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SOCS1 Links Cytokine Signaling to p53 and Senescence

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SUMMARY

SOCS1 is lost in many human tumors, but its tumor suppression activities are not well understood. We report that SOCS1 is required for transcriptional activity, DNA binding, and serine 15 phosphorylation of p53 in the context of STAT5 signaling. In agreement, inactivation of SOCS1 disabled p53-dependent senescence in response to oncogenic STAT5A and radiation-induced apoptosis in T cells. In addition, SOCS1 was sufficient to induce p53-dependent senescence in fibroblasts. The mechanism of activation of p53 by SOCS1 involved a direct interaction between the SH2 domain of SOCS1 and the N-terminal transactivation domain of p53, while the C-terminal domain of SOCS1 containing the SOCS Box mediated interaction with the DNA damage-regulated kinases ATM/ATR. Also, SOCS1 colocalized with ATM at DNA damage foci induced by oncogenic STAT5A. Collectively, these results add another component to the p53 and DNA damage networks and reveal a mechanism by which SOCS1 functions as a tumor suppressor.

INTRODUCTION

The p53 tumor suppressor exerts antitumor activities by regulating cell-autonomous processes such as DNA repair, apoptosis, cell-cycle arrest, and cellular senescence (Lowe et al., 2004). p53 also plays important roles in development, longevity, and several tissue-specific functions (Vousden and Prives, 2009). These activities of p53 are largely dependent on its ability to regulate the transcription of specific genes. In addition, p53 undergoes posttranslational modifications that cooperatively stabilize the protein and increase its transcriptional activity (Vousden and Prives, 2009). These modifications can be detected in normal cells that express oncogenic activities, suggesting that oncogenic stimuli activate signaling pathways that activate p53 via posttranslational modifications. In cells expressing oncogenes, the DNA damage-activated kinases ATM and ATR play an essential role in p53 activation, indicating that the DNA damage response links oncogenic activity to the tumor suppression functions of p53 (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007a). The mechanism of p53 activation by ATM and ATR is not completely understood. These kinases phosphorylate p53 at serine 15 (Banin et al., 1998; Canman et al., 1998) with the help of adaptor proteins such as BRCA1 and 53BP1, which are also called mediators (Wang et al., 2002). Phosphorylation at this site is required for tumor suppression in vivo (Armata et al., 2007), but most probably this modification acts in the context of other posttranslational modifications.

The suppressor of cytokine signaling SOCS1 was originally identified as a protein that limits signaling from cytokine receptors (Starr et al., 1997). SOCS1 possesses an N-terminal proline-rich domain, a central SH2 domain, and a C-terminal SOCS Box that interacts with elongin B/C and Cullin 2 (Kamura et al., 2004). SOCS1 serves as an adaptor to bring proteins involved in cytokine signaling to the elongin B/C-Cullin E3 ligase complex for ubiquitination (Kamura et al., 1998; Zhang et al., 1999). Major targets of SOCS1 in the suppression of cytokine signaling are the JAK kinases (Endo et al., 1997). SOCS1 is silenced by mutations, promoter methylation, or miRNA overexpression in several human cancers, suggesting that it acts as a tumor suppressor (Fukushima et al., 2003; Galm et al., 2003; Pichiorri et al., 2008; Sutherland et al., 2004; Weniger et al., 2006; Yoshikawa et al., 2001; Zardo et al., 2002).

We found SOCS1 as a gene highly expressed in cells undergoing senescence due to expression of constitutively active STAT5A (ca-STAT5A) at levels found in human tumors (Mallette et al., 2007b). This allele of STAT5A is oncogenic in mice and recapitulates the constitutive activation of STAT5 seen in many human tumors (Kato et al., 2005). We report here that SOCS1 is required for p53 activity and the senescence response to ca-STAT5A. SOCS1 was also required in T cells for the control of apoptosis, cell proliferation, and the expression of several p53 target genes. Finally, we provide mechanistic data that shed light on the role of SOCS1 in p53 activation. Although SOCS1 has been found to form cytoplasmic E3 ligase complexes with elongin B/C and Cullin 2, our results show that SOCS1 can also localize to the nucleus at DNA damage foci and interacts with p53 and ATM. SOCS1 affects p53

phosphorylation at serine 15, DNA binding, and transcriptional activity. These findings connect SOCS1 to p53 and have potential implications for human cancers.

RESULTS

SOCS1 Is Required for p53 Accumulation and Senescence Induced by Ca-STAT5A

Constitutive expression of STAT5 is characteristic of many human tumors, but in normal cells, oncogenic STAT5A induces senescence (Mallette et al., 2007a, 2007b). We used microarrays to profile the gene expression pattern of STAT5-induced senescence and found a high expression of several members of the SOCS family. We confirmed the array results by qPCR (Figures 1A and S1) and western blot (Figure 1B). We focus here on SOCS1, which was the most highly expressed. In fact, the levels of SOCS1 in STAT5-senescent cells were 4 times higher than in cells treated with IFNγ (Figure 1A).

Next, we asked whether inhibition of SOCS1 would affect STAT5-induced senescence using a siRNA against SOCS1, previously used to specifically deplete SOCS1 in dendritic cells (Shen et al., 2004). We found that reduction of SOCS1 expression by siRNA (Figures 1C and 1D) inhibited the number of flat cells that stained positive for the senescence marker SA-β-gal when combined with papillomavirus E7, which inactivates the Rb pathway (Figure 1E). Since senescence induced by ca-STAT5A can only be bypassed by inactivation of both the p53 and the Rb pathways (Mallette et al., 2007a, 2007b), we concluded that SOCS1 is an important player in the senescence response to oncogenic JAK/STAT signaling acting on the p53 pathway. The remaining senescent cells after SOCS1 knockdown may be explained by partial knockdown of SOCS1 by RNAi or by compensatory functions of other proteins, perhaps other members of the SOCS family also induced by STAT5A (Figure S1).

To investigate whether SOCS1 played a role in p53 accumulation during STAT5A-induced senescence, we used the siRNA against SOCS1. Transfection of this siRNA reduced p53 accumulation (Figure 1F) in cells expressing ca-STAT5A. To investigate the functional significance of SOCS1 inhibition for p53 activity, we first profiled the p53 pathway during STAT5-induced senescence using a p53 pathway microarray (Table S1). Then, we used qPCR to confirm the array results and evaluate the effects of SOCS1 inhibition. We found that STAT5A induced a unique and interesting pattern of p53 pathway genes, including *p53* itself, *RELA*, *DAPK1*, *PMP22*, *HDM2*, and *GADD45α*. Among these genes, *DAPK1*, *HDM2*, *GADD45α*, and *p53* are bona fide p53 target genes. SOCS1 was required for induction of all those genes (Figure 1G). Taken together, these data indicate that SOCS1 is linked to the activation of p53 during STAT5-induced senescence.

Socs1 Null Mice Have Defects in the p53 Signaling Pathway

The requirement of SOCS1 for activation of p53 during STAT5-induced senescence reveals a mechanism for the assigned tumor suppressor role of SOCS1 and suggests that an interaction between SOCS1 and p53 might underlie the regulation of other p53 functions. To investigate this issue, we used *socs1* null mice. Deletion of *socs1* is lethal in mice. However, concomitant ablation of *IFNγ* allows animals to survive to adulthood. First, we analyzed the apoptotic response of T cells undergoing development and maturation within the thymus after irradiation, which is known to be mediated by p53 (Lowe et al., 1993). We exposed the total thymocyte preparation to the indicated doses of γ radiation for 16 hr followed by evaluation of apoptosis by flow cytometry using annexin V. The thymocytes were labeled with anti-CD4 and anti-CD8 antibody to evaluate the apoptotic response in the four distinct sequential maturation stages of T cells within the thymus: the CD4/CD8 double-negative (DN) cells, which become CD4/CD8 double-positive (DP) cells that later differentiate into either CD4 single-positive (SP) or CD8 SP cells (Zúñiga-Pflücker, 2004). As reported earlier, $Socs1^{-/-}Ifn\gamma^{-/-}$ mice harbor more CD8 SP thymocytes than $Socs1^{+/+}Ifn\gamma^{-/-}$ or $Socs1^{+/+}Ifn\gamma^{+/+}$ control mice (Ilangumaran et al., 2003b; Ramanathan et al., 2006) (Figure 2A). We found a dramatic defect in the p53-dependent cell death response in *Socs1* null CD8 SP cells (Figure 2B), which constitutively express SOCS1 and selectively accumulate in the *Socs1* null thymus. This defect is specific to SOCS1 deficiency and is not compounded by IFNγ deficiency. SOCS1 deficiency did not affect the radiation sensitivity of CD4 SP cells, which neither express SOCS1 constitutively nor accumulate in SOCS1-deficient thymus (Ilangumaran et al., 2003b; Ramanathan et al., 2006). DN cells lacking SOCS1 showed resistance to radiation-induced apoptosis; however, they did not accumulate in SOCS1-deficient thymi, possibly due to rapid transit through this developmental stage to become DP cells (Zúñiga-Pflücker, 2004). Curiously, the p53-dependent apoptosis of DP cells (Lowe et al., 1993) was not affected by SOCS1 deficiency (Figure 2B), suggesting that other compensatory mechanisms may operate during the DP stage in mediating p53-dependent apoptosis.

Although the absence of IFNγ allows the survival of *Socs1* null mice, these double knockout mice display defective homeostasis of T cells characterized by selective increase in the subpopulation of $CD8^+$ cells (Cornish et al., 2003; Ilangumaran et al., 2003a). Therefore, we used $CD8^+$ T cells from $Socs1^{-/-}Ifn\gamma^{-/-}$ double knockout mice to test other functions of the p53 pathway in a *Socs1* null background. Stimulation of purified $CD8^+$ T cells by a combination of IL-15 and IL-21 showed increased proliferation of cells derived from $Socs1^{-/-}Ifn\gamma^{-/-}$ mice compared to those isolated from the control $Socs1^{+/+}Ifn\gamma^{-/-}$ or $Socs1^{+/+}Ifn\gamma^{+/+}$ mice (Figure 2C). Although an increased sensitivity to cytokines could explain the increased proliferation of *Socs1* null T cells (Gagnon et al., 2007; Ilangumaran et al., 2003a), we asked whether a defect in the expression of p53 target genes might also contribute to this increased proliferation. Since endogenous SOCS1 is expressed during STAT5A-induced senescence and is required for p53 activity, we speculated that p53 target genes upregulated during cytokine stimulation may also be relevant targets of SOCS1 and p53 in T cells. Among these targets, we found a significant decrease in the expression of *p53*, *MDM2*, *PMP22*, *BBC3 (PUMA)*, and *GADD45α* in $CD8^+$ T cells from *Socs1* null mice (Figure 2D). Consistent with a defect in p53 target gene

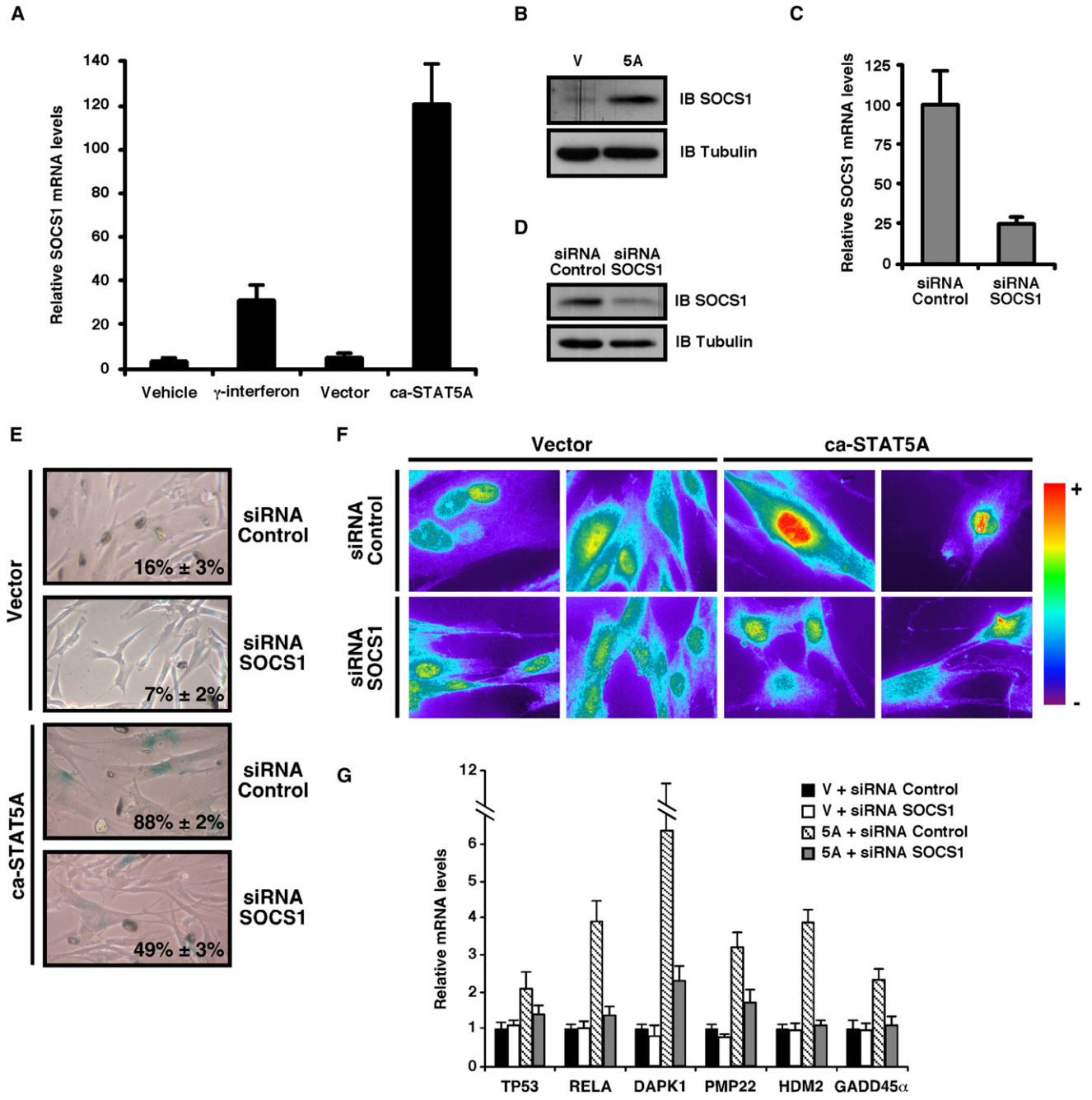

Figure 1. SOCS1 in STAT5-Induced Senescence

(A) qPCR for SOCS1 of IMR90 cells with a vector control or ca-STAT5A, or the same cells treated with IFNγ (100 ng/ml for 1 hr) or vehicle.

(B) Immunoblot for SOCS1 using cell lysates from STAT5A-senescent IMR90 cells (5A) or control cells with empty vector (V).

(C) SOCS1 mRNA levels measured by qPCR in cells expressing ca-STAT5A transfected with a siRNA control or a siRNA against SOCS1.

(D) SOCS1 protein levels in cells as in (C).

(E) SA-β-gal staining of IMR90 cells expressing E7 and infected with a control vector or its derivative expressing ca-STAT5A. After selection, cells were transfected with a siRNA control or an anti-SOCS1 siRNA. The average and standard deviation of the quantitation of SA-β-gal-positive cells is shown at the bottom right of each panel.

(F) p53 immunofluorescence of IMR90 cells expressing an empty control vector or ca-STAT5A and transfected with a siRNA control or a siRNA against SOCS1.

(G) qPCR of *p53* pathway genes in cells expressing a control vector or ca-STAT5A and then treated with a siRNA control or against SOCS1. For (A) and (G), data are presented as mean ±SEM of triplicates.

expression, lower levels of p53 or of phospho-p53^{S15} were found bound to the Mdm2 promoter in *Socs1* null T cells (Figure 2E). Not all p53 target genes were found reduced in *Socs1* null T cells. These cells have an increased JAK/STAT signaling that can activate some p53 targets independently of p53. For example, p21 can be induced by STAT1 (Chin et al., 1996), perhaps explaining why we do not find a defect in p21 expression in *Socs1* null T cells treated with IL-21 (data not shown), which activates STAT1 (Gagnon et al., 2008). Taken together, these results indicate that SOCS1 plays an active role in p53 functions in vivo and is important for the control of cell proliferation and p53 target gene expression in CD8$^+$ T cells stimulated with cytokines.

SOCS1 Induces a p53-Dependent Senescence Program in Human Fibroblasts

We showed above that SOCS1 was required for p53 activity in the context of cytokine signaling. To investigate whether SOCS1 is sufficient to activate p53 and trigger p53-dependent responses, we first used as a model the osteosarcoma cell line-U2OS, which expresses wild-type p53. Transfection with SOCS1 but not with a control vector dramatically inhibited colony formation in these cells (Figure S2A). On the other hand, transfection of SOCS1 in the p53 null cell line H1299 did not affect their proliferation (Figure S2B). The antiproliferative effect of SOCS1 in U2OS cells was accompanied with an accumulation of p53 protein and its target, p21 (Figure S2C).

To study the effects of SOCS1 in normal cells, we infected the human diploid fibroblast strain IMR90 with a retroviral vector encoding SOCS1 or a control vector. Expression of SOCS1 inhibited cell growth (Figure 3A) and induced senescence, characterized by senescence-associated β-galactosidase staining (Figure S3A) and the accumulation of PML bodies (Figure S3B). In addition, SOCS1 induced high levels of p53 and its target, p21; a reduction in Rb phosphorylation; and the inhibition of E2F target genes such as Mcm6 (Figure 3B). Intriguingly, during SOCS1-induced senescence, the increase in p53 levels was accompanied with serine 15 phosphorylation of p53 (Figure 3B) and activation of the DNA damage-regulated kinases ATM and Chk2 (Figure 3C). Staining of SOCS1-expressing cells with an antibody against γH2AX revealed numerous DNA damage foci (Figure S3C).

One of the key mechanisms by which SOCS1 functions as a tumor suppressor is by targeting oncoproteins to the ubiquitination machinery via the C-terminal SOCS Box (Ilangumaran and Rottapel, 2003). Therefore, we investigated the role of the SOCS Box on the effects of SOCS1 in normal human fibroblasts. As shown in Figures S3A and 3D, SOCS1ΔBox was not able to induce p53, p53 phosphorylation at serine 15, or senescence in IMR90 fibroblasts. These results suggest that the SOCS Box is essential for the activation of the DNA damage response, p53, and senescence by SOCS1 in normal cells.

To investigate whether activation of p53 was required for SOCS1-induced senescence, we used a cell line expressing a dominant-negative allele of p53 (p53^{H175}) that we used before to interfere with p53-dependent senescence (Mallette et al., 2004; Serrano et al., 1997). As shown in Figure 3E, the senescence response to SOCS1 was completely inhibited by the dominant-negative allele of p53. Overall, these results indicate that SOCS1 can activate p53 both in tumor cells and in normal cells and that the SOCS Box is required to induce the p53-dependent senescence process.

SOCS1 Cooperates with p53 to Transactivate p53 Target Promoters

To investigate the mechanisms of activation of p53 by SOCS1, we transfected SOCS1 or a control vector along with the p21-promoter-luciferase reporter and p53 into the p53 null cell line H1299. SOCS1 was not able to activate the p53 reporter alone, but it did increase the activity of added p53 to a similar extent as PML (Figure 4A), another regulator of p53 and senescence (Ferbeyre et al., 2000; Fogal et al., 2000). This result indicates that SOCS1 can potentiate the transcriptional activity of p53. To generalize these results, we transfected SOCS1 with one of several p53 reporter promoters into U2OS cells that express endogenous wild-type p53. We found that SOCS1 increased the ability of endogenous p53 to activate the transcription of reporters containing the p53-binding sites of *p21*, *MDM2*, *PTEN*, *NOXA*, and *BAX* in cells treated with the DNA-damaging drug doxorubicin (Figures 4B and S4A). In the absence of doxorubicin, SOCS1 did not stimulate the p21 promoter, but it did stimulate endogenous p53 on the MDM2 promoter (Figure S4B). SOCS1ΔBox did not stimulate the ability of transfected p53 to induce p21 in H1299 cells (Figure 4A), and similar results were obtained when this mutant was transfected in U2OS cells treated with doxorubicin (data not shown). Hence, the SOCS Box is required to stimulate p53 activity on the p21 promoter.

To further prove that SOCS1 stimulated the expression of p53 reporters via p53, we knocked down p53 expression in U2OS cells with a validated shRNA against p53 (Voorhoeve and Agami, 2003). This shRNA abrogated the stimulation of the p21 reporter by SOCS1 in U2OS cells treated with doxorubicin (Figure 4C). Interestingly, an shRNA against ATM (Mukhopadhyay et al., 2005) decreased significantly, but not completely, the p53 activity stimulated by SOCS1 on the p21 promoter (Figure 4C). Next, we investigated how SOCS1 affected p53 binding on the p21 and the HDM2 promoters using chromatin immunoprecipitation in U2OS cells treated with doxorubicin or vehicle. SOCS1 increased the binding of endogenous p53 to the p21 promoter by a factor of 1.8 and to the HDM2 promoter by a factor of 8.4. In addition, it stimulated the effect of DNA damage, increasing p53 binding to the p21 promoter by a factor of 5.1 and to the HDM2 promoter by a factor of 1.5 (Figure 4D). Collectively, these results suggest that SOCS1 stimulates p53 target gene expression by stimulating p53 DNA binding. To confirm this model, we used a fusion of the GAL4 DNA-binding domain with the transcriptional activation domain of p53 that binds DNA via GAL4. As expected, SOCS1 did not stimulate the activity of GAL4-p53 on a reporter promoter containing GAL4-binding sites (Figure 4E).

SOCS1 Interacts with p53

To investigate whether SOCS1 could bind p53, we cotransfected Myc-tagged SOCS1 and p53 in the p53 null cell line H1299. We found Myc-SOCS1 in the immunoprecipitate of

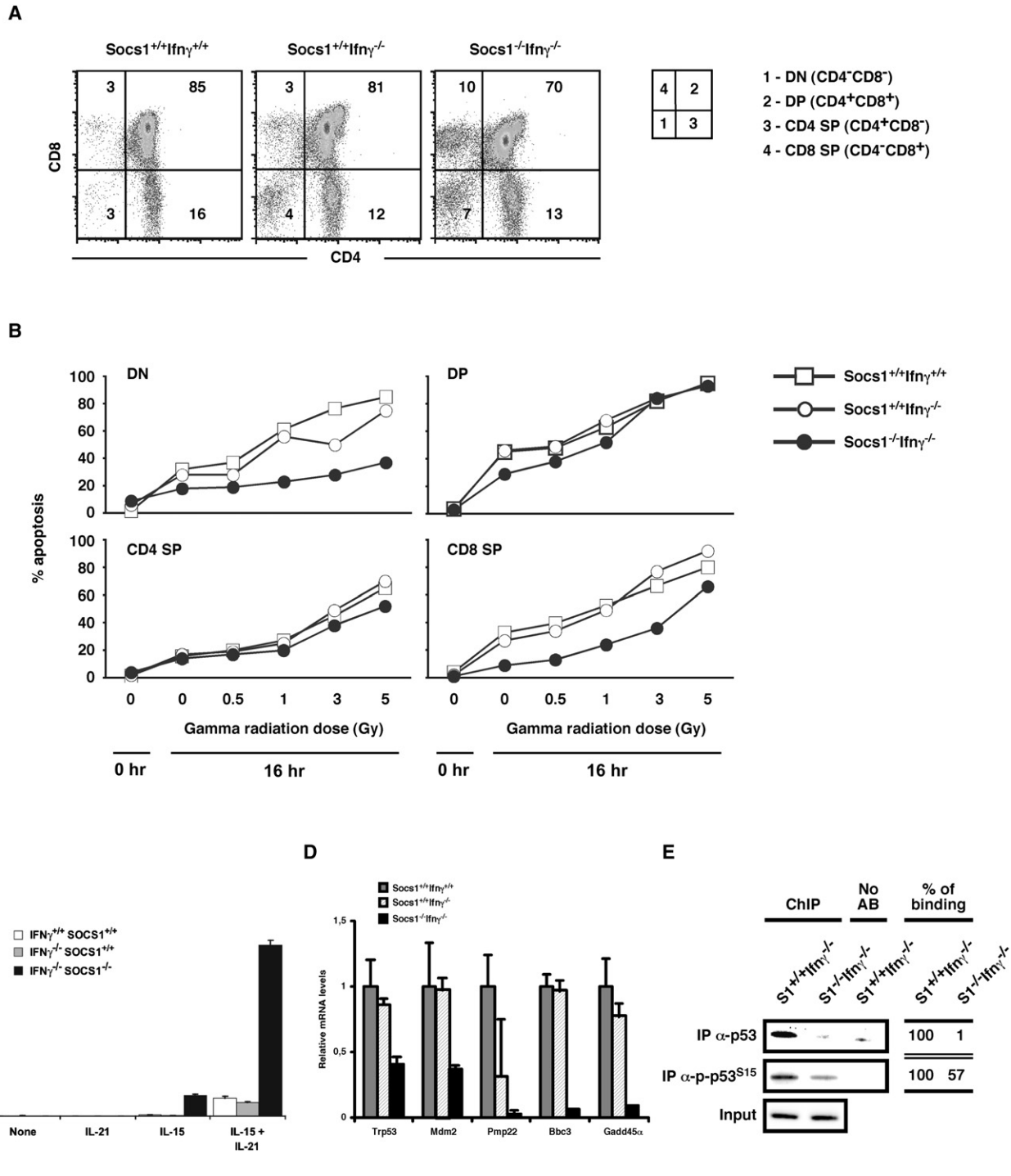

Figure 2. *Socs1* Null Mice Have Defects in p53 Pathways

(A) The four different developmental stages of thymocytes are indicated within rectangles, and the proportion of cells within these gates is indicated in parentheses.

(B) Single cell thymocyte suspensions were exposed to the indicated doses of γ radiation. After incubation at 37°C for 16 hr, the cells were stained with anti-CD4, anti-CD8, and annexin V-PE. The percent of cells stained positive for annexin V within the gated thymocyte subsets is indicated as a function of the dose of radiation. The data shown are representative of four different experiments, and the flow cytometer charts are available as Figure S8. The flow cytometry profile of irradiated thymocytes from C57BL/6 controls was similar to that of *Ifnγ*$^{-/-}$ control (data not shown).

(C) Cell proliferation of T cells, as measured by ^{3}[H]thymidine incorporation, from *Socs1*$^{-/-}$*Ifnγ*$^{-/-}$ mice, control *Ifnγ* null mice, or wild-type BL6 mice treated with IL-21, IL-15, or both cytokines.

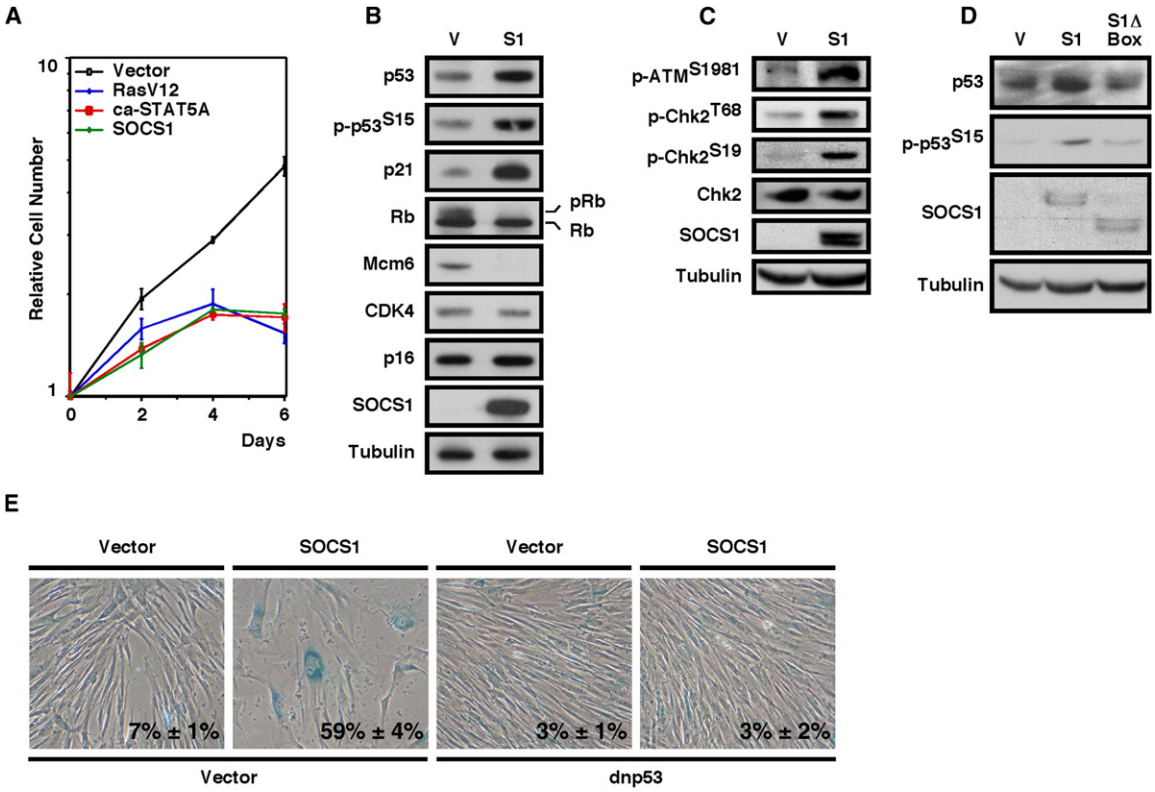

Figure 3. SOCS1 Induces Cellular Senescence

(A) Growth curves of IMR90 cells infected with an empty control vector, RasV12, ca-STAT5A, or SOCS1. Data are presented as mean ±SD of triplicates.

(B) Immunoblots of cell-cycle regulators in IMR90 cells expressing an empty control vector (V) or SOCS1 (S1).

(C) As in (B), but using antibodies for markers of the DNA damage response.

(D) Immunoblots of p53 and phospho-p53^{S15} in IMR90 cells infected with an empty control vector (V), SOCS1 (S1), or SOCS1ΔBox (S1ΔBox).

(E) SA-β-gal staining of IMR90 cells expressing an empty control vector or dominant-negative p53 (dnp53) and then infected with an empty control vector or SOCS1.

a p53-specific antibody but not in the immunoprecipitate of an anti-cyclin A antibody (Figure S5A). Next, we repeated the experiments in U2OS cells that express endogenous p53. We transfected these cells with a Flag-tagged SOCS1 or a Flag-tagged SOCS1ΔBox and treated them with doxorubicin to increase the expression of p53. We found both SOCS1 and SOCS1ΔBox in p53 immunoprecipitates (Figure 5A), suggesting that the interaction between SOCS1 and p53 involves the N-terminal proline-rich domain or the SH2 domain of SOCS1 and not the SOCS Box, as reported for VHL, another SOCS Box protein interacting with p53 (Roe et al., 2006). This interaction between SOCS1 and endogenous p53 was also revealed in cells that were not treated with doxorubicin (Figure S5B), indicative of some level of interaction in the absence of strong external stimuli.

To clarify if the SOCS1-p53 complex found in vivo is the result of direct interaction, we performed GST pull-down assays using GST-p53 fusion proteins (Figure 5B) and in vitro transcribed/translated SOCS1. SOCS1 interacted with full-length p53 and a fragment containing the N-terminal region of p53 (amino acids 1–160) (Figure 5C, top panel). Next, we determined that the interaction region between p53 and SOCS1 involves the first 80 amino acids of p53 (Figure 5C, bottom panel), known to contain the transcriptional activation domains (Laptenko and Prives, 2006). In fact, a construct containing only the two transactivation domains of p53, GST-p53(1–67), was sufficient to bind the SH2 domain of SOCS1 (Figures 5D and 5E). This interaction must be phosphotyrosine independent, because there are no tyrosines in the p53 fragment p53-1–67. Consistent with this explanation, a mutant of

(D) qPCR of p53 pathway genes from CD8$^+$ T lymphocytes from *Socs1*/*Ifnγ*$^-$ null mice, control *Ifnγ* null mice, or wild-type BL6 mice after stimulation with IL-15 and IL-21. Data are presented as mean ±SEM of triplicates. Statistical significance was determined with Student's t test.

(E) Chromatin immunoprecipitation of p53 and phospho-p53^{S15} on the Mdm2 promoter in T cells from *Socs1*$^{-/-}$*Ifnγ*$^{-/-}$ mice, control *Ifnγ* null mice, or wild-type BL6 mice after stimulation with IL-15 and IL-21.

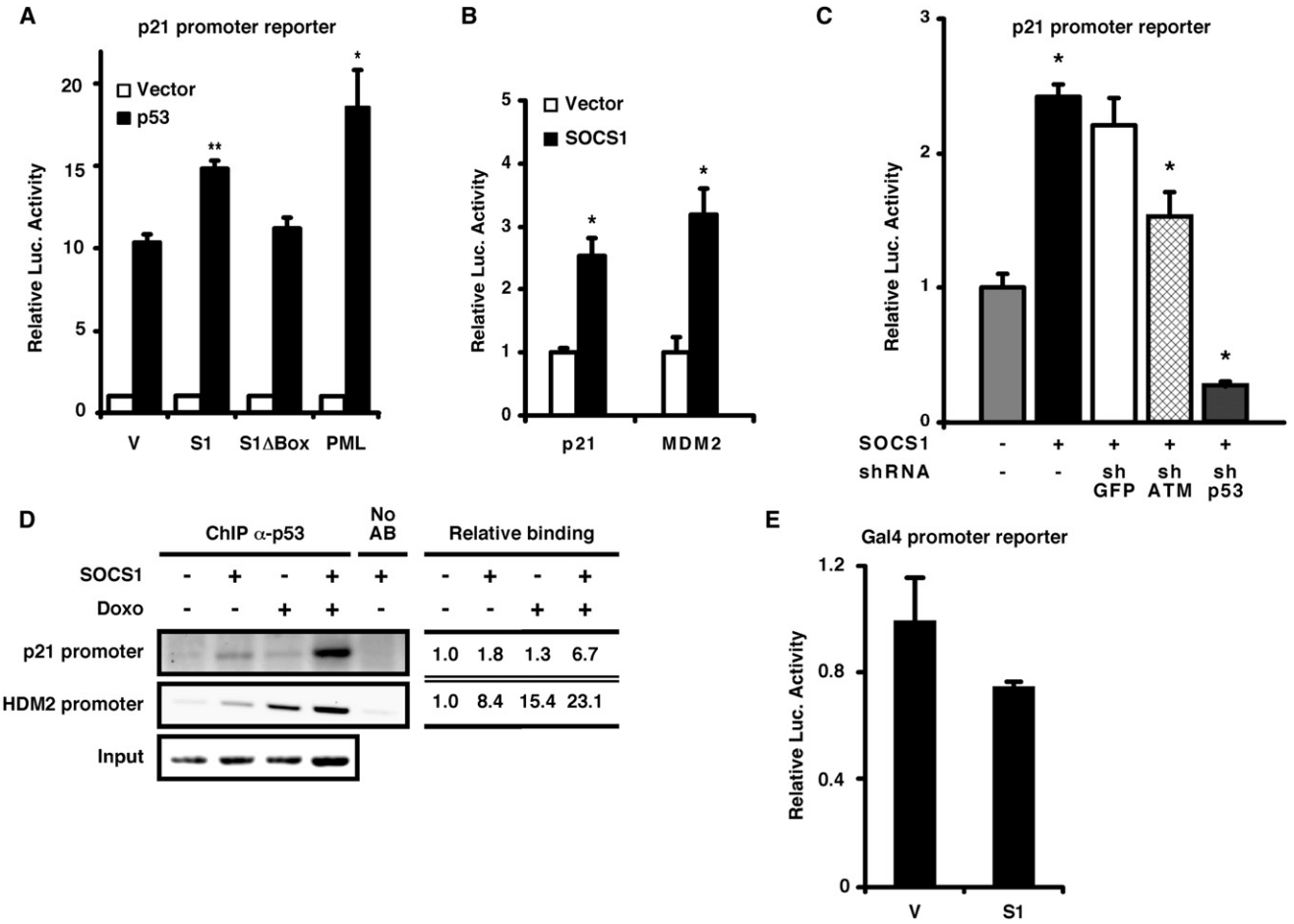

Figure 4. SOCS1 Stimulates the Transcriptional Activity of p53

(A) Luciferase assay in H1299 cells transiently transfected with the p21 promoter luciferase plasmid, an empty control vector, or p53 and an empty control vector (V), SOCS1 (S1), SOCS1ΔBox (S1ΔBox), or PML.

(B) Luciferase assay in U2OS cells transiently transfected with the p21 or MDM2 promoter luciferase reporter and an empty control vector or SOCS1. The cells were treated for 24 hr with doxorubicin (300 ng/ml) 24 hr posttransfection.

(C) Luciferase assay in U2OS cells transiently transfected with the p21 promoter luciferase plasmid and an empty control vector, SOCS1, or SOCS1 with shRNA against ATM (shATM), p53 (shp53), or GFP (shGFP). The cells were treated for 24 hr with doxorubicin (300 ng/ml) 24 hr posttransfection. The data represent the mean and standard deviation of three independent experiments. Asterisks represent a significant increase of luciferase activity (paired t test: *$p < 0.01$; **$p < 0.001$) compared to control cells.

(D) Chromatin immunoprecipitation of p53 on the p21 and HDM2 promoters in U2OS cells transfected with SOCS1 or control plasmid and treated with doxorubicin (300 ng/ml) or vehicle.

(E) Luciferase assay in H1299 cells transiently transfected with the Gal4 promoter plasmid, a Gal4-p53 fusion, and either an empty control vector (V) or SOCS1 (S1). Data are presented as mean ±SD of triplicates.

the phosphotyrosine-binding pocket of SOCS1 (SOCS1R105E) unable to inhibit Jak functions was able to interact with p53, induce senescence in fibroblasts, and reduce colony formation in U2OS cells (Figure S6).

Finally, to test whether the interaction of p53 and SOCS1 could be shown with both endogenous proteins, we used again the model of STAT5A-induced senescence in normal human fibroblasts. In these cells, the immunoprecipitation of endogenous SOCS1 pulled down endogenous p53 (Figure 5F). Taken together, these biochemical studies suggest that SOCS1 is engaged in direct protein-protein interaction with the N-terminal transactivation domain region of p53.

SOCS1 Interacts with the DNA Damage-Responsive Kinases ATM and ATR

The stimulation of p53 reporters by SOCS1 was partially inhibited by knocking down ATM (Figure 4C). This suggested that SOCS1, like VHL (Roe et al., 2006) or BRCA1 (Fabbro et al., 2004), might facilitate the interactions between p53 and ATM. To examine if this was indeed the case, we probed the immunoprecipitates of endogenous ATM or its homolog kinase ATR by western blot using an anti-SOCS1 antibody. We found SOCS1, but not SOCS1ΔBox, in both ATM and ATR immunoprecipitates from U2OS cells treated with doxorubicin (Figure 6A, top panel). This interaction between SOCS1 and ATM was

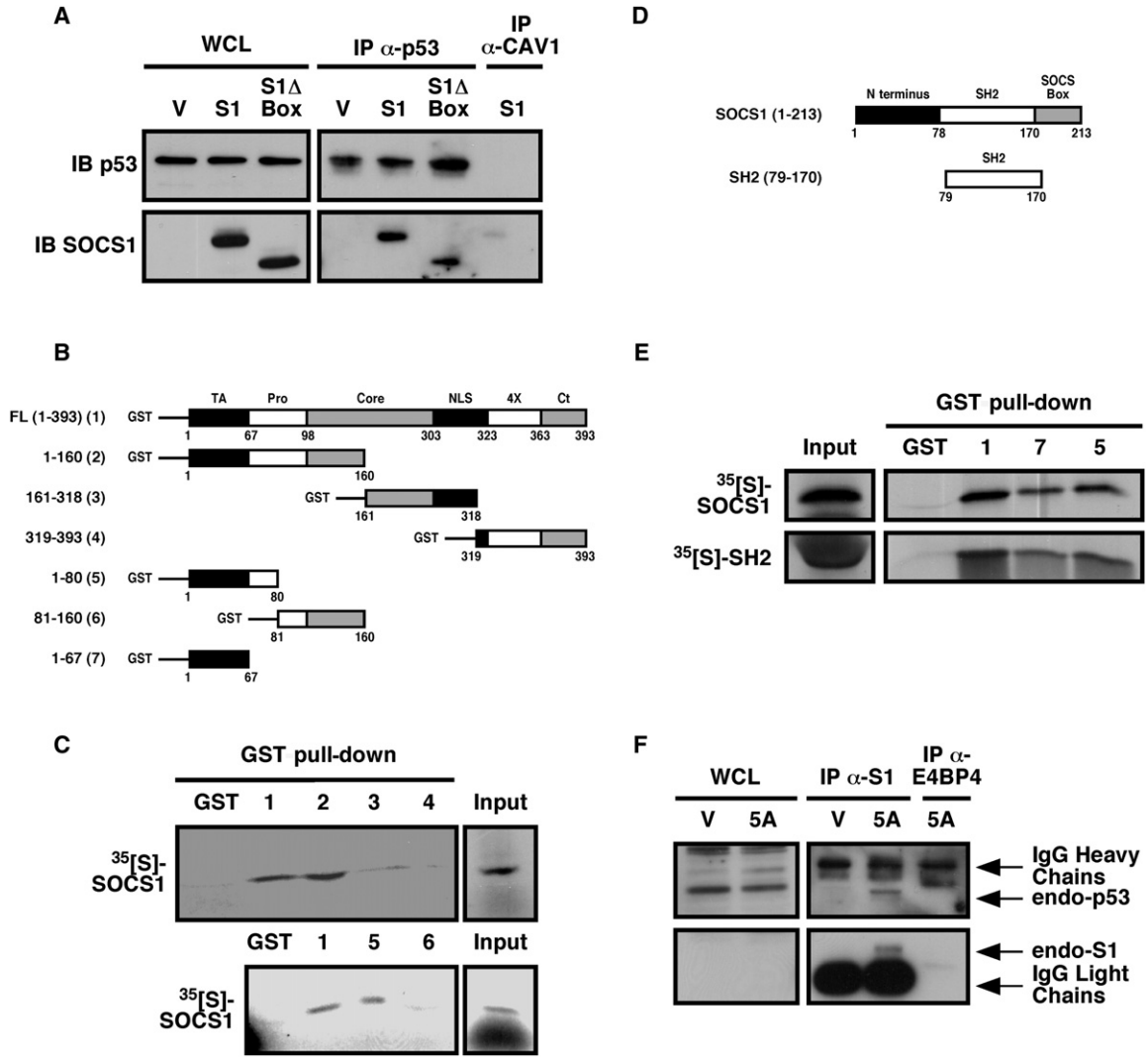

Figure 5. SOCS1 Directly Interacts with p53

(A) U2OS were transiently transfected with an empty control vector (V), SOCS1 (S1), or SOCS1ΔBox (S1ΔBox). Twenty-four hours after transfection, cells were treated with doxorubicin (300 ng/ml) for 24 hr. Lysates were immunoprecipitated with anti-p53 antibody and immunoblotted with anti-SOCS1 antibody. Immunoprecipitation against caveolin (CAV1) was used as a negative control.

(B) Schematic representation of the GST-p53 fusion proteins used in (C). Numbers indicate the p53 amino acids starting and ending every fragment.

(C) Autoradiography of ^{35}[S]Flag-SOCS1 pull-down with GST-hp53 full-length (FL) or fragments bound to glutathione Sepharose-4B. Input is 50% of the amount of protein used for immunoprecipitation. Numbers 1–6 correspond to constructs in (B).

(D) Schematic representation of SOCS1 and its domain structure.

(E) GST pull-down assays as in (C); numbers 1, 7, and 5 correspond to constructs in (B).

(F) STAT5A-senescent IMR90 cell lysates were immunoprecipitated with anti-SOCS1 antibody and immunoblotted with anti-p53 antibody 4 days postinfection. Immunoprecipitation against E4BP4 was used as a negative control.

independent of p53, because it was also observed in the p53 null cell line H1299 (Figure 6A, bottom panel). Hence, SOCS1 interacts with p53 via its N terminus and with ATR/ATM through the SOCS Box. Finally, we immunoprecipitated ATM phosphorylated at serine 1981 with a specific antibody in U2OS cells, taking advantage of a constitutively activated DNA damage response in these cells. Again, we found SOCS1 in the immunoprecipitates (Figure S7).

To determine whether the interaction between SOCS1 and ATM occurred in vitro as a result of direct protein-protein interactions, we performed GST pull-down assays using GST-ATM fragments purified from *E. coli* and in vitro transcribed/translated SOCS1. We found that SOCS1 interacted more strongly with the FAT domain of ATM, a region that is also present in the PIK-related kinases ATR and DNA-PK (Bosotti et al., 2000) (Figure 6B). Two additional fragments of ATM interacted with purified SOCS1, including the fragment that contains the kinase domain. None of these interacting regions overlap with the region of ATM that binds p53, which was mapped to the N terminus of ATM (Khanna et al., 1998). Overall, our functional

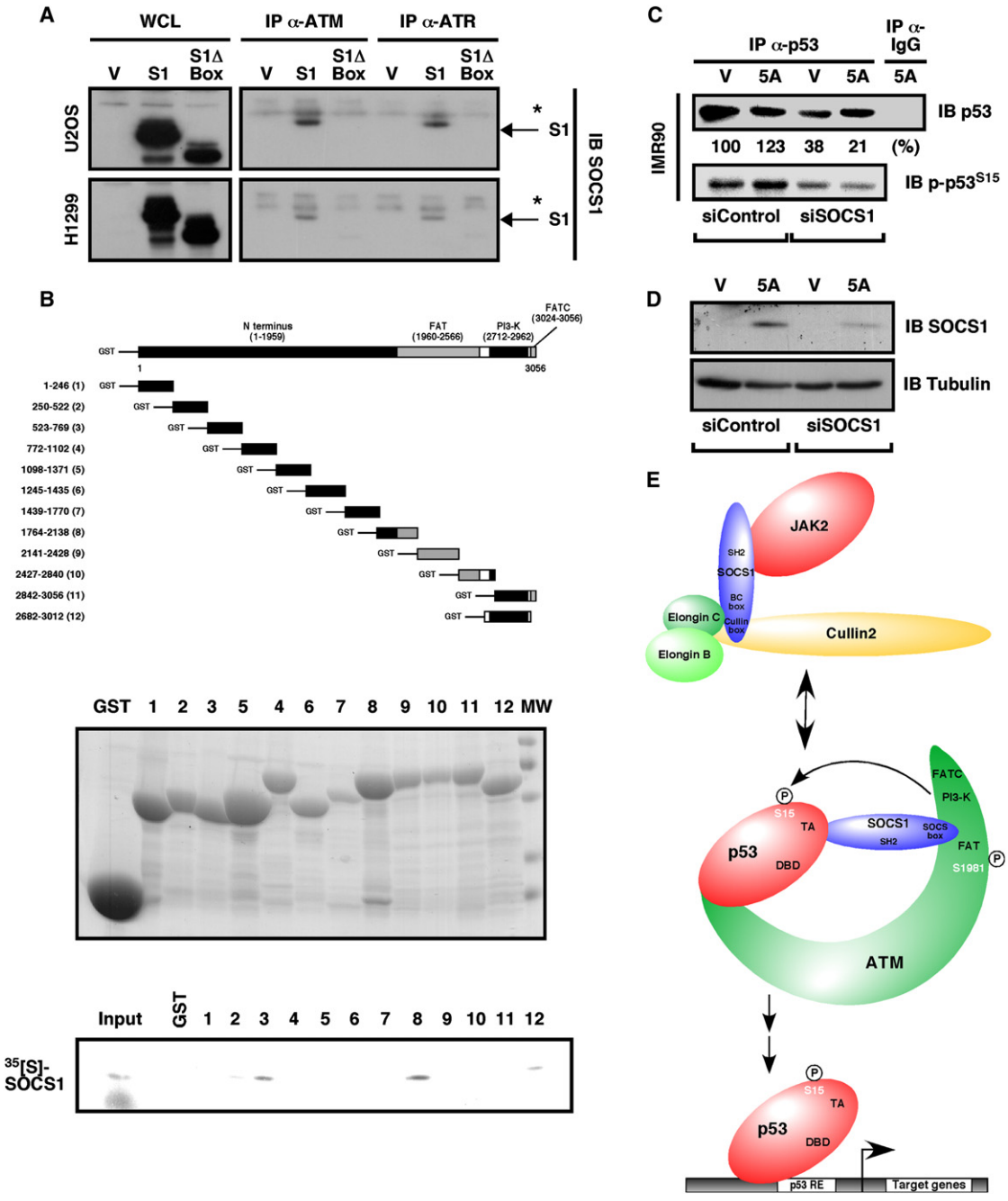

Figure 6. SOCS1 Interacts with ATM and ATR

(A) SOCS1 immunoblots of ATM or ATR immunoprecipitates from U2OS or H1299 cells transfected with a control vector (V), SOCS1 (S1), or SOCS1ΔBox (S1ΔBox). Cells were treated with doxorubicin (300 ng/ml) 24 hr after transfection. * denotes a nonspecific band.

(B) Equal amounts of each GST-ATM fragment (top panel) were estimated from Coomassie blue staining (middle panel), bound to glutathione Sepharose-4B, and incubated with ^{35}[S]Flag-SOCS1. After washing and SDS PAGE, ^{35}[S]Flag-SOCS1 was detected by autoradiography (bottom panel). Numbers 1–12 correspond to GST-ATM fragments indicated at the top of the panel.

(C) Phospho-p53^{S15} and total p53 immunoblots of p53 immunoprecipitates from extracts of IMR90 cells expressing an empty control vector or ca-STAT5A and transiently transfected with a siRNA control or a siRNA against SOCS1. The percent of phospho-p53^{S15} is indicated.

(D) SOCS1 immunoblot from total lysates of the experiment shown in (C).

(E) SOCS1 forms part of two protein complexes. Top: SOCS1 regulates cytokine signaling as part of an E3 ligase complex with elongin B/C and Cullin 2. Bottom: SOCS1 serves as a mediator of the DNA damage response by binding to ATM and p53, enhancing the phosphorylation of p53 at serine 15.

analysis of SOC1 and our protein-protein interaction data suggests that SOCS1 could facilitate the phosphorylation of p53 at serine 15 by PIK-related kinases (Figure 3B). To test this model, we evaluated the ATM/ATR-dependent phosphorylation of p53 at serine 15 in human fibroblasts expressing ca-STAT5A and transfected with the siRNA against SOCS1 or a siRNA control. Consistent with the ability of this siRNA to inhibit p53 activity in STAT5A-expressing cells (Figure 1F), we also observed a reduction in the levels of p53 phosphorylation at serine 15 in cells treated with the anti-SOCS1 siRNA in comparison with the siRNA control (Figure 6C). Taken together, our results are consistent with a model proposing that SOCS1 can form two different complexes, one as an E3 ligase with elongin B/C and another acting as an adaptor for phosphorylation of p53 by ATR/ATM (Figure 6E).

SOCS1 Can Localize to the Nuclear Compartment and Can Be Found at DNA Damage Foci Induced by Oncogenic STAT5

Most of the known SOCS1 targets in cytokine signaling are cytoplasmic. However, some studies have indicated that a fraction of SOCS1 is nuclear when it is overexpressed (Ben-Zvi et al., 2006). We confirmed the nuclear localization of SOCS1 in U2OS cells by indirect immunofluorescence of cells transfected with a Flag-tagged SOCS1 (Figure 7A) or by direct immunofluorescence using a GFP-SOCS1 fusion protein (Figure 7B).

Interestingly, immunolocalization of endogenous SOCS1 in cells expressing ca-STAT5A revealed a pattern of foci that colocalized with foci stained by an antibody recognizing phosphorylated ATM and were largely reduced by a siRNA against SOCS1 (Figures 7C and 7D). These oncogene-induced DNA damage foci are part of a DNA damage response necessary to mediate oncogene-induced senescence (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007a). Hence, the localization of SOCS1 at these foci further strengthens the proposition that SOCS1 plays a role as a mediator of the DNA damage response.

DISCUSSION

The numerous functions of p53 (Vousden and Prives, 2009) imply the existence of multiple cellular regulators of this protein. Here, we report a link between SOCS1 and the p53 pathway relevant for three important physiological processes. First, SOCS1 connects aberrant STAT5 signaling to p53, facilitating the process of cellular senescence, which is an important tumor suppressor mechanism in vivo (Braig et al., 2005). Interestingly, SOCS1 can be induced by many cytokines, which play an important role in maintaining the senescent cell-cycle arrest (Acosta et al., 2008; Kuilman et al., 2008). Second, SOCS1 was required in T cells for radiation-induced apoptosis, a p53-dependent process (Lowe et al., 1993). Finally, we also showed that SOCS1 was required to control T cell proliferation in response to cytokines, in part because it controls the expression of several p53 target genes.

Structural-functional analysis of SOCS1 points to an adaptor function of SOCS1 in the DNA damage response. The SH2 domain of SOCS1 binds the N terminus of p53 containing the transcriptional activation domains, while the SOCS Box mediates binding to ATM and ATR. In this way, SOCS1 can facilitate the interaction between the ATM/ATR kinases and p53 (Figure 6E). In agreement, SOCS1 colocalizes with ATM at DNA damage foci. Also, inhibition of SOCS1 expression by RNAi decreased $p53^{S15}$ phosphorylation and the expression of several p53 target genes during STAT5-induced senescence. In addition, a mutant of SOCS1 without the SOCS Box and therefore unable to bind ATM/ATR was not able to induce senescence or $p53^{S15}$ phosphorylation. Hence, we propose that SOCS1 can link the DNA damage signals characteristic of oncogene-expressing cells to p53, serving as a mediator for p53 phosphorylation by ATM and ATR (Figure 6E). $p53^{S15}$ phosphorylation has been previously linked to oncogene-induced senescence (Ferbeyre et al., 2002, 2000). This modification affects the conformation of the p53 N terminus (Kar et al., 2002), facilitating binding to coactivators and increasing the transactivation potential of p53 (Lambert et al., 1998). It is also plausible that SOCS1 can shape the pattern of ATM/ATR targets phosphorylated after DNA damage and influence the p53 response to the extent that other components of the p53 pathway can be recruited to ATM/ATR by SOCS1.

The decrease in expression of p53 targets in *Socs1/Ifn*γ null animals may have implications for diseases characterized by hyperproliferation of T cells, such as autoimmunity and lymphomas. In particular, SOCS1 was required for the expression of *Gadd45*α, and *Gadd45*α null mice display signs of hyperproliferation of T cells and autoimmune disease (Salvador et al., 2002, 2005). Also, *Bbc3/Puma*, a proapoptotic p53 target decreased in *Socs1* null T cells, is known to control T cell survival (Bauer et al., 2006). Consistent with our data, it has been reported that the p53 pathway plays a role in controlling the proliferation of $CD8^+$ T cells (Zhou et al., 1999). The impact of these defects in the expression of p53 target genes for T cells is not only a hyperproliferative response to cytokines but also a higher susceptibility to accumulate genetic alterations that may lead to transformation.

Concluding Remarks

SOCS1 has been considered almost exclusively as a gene that blocks cytokine signaling via the JAK/STAT pathway. The results presented here add another aspect of SOCS1 function: namely, its ability to interact with ATM/ATR and modulate the p53 pathway. In this way, SOCS1 can block an excessive cytokine response not only by blocking cytokine signaling in the cytoplasm, but also by activating p53 in the nucleus, preventing the proliferation of proinflammatory cells. Other negative regulators of cytokine signaling may share this paradigm. For example, in STAT5 senescence, we found multiple members of the SOCS family highly upregulated. Also, the E3-Sumo ligase PIASy regulates p53-dependent apoptosis and senescence (Bischof et al., 2006). These p53-activating activities can engage antiproliferative and anti-inflammatory programs in cells exposed to aberrant cytokine stimulation.

EXPERIMENTAL PROCEDURES

Cells and Tissue Culture

U2OS, H1299, and IMR90 cells were obtained from American Type Culture Collection (ATCC) and cultured in DMEM (GIBCO; Burlington, ON, Canada)

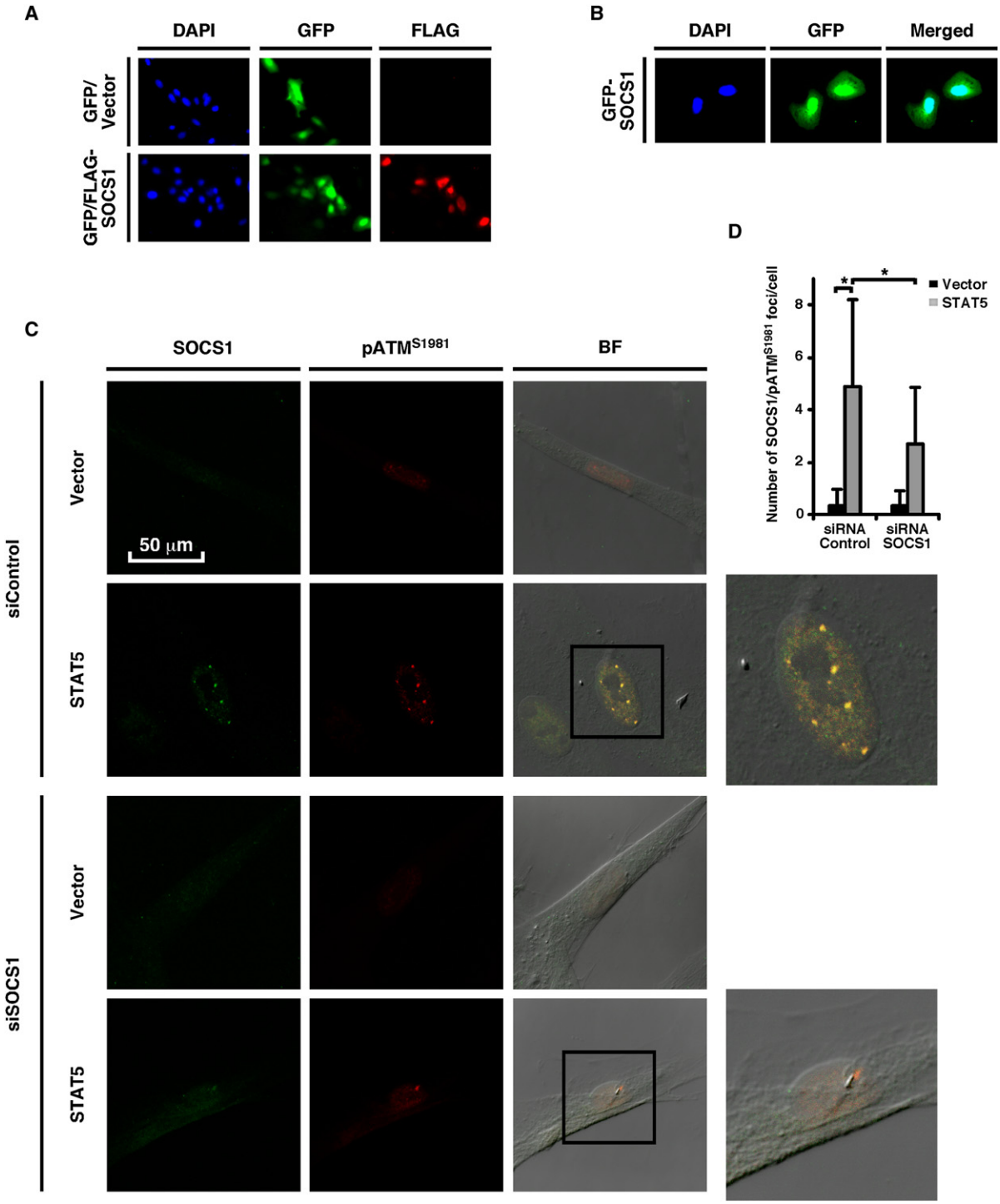

Figure 7. Cellular Localization of SOCS1

(A) Indirect Flag immunofluorescence of U2OS transiently transfected with GFP as a transfection control and an empty control vector or FLAG-SOCS1.

(B) Direct GFP fluorescence of U2OS cells transiently transfected with GFP-SOCS1. All cells (100%) transfected with the SOCS1 constructs in (A) and (B) were positive for SOCS1 in the nucleus.

(C) Confocal immunofluorescence of IMR90 fibroblasts expressing a vector control or its derivative expressing ca-STAT5A and transfected with siRNA control or against SOCS1. Cells were fixed 4 days after selection and stained with the antibodies recognizing SOCS1 and phospho-ATM^{S1981}.

(D) Quantitation of the percentage of cells having SOCS1/phospho-ATM^{S1981} foci. Data are presented as mean ±SD of triplicates. Asterisks represent a significant change in the number of SOCS1/phospho-ATM^{S1981} foci according to the paired t test: *$p < 0.001$.

supplemented with 10% FBS (Hyclone; Logan, UT) and 1% penicillin G/streptomycin sulfate (GIBCO). Retroviral-mediated gene transfer, BrdU incorporation, cell-cycle analysis, senescence assays, and fluorescence microscopy were done as described previously (Ferbeyre et al. 2000).

Mice

Socs1$^{+/-}$*Ifnγ*$^{-/-}$ mice (Marine et al., 1999) (generous gifts from J. Ihle) have been backcrossed onto the C57BL/6 background for more than ten generations in our animal facility. *Socs1*$^{-/-}$*Ifnγ*$^{-/-}$ mice were generated by crossing *Socs1*$^{+/-}$*Ifnγ*$^{-/-}$ parents. The *Socs1*$^{+/+}$*Ifnγ*$^{-/-}$ and C57BL/6 (*Socs1*$^{+/+}$*Ifnγ*$^{+/+}$) control mice were purchased from the Jackson Laboratory. All experiments using mice have been carried out with strict adherence to institutional guidelines.

T Cell Apoptosis

Single-cell suspensions of thymocytes from C57BL/6, *Ifnγ*$^{-/-}$, and *Socs1*$^{-/-}$ *Ifnγ*$^{-/-}$ mice were exposed to different doses of γ radiation as described (Clarke et al., 1993). Irradiated cells were incubated in RPMI-1640 culture medium containing 10% FCS at 37°C for 16 hr. The cells were stained with anti-mCD4-FITC and anti-mCD8-biotin (BD Biosciences; Mississauga, ON, Canada) followed by streptavidin SPRD (Southern Biotechnologies; Birmingham, AL) for 10 min each. After the final wash, the cells were stained with annexin-PE (BioLegend; San Diego, CA) following the manufacturer's instructions. The flow cytometry data were acquired using the FACSCalibur flow cytometer (BD Biosciences) and analyzed using the FlowJo software.

In Vitro Transcription/Translation

Flag-mSOCS1, Flag-mSOCS1ΔBox, and Flag-mSH2-SOCS1 were transcribed and translated in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega; Madison, WI) according to the manufacturer's instructions.

Coimmunoprecipitations

H1299 and U2OS were transfected with 20 μg of DNA using the calcium phosphate precipitation method. Twenty-four hours posttransfection, U2OS were treated with doxorubicin (300 ng/ml) for 24 hr. To prepare cell lysates, cells were washed twice with PBS and collected by scrapping in cold Cell Lysis Buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium Pyrophosphate, 1 mM β-glycerolphosphate, protease inhibitor cocktail [Roche; Mississauga, ON, Canada]) after 5 min of incubation on ice. Immunoprecipitations were performed with 1 or 2 mg of total cell proteins overnight at 4°C with a suitable antibody. The antibodies used for immunoprecipitation were as follows: anti-p53 (9282, 1:200, Cell Signaling; Beverly, MA), anti-SOCS1 (4H1, 1:200, MBL; Woburn, MA), anti-ATM (c-20, 1:200, Santa Cruz), anti-ATR (N-19, 1:200, Santa Cruz), anti-pATM^{S1981} (4526, 1:200, Cell Signaling), anti-Cyclin A (C-19, 1:200, Santa Cruz Biotechnology; Santa Cruz, CA), anti-Caveolin (BD, 1:200, Santa Cruz), and anti-E4BP4 (V-19, 1:200, Santa Cruz). Immunoprecipitates were recovered by 1 hr of incubation at 4°C with protein A/G Sepharose beads (Amersham/GE Healthcare; Baie d'Urfé, QC, Canada). Precipitates were washed three times with Cell Lysis Buffer, one time with PBS, and one time with H_2O. Precipitates were eluted in 30 μl of SDS Sample Buffer, 30 μl of H_2O, and 10 μl of Bromophenol Blue and boiled for 5 min. Half of elutions (35 μl) were separated on SDS-PAGE and transferred to Immobilon-P membranes (Millipore; Billerica, MA). For whole-cell lysates, 50–75 μg was loaded.

siRNA Transfections

Twenty-four hours before transfection, 1 × 10^4 IMR90 cells stably expressing pBabe or pBabe ca-STAT5A were plated in 6-well plates. siRNA against SOCS1 (siSOCS1) or siRNA against GFP (siGFP) (300 pmol each) (Supplemental Experimental Procedures) was transfected with Oligofectamine (Invitrogen; Burlington, ON, Canada) according to the manufacturer's instructions.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, eight figures, and one table and can be found online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00822-3.

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