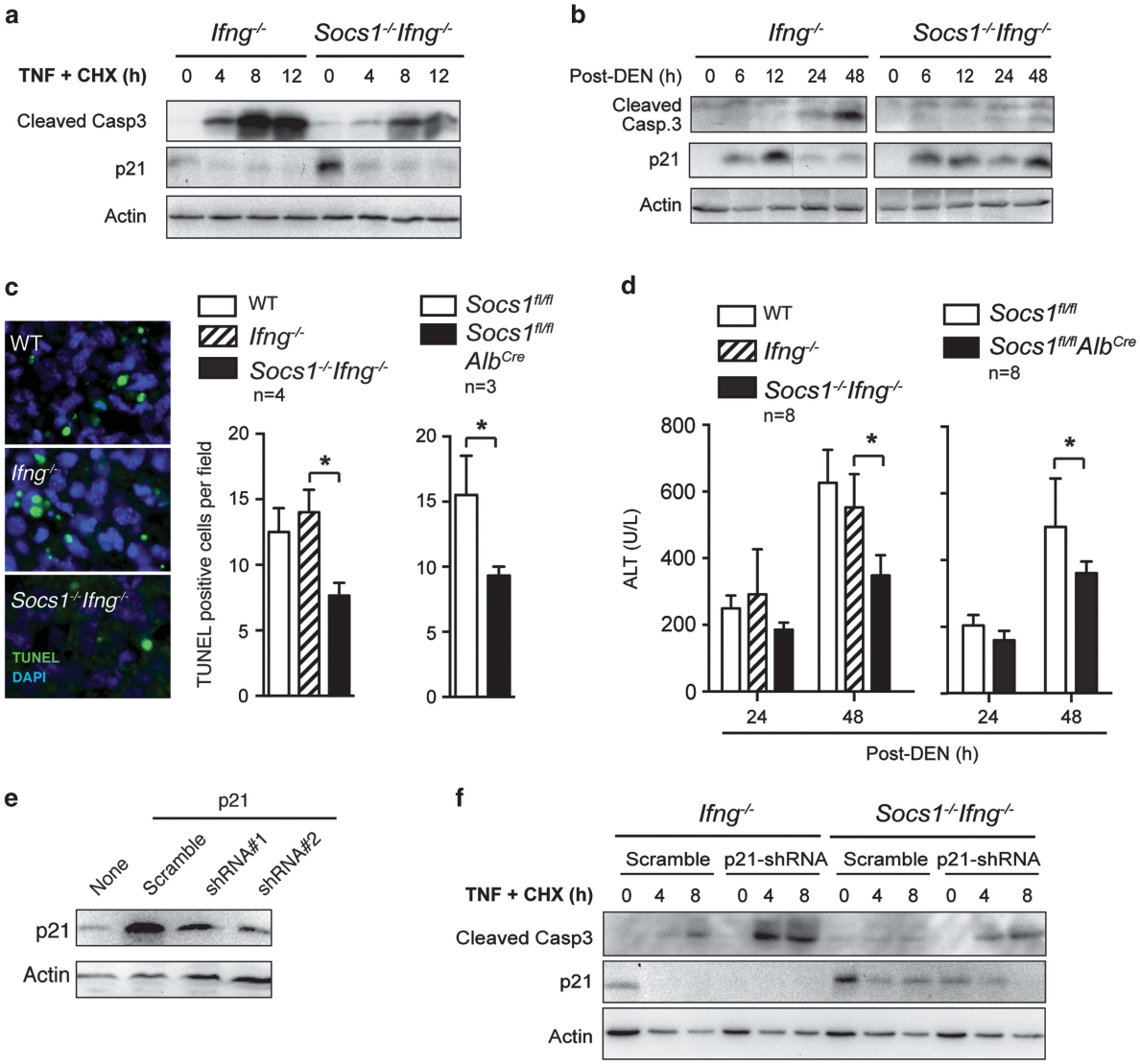

Figure 7. Increased p21 expression in SOCS1-deficient hepatocytes confers resistance to apoptosis. (**a**) Primary hepatocytes from *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice were starved in 0.25% serum for 24 h and treated with TNFα (10 ng/ml) and CHX (100 μM). At the indicated time points, cells were lysed and evaluated for cleaved caspase-3 and p21. Data represent three independent experiments. (**b**) *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and control mice were treated with high-dose DEN and killed at the indicated time points, and p21 and cleaved caspase-3 levels were evaluated in liver lysates. Data represent two independent experiments. (**c**) *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Socs1*$^{fl/fl}$*Alb*^{Cre} mice and corresponding control groups were treated with high-dose DEN. TUNEL-positive hepatocytes in the liver were evaluated at 48 h post-DEN. Left: Representative images from *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and control mice. Right: Quantification of TUNEL-positive cells in five random fields. (**d**) Serum alanine transferase (ALT) levels from eight mice per group from three different experiments. Mean ± s.e.m. Analysis of variance with Tukey's test: $^*P < 0.05$. (**e**) COS-7 cells were transfected with mouse p21 expression construct along with scramble or p21 shRNA. p21 expression was evaluated after 48 h. (**f**) *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ primary hepatocytes were transfected with p21-shRNA or control plasmid and serum starved. Following TNFα/CHX treatment for the indicated periods, caspase-3 cleavage and p21 expression were analyzed. Data represent two independent experiments.

which signals via EGFR to induce hepatocyte proliferation during liver regeneration.[9,26] The cytosolic p21 accumulation in SOCS1-deficient hepatocytes following TGFα stimulation was also confirmed by immunofluorescence staining (Figure 6d). TGFα-stimulated *Socs1*-null hepatocytes also showed phosphorylation of p21 on Thr145 (Figure 6e). Accordingly, stable SOCS1 expression attenuated TGFα-induced phosphorylation of AKT and p21 in HepG2 cells (Supplementary Figure S9). Inhibition of PI3K upstream of AKT using LY294002 prevented TGFα-induced cytosolic accumulation of p21 in SOCS1-deficient hepatocytes without affecting nuclear p21 levels (Figure 6c, lanes 7 and 8). These findings show that SOCS1 controls PI3K-dependent, AKT-mediated cytosolic retention of p21 downstream of growth factor receptors.

Elevated p21 expression in SOCS1-deficient hepatocytes confers resistance to apoptosis

The paradoxical oncogenic activity of cytosolic p21 is attributed partly to inhibition of apoptosis pathways.[13–16] Therefore, we examined apoptosis induction by TNFα and CHX in SOCS1-deficient hepatocytes. *Socs1*-null hepatocytes showed reduced levels of cleaved caspase-3 compared with control cells (Figure 7a), indicating increased resistance to apoptosis. Consistent with these *in vitro* findings, *Socs1*$^{-/-}$*Ifng*$^{-/-}$ *and Socs1*$^{fl/fl}$*Alb*^{Cre} mice treated with high-dose DEN showed less caspase-3 cleavage and fewer TUNEL (terminal deoxinucleotidyl transferase-mediated dUTP-fluorescein nick end labeling)-positive cells in the liver than control mice (Figures 7b and c). This was accompanied by reduced serum alanine transferase levels in SOCS1-deficient mice

(Figure 7d), indicating less severe liver damage. To determine whether increased p21 expression in *Socs1*-null hepatocytes conferred resistance to apoptosis, we used shRNA to down-modulate p21 prior to TNFα/CHX treatment. The p21-shRNAs were tested for their ability to reduce overexpressed p21 in COS-7 cells (Figure 7e). As shown in Figure 7f, shRNA-mediated p21 knock-down in primary hepatocytes increased caspase-3 cleavage in control cells and restored it in *Socs1*-null cells (Figure 7f). These findings indicate that SOCS1 deficiency renders hepatocytes resistant to apoptosis via upregulating p21.

Increased p21 expression in *Socs1*-null hepatocytes promotes DNA synthesis following growth factor stimulation

We observed increased phosphorylation of EGFR in *Socs1*-null hepatocytes following TGFα stimulation, accompanied by increased phosphorylation of ERK and induction of cyclinD proteins (Figure 6e). EGFR has an important role in liver generation, and p21 induced by EGFR signaling exerts pro-mitogenic effect in hepatocytes.[26,27] Besides, the steady-state liver displays a basal level of EGFR phosphorylation that decreases after partial hepatectomy (PH) due to ubiquitination, internalization and degradation of EGFR.[28] We observed higher baseline EGFR phosphorylation and EGFR expression in the livers of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice that declined more slowly than in *Ifng*$^{-/-}$ mice during liver regeneration following PH (Figure 8a). The SOCS1-deficient livers also showed elevated levels of AKT phosphorylation at steady state that declined after PH in parallel to EGFR downmodulation (Figure 8a). The regenerating livers of *Socs1*-null mice showed increased levels of p21 at 24–72 h post-PH that was preceded by elevated expression of cyclinD isoforms and was associated with earlier and higher expression of proliferating cell nuclear antigen, although CDK2 expression was not affected (Figure 8a). As p21 is also implicated in cell cycle progression,[16,29] we compared DNA synthesis in primary hepatocytes from the SOCS1-deficient and control livers following hepatocyte growth factor, TGFα or EGF stimulation, with or without prior down-modulation of p21 using shRNA. *Socs1*-null primary hepatocytes showed increased proliferation in response to growth factors, which was completely reversed by p21 knockdown (Figure 8b). These results indicate that SOCS1-dependent control of hepatocyte proliferation is mediated partly via regulation of p21 expression.

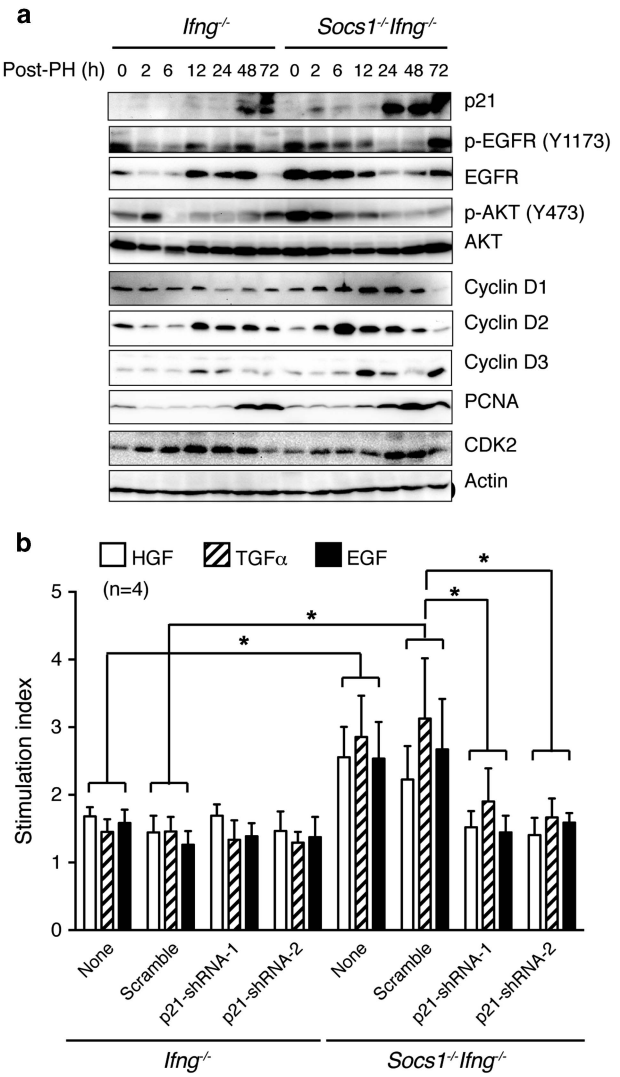

Figure 8. Elevated p21 expression in SOCS1-deficient hepatocytes promotes DNA synthesis in response to growth factor stimulation. (**a**) Following PH, expression of the indicated proteins in the regenerating livers of *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice was analyzed at the indicated time points. Data represent two independent sets of experiments. (**b**) Primary hepatocytes from *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice were transfected with control or p21 shRNA. After 24 h, cells were cultured in serum-free medium supplemented with hepatocyte growth factor (HGF), TGFα or EGF (10 ng/ml) for 48 h. [^{3}H]-thymidine was added during the last 12 h and the incorporated radioactivity was measured (cpm, counts per minute). Stimulation index (cpm for growth factor-stimulated cultures/cpm for non-stimulated cultures) was calculated from four independent experiments. Mean ± s.e.m. Analysis of variance with Tukey's test: *$P < 0.05$.

DISCUSSION

Despite compelling evidence for a tumor-suppressor role of SOCS1 in the liver, the underlying molecular mechanisms remain obscure. In rodent models of HCC, increased levels of inflammatory cytokines, particularly IL-6, has an essential role in hepatocarcinogenesis.[10] Here we have shown that SOCS1 deficiency promotes HCC without augmenting circulating IL-6 levels or IL-6 signaling in the liver. Intriguingly, circulating IL-6 level is reduced in SOCS1-deficient mice compared with controls, despite increased hepatic *Il6* transcript levels 48 h post-DEN (Figure 1d and Supplementary Figure S1). As pro-inflammatory cytokine secretion is regulated by posttranscriptional control mechanisms,[30] further studies are needed to determine how SOCS1 modulates IL-6 and other inflammatory cytokine production in the liver. Notwithstanding the role of SOCS1 in regulating hepatic inflammatory cytokine production, our findings indicate that antitumor function of SOCS1 in the liver is mediated, at least partly, via inhibiting the potential oncogenic functions of p21 (Figure 9). SOCS1 deficiency increased the expression and cytosolic localization of p21 in hepatocytes, accompanied by increased resistance to apoptosis and enhanced cell proliferation. Our findings show that SOCS1 regulates p21 expression in the liver at three levels, namely, gene expression, protein stability and subcellular localization.

First, induction of *Cdkn1a* mRNA was increased by SOCS1 deficiency. SOCS1 appears to regulate transcription rather than mRNA stability because *Cdkn1a* transcript levels are low in the steady-state livers of *Socs1*-null mice (Supplementary Figure S10). *Cdkn1a* transcription is regulated by many positive and negative transcription factors.[16] Although *Cdkn1a* transcription following DNA damage is mediated by p53, STAT molecules activated by growth factors and cytokines can also induce *Cdkn1a* expression. The elevated p21 levels in the regenerating livers of *Socs1*-null mice (Figure 8a) and in growth factor-stimulated SOCS1-deficient

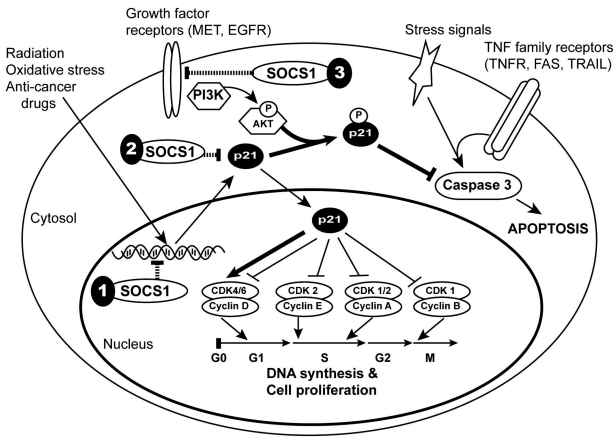

Figure 9. Proposed mechanisms of SOCS1-dependent regulation of p21 and its potential oncogenic functions. Generally p21 functions as a tumor suppressor in the nucleus by inhibiting CDKs. However, phosphorylation of p21 downstream of aberrant growth factor signaling has been shown to promote its cytosolic accumulation and inhibition of apoptosis pathways. Besides, p21 is implicated in promoting cell cycle progression at G1/S and G2/M transition.[13,27] Our findings show that SOCS1 regulates the expression of p21 at the (1) transcriptional level, (2) protein stability and (3) subcellular distribution by attenuating growth factor signaling and thereby controls the paradoxical oncogenic function of p21 (bold lines) namely, inhibition of apoptosis and promoting cell proliferation.

hepatocytes (Figures 6c–e) could result from increased STAT-mediated transcription. STAT-mediated transcription may also contribute to *Cdkn1a* transcription in the DEN-treated livers, as DEN-induced liver damage also elicits compensatory hepatocyte proliferation driven by growth factors. Even though SOCS1–p53 co-operation[12] is not expected to occur in SOCS1-deficient cells, we observed that siRNA-mediated p53 knockdown abrogated the *Cdkn1a* gene expression induced by DNA damage (Supplementary Figure S4). This indicated that p53 is required for the induction of *Cdkn1a* gene in SOCS1-deficient cells following DNA damage, although other p53 target genes except *Tfnrfs10b* were not affected (Supplementary Figure S5). We posit that either SOCS1 is dispensable for p53-mediated induction of *Cdkn1a* or SOCS3 may compensate for the loss of SOCS1, as SOCS3 has been shown to interact with p53 and induce *Cdkn1a* in doxorubicin-treated mesothelioma cells and IL-22-stimulated hepatic stellate cells.[31,32] In support of this idea, we observed increased *Socs3* expression in the SOCS1-deficient livers following PH or DEN treatment (Supplementary Figure S11) and in TGFα-stimulated primary hepatocytes (Supplementary Figure S13). Clearly, further studies are needed to determine whether increased expression of *Cdkn1a* in SOCS1-deficient hepatocytes is indeed mediated by SOCS3 and why this occurs only for *Cdkn1a* and *Tfnrfs10b* but not for other p53 target genes.

Given that SOCS1 interacts with p53 and promotes ATM/ATR-mediated p53 phosphorylation,[12] it is possible that SOCS1 deficiency may compromise other p53-dependent tumor-suppression pathways. For instance, the ARF tumor-suppressor pathway, which is induced by excessive mitogenic stimuli, is dependent on p53.[33] We observed increased induction of *Cdkn2a* coding for p19ARF in the SOCS1-deficient livers, although this increase was not statistically significant (Supplementary Figure S12). The ATM kinase has been shown to regulate ubiquitination and stability of p14ARF in human cancer cell lines and primary lung cancer tissues.[34] Paradoxically, ARF may promote cancer progression by oncogenic STAT3.[35] As SOCS3 is an important regulator of STAT3 activation[6] and SOCS3 is induced at a higher level in the SOCS1-deficient liver (Supplementary Figure S12), further studies are needed to determine how SOCS1 and SOCS3 modulate the ARF and other p53-dependent tumor-suppression pathways.

Second, even though increased p53-dependent gene transcription likely contributes to elevated p21 protein expression in the SOCS1-deficient livers, we have provided many lines of evidence to show that SOCS1 has an important role in regulating p21 at the protein level. The induction of *Cdkn1a* during liver regeneration occurred in a biphasic manner, with significant upregulation at 2–6 h post-PH (Supplementary Figure S11a). However, p21 protein was only moderately elevated during this period in the SOCS1-deficient livers but reached higher levels after *Cdkn1a* mRNA levels had returned to basal levels (Figure 8a; Supplementary Figure S11a). This suggested a role for SOCS1 in posttranslational regulation that was confirmed by increased half-life of p21 in SOCS1-deficient hepatocytes (Figure 4c). We have shown that SOCS1 interacts with p21 and promotes its ubiquitination in a SOCS box-dependent manner. Although the molecular determinants of this interaction remain to be elucidated, our data adds CRL5^{SOCS1} Ub-ligase to five other Ub-ligases that regulate p21 at different stages of the cell cycle.[16,21,22] These include four nuclear Ub-ligases, MDM2/X (G1 phase), SCF^{SKP2} (G1/S transition and S phase), CRL4^{CDT2} (S phase when p21 is bound to proliferating cell nuclear antigen) and APC/C^{CDC20} (G2/M transition), and CRL2^{LRR1} implicated in degrading cytosolic p21.[21] Although the nuclear Ub-ligases are implicated in cell cycle regulation, CRL2^{LRR1} is implicated in regulating actin remodeling and cell motility.[21] Whether SOCS1-dependent regulation of p21 occurs at specific stages of the cell cycle and is associated with specific cellular functions remain to be elucidated.

Third, SOCS1 deficiency increased cytoplasmic distribution of p21 in a PI3K-dependent manner. Cytoplasmic localization of p21 is promoted by AKT-mediated phosphorylation downstream of growth factor signaling.[16,25] EGFR signaling is critical for liver regeneration.[26] We found that EGFR and AKT are constitutively phosphorylated in the *Socs1*-null liver (Figure 8a). Additionally, we detected increased EGFR expression and downstream signaling in SOCS1-deficient hepatocytes. We have previously shown that SOCS1 is also an important regulator of MET receptor signaling.[11,36] It is likely that SOCS1 may exert wide, and possibly redundant, regulatory functions on receptor tyrosine kinases.[7] Increased EGFR expression in the absence of SOCS1 (Figure 8a) is an intriguing observation that warrants further investigation. SOCS3 has been reported to regulate EGFR signaling in hepatocytes.[37] We observed that decreased expression of EGFR in the *Socs1*-null livers coincided with increased *Socs3* expression (Supplementary Figure S11b). Therefore, it is likely that SOCS1 and SOCS3 may exert complementary roles in regulating EGFR, MET and other receptor tyrosine kinases during physiological and neoplastic proliferation of hepatocytes.

Elevated p21 levels in SOCS1-deficient hepatocytes, which contributes to their increased growth factor responsiveness (Figure 8b), might also underlie increased liver regeneration in *Socs1*-null mice.[11] Cell cycle progression through the G1 phase requires cyclinD-dependent activation of CDK4/6.[38] Indeed, mitogen-induced cyclinD1 forms complex with p21 and CDK4 in hepatocytes.[39] Furthermore, p21 is required for EGFR-induced adaptive proliferation of intestinal epithelial cells.[40] These studies provide firm evidence that p21 has a permissive role during cell cycle progression. We found increased expression of both p21 and cyclinD in the SOCS1-deficient livers following PH (Figure 8a). Moreover, increased growth factor-induced DNA synthesis in *Socs1*-null hepatocytes was reversed by p21 knockdown (Figure 8b). In this context, recent studies using p21-deficient mice have shown a critical role for p21 in liver regeneration.[41,42] Determining whether p21 has an essential role in accelerated regeneration in SOCS1-deficient liver[11] requires further investigation.

Under conditions that induce cell cycle arrest, p21 mediates its functions by inhibiting CDKs within the nucleus.[16] On the other hand, retention of p21 within the cytosol inhibits apoptosis by binding to and inactivating apoptosis-inducing proteins such as caspases, stress-activated protein kinase and apoptosis signal regulating kinase 1.[13,16] Although the hepatic nuclear p21 content relative to cytosolic p21 levels increased in control mice following DEN treatment, the SOCS1-deficient livers showed much less nuclear and abundant cytosolic p21 (Figure 6b). Although the increased cytosolic p21 levels inhibited apoptosis as shown by decreased caspase-3 cleavage, reduced TUNEL+ cells and less severe liver damage (Figures 8a–d), the reduced nuclear p21 content may also compromise its ability to inhibit CDKs and cause cell cycle arrest. Apoptosis inhibition by p21 was demonstrated mostly under conditions that dissociate its effects on the cell cycle.[13,16] However, this is difficult to achieve during DEN-induced liver damage, which not only causes cell death but also elicits compensatory proliferation. Although our findings support the idea that altered subcellular distribution of p21 in SOCS1-deficient hepatocytes inhibits apoptosis, we do not exclude the possibility that this may also compromise p21-dependent cell cycle arrest.

Contrary to our finding that SOCS1 deficiency protects hepatocytes from apoptosis, SOCS1 overexpression was reported to protect Jurkat T cell from apoptosis induction by reactive oxygen species via attenuating p38 mitogen-activated protein kinase activation.[43] However, we did not find significant change in phospho-p38 levels in SOCS1-deficient hepatocytes following TNFα stimulation (Supplementary Figure S14). On the other hand, increased p21 expression in SOCS1-deficient hepatocytes may promote cell survival by other mechanisms such as protection against reactive oxygen species-induced DNA damage and apoptosis.[13] It has been shown that p21, upregulated by reactive oxygen species, interacts with NRF2 (nuclear factor erythroid 2-related factor) and prevent its constitutive ubiquitination by KEAP1, thus promoting NRF2-dependent transcription of antioxidant enzymes.[44] Liver tissues of DEN-treated SOCS1-deficient mice slightly increased *Nrf2* mRNA expression (Supplementary Figure S12) and NRF2 protein, which showed increased cytosolic distribution (Figure 6b). Increased NRF2 expression also occurred in the livers of *Ifng*$^{-/-}$ mice, where it showed prominent nuclear distribution (Figure 6b). The basis for this differential subcellular distribution of NRF2 in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice and whether increased NRF2 expression in the SOCS1-deficient livers arises from elevated p21 levels and confers resistance to oxidative damage await further investigations.

Even though the tumor-suppressor role of p21 has been documented in human HCC cells and in rodent models, recent studies have clearly demonstrated the oncogenic potential of p21 in the liver.[41,42,45] In fact, loss of p21 can protect against HCC after modest, but not severe, liver damage.[41] Furthermore, during chronic liver damage, p21 promotes hepatocarcinogenesis and serves as a negative prognostic factor.[42] However, physiological mechanisms that regulate the oncogenic potential of p21 have not yet been elucidated. Our findings indicate that SOCS1 is an important regulator of the oncogenic functions of p21, although a genetic proof requires the generation of SOCS1-deficient mice lacking p21 in hepatocytes. Nevertheless, given the frequent loss of SOCS1 expression in HCC, we propose that evaluating epigenetic repression of *SOCS1* along with p21 levels in HCC biopsies may have a prognostic value and guide personalized therapy.

MATERIALS AND METHODS

Mice, DEN treatment, PH and primary hepatocyte culture

Socs1$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice in C57BL/6 background have been previously described.[11] C57BL/6 and *Alb*^{Cre} mice were purchased from the Jackson Laboratory (Bar Arbor, ME, USA). *Socs1*$^{fl/fl}$ mice[46] were crossed with *Alb*^{Cre} mice to ablate the *Socs1* gene in hepatocytes. All animal experiments were approved by the institutional ethics committee. Induction of HCC by DEN, PH and primary hepatocytes isolation are detailed in Supplementary Methods.

Serum alanine transferase and IL-6 levels

Serum alanine transferase levels were measured using a kinetic assay (Pointe Scientific, Brussels, Belgium). Serum IL-6 was measured by sandwich enzyme-linked immunosorbent assay using antibodies from BD Pharmingen Biosciences (San Diego, CA, USA).

Cell lines and plasmids

HepG2, Hepa1-6 and COS-7 cells were purchased from ATCC (Manassas, VA, USA). Stable lines of SOCS1-expressing cells were established using a lentiviral vector.[11] Expression constructs of SOCS1, p21 and ubiquitin and shRNA targeting p21 are detailed in Supplementary Methods.

Cell assays

To activate p53, primary hepatocytes and HepG2 cells were treated with cisplatin (Cis-Pt, 10μM; Sigma-Aldrich, Oakville, ON, Canada) for 3 h, washed with phosphate-buffered saline and replenished with fresh medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum) or exposed to gamma irradiation (10 Gy). For growth factor stimulation, cells were serum-starved (Dulbecco's modified Eagle's medium–0.25% fetal calf serum) overnight before adding TGFα, EGF or hepatocyte growth factor (10 ng/ml, PeproTech, Ricky Hill, NJ, USA). Cell proliferation was measured by thymidine incorporation.[11]

Gene and protein expression analysis and ubiquitination assay

Total RNA was isolated using RNeasy (Qiagen, Mississauga, ON, Canada) from cultured hepatocytes and the livers were fixed in RNAlater. Quantitative PCR analysis of gene expression is detailed in Supplementary Methods. Preparation of cell and tissue lysates, western blotting, immunoprecipitation and GST pull-down and ubiquitination assays are described in Supplementary Methods.

Immunofluorescence microscopy, immunohistochemistry and TUNEL assay

Detailed methodology is described in Supplementary Methods.

Statistical analysis

Data were analyzed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA), and the statistical significance was calculated by analysis of variance or Mann–Whitney test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

MY was a recipient of FRQNT graduate scholarship. YG was supported by a graduate scholarship from the Canadian Liver Foundation. RK is supported by a graduate scholarship from the Faculté de médecine et des sciences de la santé, Université de Sherbrooke. DB is a recipient of a postdoctoral fellowship from FRQS. CR-CHUS is an FRQS-funded research center. This work was supported by operating grants from the Canadian Institutes of Heath research (to SI, MOP-84234) and Cancer Research Society, Montreal, Canada (to SI and GF, PIN: 17009).

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)