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The heat shock transcription factor Hsf1 is downregulated in DNA damage–associated senescence, contributing to the maintenance of senescence phenotype

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Summary

Heat shock response (HSR) that protects cells from proteotoxic stresses is downregulated in aging, as well as upon replicative senescence of cells in culture. Here we demonstrate that HSR is suppressed in fibroblasts from the patients with segmental progerioid Werner Syndrome, which undergo premature senescence. Similar suppression of HSR was seen in normal fibroblasts, which underwent senescence in response to DNA damaging treatments. The major DNA-damage-induced signaling (DDS) pathways p53–p21 and p38-NF-kB-SASP contributed to the HSR suppression. The HSR suppression was associated with inhibition of both activity and transcription of the heat shock transcription factor Hsf1. This inhibition in large part resulted from the downregulation of SIRT1, which in turn was because of decrease in the expression of the translation regulator HuR. Importantly, we uncovered a positive feedback regulation, where suppression of Hsf1 further activates the p38–NF-κB-SASP pathway, which in turn promotes senescence. Overexpression of Hsf1 inhibited the p38–NFκB-SASP pathway and partially relieved senescence. Therefore, downregulation of Hsf1 plays an important role in the development or in the maintenance of DNA damage signaling-induced cell senescence.

Keywords

heat shock response; Hsp70; HuR; inflammation; p38; p53; SIRT1

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Introduction

Aging is the most important risk factor in the development of neurodegenerative and cardiovascular diseases, diabetes, osteoporosis, and many types of cancer. Various age-associated disorders are characterized by accumulation of damaged proteins (Jana *et al.*, 2000; Koyama *et al.*, 2006; Rahman *et al.*, 2010; Sakellariou *et al.*, 2011). Accordingly, the reduced capacity to handle misfolded protein has been implicated in the etiology of the disease process (Sherman & Goldberg, 2001). Protein aggregation and accumulation of damaged species in aging reflect the collapse in protein homeostasis (proteostasis; Ben-Zvi *et al.*, 2009; Taylor & Dillin, 2011), which in part results from the reduced ability to induce heat shock proteins that function as molecular chaperones in protein folding and degradation (Wu *et al.*, 1993; Heydari *et al.*, 1994). In turn, malfunction of the heat shock response (HSR) can shorten lifespan of the organism (Hsu *et al.*, 2003). In fact, in *Caenorhabditis elegans* model, suppression of HSR by downregulation of the heat shock transcription factor Hsf1 shortens the lifespan, while overexpression of Hsf1 extends it (Morley & Morimoto, 2004; Ben-Zvi *et al.*, 2009). As Hsf1 has been implicated both in aging and in disease, understanding its regulation as well as the effects of Hsf1 on aging and disease is of primary importance.

A NAD+-dependent deacetylase SIRT1 has been implicated in control of Hsf1. SIRT1 was found to deacetylate Hsf1 and keep it associated with the promoter region of heat shock proteins, thus enhancing the overall transcription from the Hsf1-responsive promoters (Westerheide *et al.*, 2009). In line with this finding, caloric restriction, which delays aging by increasing SIRT1 activity, increased HSR in aging rats (Heydari *et al.*, 1993). This finding provides a link between the organism aging and downregulation of HSR.

Certain features of the aging paradigm can be recapitulated in cell culture, where cells undergo replicative senescence. For example, downregulation of HSR was demonstrated in fibroblasts that underwent senescence following multiple passages in culture (Lee *et al.*, 1996). Interestingly, SIRT1 levels decrease in replicatively senescent cells (Sasaki *et al.*, 2006), suggesting that this factor may be involved in downregulation of HSR in senescence. Among various factors that regulate SIRT1, HuR binds to SIRT1 mRNA and increases its stability, and ultimately its translation (Fan & Steitz, 1998; Abdelmohsen *et al.*, 2007). Levels of HuR also diminish in senescent cells, suggesting that HuR may mediate the effects of senescence on SIRT1, and consequently on Hsf1.

Cell senescence results from the activation of DNA damage signaling (DDS) (Vaziri *et al.*, 1997; Robles & Adami, 1998; Chang *et al.*, 1999). Among DDS pathways, the p53–p21 pathways trigger the senescence, while the so-called senescence-associated secretion phenotype (SASP) is responsible for senescence maintenance (Rodier *et al.*, 2009; Freund *et al.*, 2011). SASP represents secretion of a variety of signaling molecules, for example, IL-6, IL-1 or IL-8, controlled by the activation of p38MAPK and NF-κB (Acosta *et al.*, 2008; Orjalo *et al.*, 2009). In addition, p38MAPK regulates a distinct senescence factor p16 (Du *et al.*, 2009).

As a model of aging, we chose fibroblasts from patients with Werner Syndrome (WS). WS is caused by a mutation in the *WRN* gene that presents with premature aging (Goto, 1997). Fibroblasts from patients with WS demonstrate premature senescence and show accumulation of DNA damage (Wyllie *et al.*, 2000).

Here, we investigated the role of DNA damage in the suppression of HSR. Specifically, we probed the main DDS pathways as potential modulators of HSR. We found that Hsf1 activity decreases in DDS-induced senescence in part because of the changes in SIRT1 and

HuR. Furthermore, we found a new role of Hsf1 as a modulator of senescence phenotype (Experimental procedures are given in Supporting information).

Results

DNA-damage-induced senescence is associated with suppression of the HSR

To understand how HSR is modulated in aging, we investigated HSR in skin fibroblasts isolated from prematurely aging patients with WS. We used fibroblasts isolated from three patients with WS aged between 19 and 30 at passages 4 to 11 and fibroblasts from normal individuals as age-matched controls, ages between 22 and 27 at passage 14 to 16. Previously, WS fibroblasts were found to undergo premature senescence (Goto, 1997). We observed the typical senescent morphology, including enlargement, flattening, and vacuolization, in WS fibroblasts, which was of a stark contrast to fibroblasts from an age-matched subject (Fig. S1). To assess the HSR in WS, cells were incubated at 43 °C for 35 min, left to recover for 6 h, and expression of the major inducible heat shock protein Hsp70 was measured by immunoblotting. These conditions for HSR were used throughout the study. Compared to fibroblasts from age-matched healthy subjects, WS fibroblasts demonstrated strong decrease in the induction of Hsp70 (Fig. 1A). Unlike finite lifespan WS fibroblasts, hTERT immortalized WS cells did not show suppressed HSR compared with normal hTERT-immortalized fibroblasts (Fig. S2). Therefore, we suggested that downregulation of the HSR in WS fibroblasts could be associated with premature senescence.

The premature aging phenotype of WS, which is caused by the mutation in the DNA helicase *WRN* gene, is associated with DNA instability (Rossi *et al.*, 2010). Diminished induction of Hsp70 in WS fibroblasts could be connected to the DNA damage. Accordingly, we tested whether DNA damaging treatments in normal cells could have a similar suppressive effect on the HSR. Normal human diploid fibroblasts TIG-1 in early passage (between p12 and p15) were treated with 100 nM doxorubicin (Dox) overnight or exposed to 10 Gy γ–irradiation (IR) and then cultured for 5 days. By day 4, such treatments led to withdrawal from the cell cycle, as indicated by reduced Ki-67 staining, and increased senescence-associated β-galactosidase activity (SA-β-gal). These features corresponded to the appearance of senescence phenotype, well in agreement with previous reports (Chang *et al.*, 1999; Fig. S2A,B). As observed with WS fibroblasts, normal fibroblasts exposed to DNA damaging agents showed significantly suppressed induction of Hsp70 (Fig. 1B). Therefore, similar to replicative senescence, premature senescence by DNA damage causes suppression of the HSR in both normal and disease, and establishes a useful model to investigate the relationship between senescence and the HSR (Fig. 6I).

DNA-damage-induced signaling (DDS) pathways regulate HSR

DNA damage can activate the p53 and the p38MAPK signaling pathways, both of which contribute to the development of senescence (Rodier *et al.*, 2009; Freund *et al.*, 2011). Here, we tested the contribution of each pathway in the suppression of HSR. By day 5 post-treatment, γ-irradiated TIG-1 cells showed strong activation of p53 pathway (Fig. 1C), as well as activation of p38MAPK pathway (Fig. 2A).

To further characterize the role of the p53 signaling pathway in the suppression of HSR, we up regulated p53 without DNA damage by treating the cells with 10 μM nutlin-3, a compound that stabilizes and activates p53 (Vassilev, 2004). By day 4, early passage cells exhibited increased p53 and p21 levels, and increased SA-β-gal staining, indicating induction of the senescent phenotype (Fig. S4A,B). Induction of Hsp70 by heat shock was strongly reduced in cells treated with nutlin-3 compared to control cells (Fig. 1D), indicating

that upregulation of p53 and downstream activation of the DDS even without DNA damage are sufficient for the HSR suppression.

Then we sought to determine the effects of upregulating p21, a downstream target of p53, and an important regulator of cell senescence. The p21 expression by retroviral infection in early passage cells led to the development of the senescence phenotype, which appeared 5–6 days after retroviral infection (Fig. S4A,B). As in the experiment with nutlin-3, expression of recombinant p21 caused significant decrease in induction of Hsp70 (Fig. 1E). All together, these results demonstrated that prolonged activation of the p53–p21 signaling is sufficient to suppress the HSR, (see the scheme on Fig. 6I).

To test the possibility that inhibition of cell cycle suppresses HSR, we tested HSR in cells that have been growth arrested at G1 without activating DDS pathway, and without senescence. Accordingly, early passage TIG-1 was treated with reversible inhibitors of cyclin D/CDK4 and 6 complexes, 1 μM of CDK4 inhibitor (2-Bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione) and 10 μM of CDK4/6 inhibitor IV (trans-4-((6-(ethylamino)-2-((1-(phenylmethyl)-1H-indol-5-yl)amino)-4-pyrimidinyl)amino)-cyclohexanol). After 3 days, treatments with either inhibitor led to G1 arrest (as judged by the G1 reporter (Sakaue-Sawano *et al.*, 2008) (Fig. S5). Similar cell cycle arrest was seen in cells overexpressing p21 or treated with nutlin-3 (Fig. S5). However, unlike the latter, cells treated with CDK inhibitors did not demonstrate SA-β-gal staining, indicating that they are not senescent (Fig. S6). Importantly, these growth-arrested but not senescent cells showed normal HSR (Fig. S7)

To examine whether the p53–p21 signaling was necessary for suppression of HSR by DDS, we depleted either of these proteins by retroviral shRNA expression. First, p53-depleted TIG-1 cells were treated with nutlin-3, as described earlier, and HSR was assessed. p53 depletion prevented p21 accumulation and the SA-β-Gal staining (Fig. S4A,C) and prevented suppression of HSR following exposure to nutlin-3 (Fig. 1F). Therefore, the inhibitory effect of nutlin-3 on HSR requires p53. More importantly, we observed reversion of HSR suppression in cells that were exposed to IR (Fig. 1G). These data demonstrated that p53 is an important factor in suppression of HSR in DNA-damage-induced senescent cells. Interestingly, p53-depleted fibroblasts showed both higher basal levels of Hsp70 and enhanced HSR compared to control empty-vector-infected cells. In contrast, depletion of p21 did not reverse the effect of nutlin-3 on HSR (Fig. S8), while, as shown earlier, expression of recombinant p21 had effectively suppressed HSR. This apparent contradiction indicated that p53 could affect HSR both via p21-dependent and p21-independent mechanisms. The p21-independent effect of p53 on HSR could result from the crosstalk between the p53 and p38MAPK pathways. In fact, we observed that nutlin-3 treatment alone caused activation of p38MAPK pathway (Fig. S9).

As such, we further investigated the contribution of the p38MAPK pathway in the suppression of HSR. In addition to the p53 pathway, IR of TIG-1 cells activated the p38MAPK signaling pathway, which plays a major role in maintenance of senescent phenotype (Rodier *et al.*, 2009). By day 5 following exposure to 10 Gy IR, we observed increased phosphorylation of p38MAPK and phosphorylation of its substrate Hsp27 (Fig. 2A). Activation of p38MAPK led to the activation of its downstream target NFκB, as measured by the NFκB luciferase reporter (Fig. S10A), which in turn caused a dramatic rise (8- to 10-fold) in mRNA levels of IL-6 and IL-8 cytokines (Fig. S10B), which are necessary for senescence maintenance. Another downstream target of the p38MAPK pathway, the cyclin-dependent kinase inhibitor p16, was also induced in TIG-1 cells following DNA damage (Fig. S11A). In line with this observation, expression of recombinant p16 led to an

increased SA-β-gal staining and suppression of Hsp70 induction (Fig. 2B; Figs S3B and S11B).

To determine the role of p38MAPK in DDS-induced HSR downregulation, we tested whether this downregulation is affected by inhibition of p38MAPK activity. TIG-1 cells were exposed to 10 Gy IR and cultured for 6 days in the presence or absence of specific p38MAPK inhibitor SB203580 (SB) (Davies *et al.*, 2000). SB blocked p38MAPK kinase activity induced by IR as demonstrated by suppression of Hsp27 phosphorylation and IL-6 and IL-8 transcription (Fig. 2A; Fig. S10B). Importantly, inhibition of p38MAPK significantly improved HSR in IR-induced senescent cells (Fig. 2C). Similar to the effects on Hsp70 levels after p53 depletion, p38MAPK inhibition led to significant increase in the basal levels of Hsp70, as well. Together these data indicate that both the p53–p21 and p38MAPK–SASP pathways contribute to the downregulation of the HSR in DDS-induced senescence (see Fig. 6I).

Role of SIRT1 in regulation of Hsf1 in senescence

Next we explored which step of HSR was compromised in DDS-induced senescent cells. Activation of HSF1 and its binding to heat shock elements (HSE) is the major event to induce the transcription of Hsp70 and other heat shock proteins. To assay this activity, lentiviral reporter construct was prepared by inserting six tandem repeats of HSE upstream of firefly luciferase controlled by minimal CMV promoter. To control efficiency of infection, along with the HSE luciferase virus, we co-infected the cells with retrovirus expressing GFP under the constitutive CMV promoter. Accordingly, the ratio between HSE luciferase activity and GFP expression represented the Hsf1 activity. Luciferase activity increased by almost 30-fold in heat shocked cells (Fig. 3A). However, Dox or IR treatment prior to HS dramatically reduced (by 65–80%) the Hsf1-dependent luciferase activity (Fig. 3A). Moreover, upregulation of one of the elements in the DDS pathways, namely p53, p21 or p16, similarly suppressed the Hsf1-driven luciferase activity (Fig. 3A). Hence, suppression of HSR in DDS-induced senescence is caused by the downregulation of Hsf1.

Here we assessed Hsf1 activity and its levels. The histone decetylase SIRT1 was previously shown to form complex with Hsf1 during heat shock and regulate its activity (Westerheide *et al.*, 2009). We hypothesized that in senescent TIG-1 fibroblasts, Hsf1 transcriptional activity was decreased because of reduced association with SIRT1.

Accordingly, we expressed FLAG-tagged Hsf1 using retroviral expression system. Hsf1 was immunoprecipitated (IP) with anti-FLAG antibody from lysates of heat-shocked control and nutlin-3-treated cells. The amount of SIRT1 in complex with Hsf1 assessed by immunoblotting with anti-SIRT1 antibody was dramatically reduced in nutlin-3-treated fibroblasts compared to control cells (Fig. 3B). This reduction was associated with significant decrease in the SIRT1 levels (by 90%) in the nutlin-3-treated cells (Fig. 3C). Similarly, a remarkable decrease in the SIRT1 levels was observed in senescent cells induced by p21 or p16 expression and in cells subjected to IR (Fig. 3D; Fig. S12A). Thus, upon premature senescence induced by DDS, expression levels of the critical Hsf1 regulator SIRT1 are diminished.

To understand how SIRT1 is downregulated in premature senescence, we compared SIRT1 mRNA levels measured by qRT-PCR. Indeed, SIRT1 mRNA levels were reduced by 60% in the DDS-induced senescent cells compared to control (Fig. 3E; Fig. S12B), indicating that transition to senescence affects either SIRT1 transcription or stability of its mRNA. We further assessed decay of this mRNA following addition of actinomycin D to inhibit transcription. SIRT1 mRNA half-life was >5 h in control cells while in nutlin-3-induced senescent cells, half-life dropped to <45 min (Fig. 3F). This indicated that the reduction in

the SIRT1 levels in prematurely senescent cells is associated with decreased mRNA stability.

Role of HuR in SIRT1-mediated regulation of Hsf1 activity in senescence

The RNA binding protein HuR (ELAV1) is the major regulator of SIRT1 mRNA stability (Abdelmohsen *et al.*, 2007). Using the shRNA approach, we confirmed that depletion of HuR in TIG-1 cells led to a dramatic drop in SIRT1 levels (Fig. S13). Because HuR levels decrease in replicative senescence (Marasa *et al.*, 2010), we hypothesized that HuR expression may also be reduced in cells that underwent senescence in response to DNA damage signaling. Indeed, HuR protein levels were significantly reduced in TIG-1 cells following treatments with Dox, IR or nutlin-3 as well as expression of p21 or p16 (Fig. 3G; Fig. S14A). In all these models of premature senescence, HuR downregulation resulted from decrease in mRNA levels (Fig. 3H; Fig. S14B). There was no significant change in half-life of HuR mRNA (Fig. S15), suggesting that effects of senescence on HuR were mostly because of transcription inhibition.

Furthermore, as shown in Fig. 3I, we observed a dramatic drop of both HuR and SIRT1 levels in WS fibroblasts suggesting that pathways of suppression of the HSR are similar in DDS-induced senescence and in the disease.

Hsf1 levels are decreased in DNA-damage signaling-induced senescence

To determine the contribution of SIRT1 in the HSR regulation in TIG-1 fibroblasts, we evaluated effects of the SIRT1 inhibitor nicotinamide on activity on Hsf1 in early passage cells. In line with previous reports, SIRT1 inhibition led to nearly 40% decrease in the Hsf1 activity measured with the HSE luciferase reporter (Fig. 4A), or by immunoblotting for Hsp70 (Fig. S16). Similarly, depletion of SIRT1 strongly (by 60%) inhibited Hsf1 activity (Fig. 4A). Analogous effect was seen in cells with depleted HuR (Fig. 4A), demonstrating that the HuR–SIRT1 pathway regulates Hsf1 in this system. Of note, treatment with nicotinamide or SIRT1 depletion did not cause premature senescence as measured by SA-β-gal or p53 and p21 (Figs S17 and S18A,B).

Beyond regulating Hsf1 activation, depletion of either HuR or SIRT1 consistently caused reduction (about 20%) of Hsf1 protein levels, suggesting that the histone deacetylase activity of SIRT1 contributes to Hsf1 expression (Fig. 4B). In line with these data, we discovered that Hsf1 expression levels were significantly decreased in TIG-1 fibroblasts treated with DNA damaging agents or nutlin-3, and in WS fibroblasts (Fig. 4B,C). In contrast to senescent fibroblasts, lack of HSR downregulation in growth-arrested fibroblasts correlated with the lack of downregulation of HuR, SIRT1, and Hsf1 (Fig. S19). Similar correlation was seen in control and hTERT-immortalized WS fibroblasts (Fig. S20). These data further demonstrate that both Hsf1 activity and the levels are regulated in senescence in HuR–SIRT1-dependent manner. Importantly, nutlin-3 treatment had a stronger inhibitory effect on HSF1 activity (90% inhibition) and levels (40% downregulation) compared to SIRT1 or HuR depletions (Fig. 4A,B). Furthermore, combination of either of these depletions and nutlin-3 treatment did not inhibit the HSR beyond the inhibition by nutlin-3 alone (Fig. 4A,B). These results indicate that nutlin-3 treatment downregulates Hsf1 via both HuR–SIRT1-dependent and HuR–SIRT1-independent mechanisms, and the latter mechanism(s) is likely to affect Hsf1 levels.

The effects of nutlin-3 on HSR were associated with reduction of the Hsf1 mRNA (Fig. 4D), but not the stability of mRNA (Fig. 4E) or protein (not shown). Taken together, these results demonstrate that the stress-induced senescence downregulates Hsf1 via multiple

mechanisms, including HuR–SIRT1 pathway, which ultimately leads to suppression of the HSR (see Fig. 6I).

Major DNA-damage signaling pathways are involved in regulation of HuR, SIRT1, and Hsf1

As DDS-induced senescence led to decreased HuR–SIRT1–Hsf1 levels, we tested whether inhibition of either p53 or p38MAPK pathways can restore levels of HuR–SIRT1 and expression of Hsf1. Depletion of p53 significantly increased the levels of HuR, SIRT1 and Hsf1 in naïve cells. Moreover, p53 depletion averted downregulation of the levels of these proteins after nutlin-3 or IR treatments (Fig. 4F; Fig. S21), indicating that p53 significantly contributed to the suppression of the HuR–SIRT1–Hsf1 pathway in senescence (see Fig. 6I). Similarly, we inhibited the p38MAPK pathway with SB and tested HuR, SIRT1 and Hsf1 levels. Cells were treated with nutlin-3 or Dox with or without SB, Dox was removed after 2 days, and all samples were grown for 5 days. Figure 4G and Figure S22(A) show that inhibition of p38MAPK increased the levels of HuR, SIRT1 and Hsf1 in naïve cells and partially restored these levels in DDS-induced senescent cells. In line with these data, we observed restoration of levels of Hsp70 (Fig. S22B,C), which further indicated that HuR–SIRT1–Hsf1 pathway can be regulated by the p38MAPK–SASP signaling (see Fig. 6I).

To investigate the relevance of these findings to the disease, we asked whether HuR–SIRT1–Hsf1 pathway can be restored in WS fibroblasts by the p38MAPK inhibition. This possibility was in line with the previously published study showing that extended inhibition of p38MAPK improved proliferation and reduced indicators of premature senescence in WS fibroblasts (Fleming *et al.*, 2005). We observed that incubation of WS fibroblasts with SB for 24 days led to a restoration of the levels of HuR, SIRT1 and Hsf1 (Fig. 4H). In these cells, we also observed an impressive restoration of HSR measured by induction of Hsp70 (Fig. 4I). These data indicate that the p38MAPK–SASP pathway contributes to suppression of Hur–SIRT1–Hsf1 pathway in WS.

HSF1 regulates HuR–SIRT1 pathway via a positive feedback

Using *C. elegans*, it was demonstrated that Hsf1 plays a major role in progression of aging: knockdown of Hsf1 accelerated this process, while overexpression delayed it (Hsu *et al.*, 2003; Ben-Zvi *et al.*, 2009; Anckar & Sistonen, 2010). The finding that SIRT1 is involved in the regulation of Hsf1 in senescence suggests that Hsf1 may act downstream and contribute to anti-aging effects of SIRT1. Alternatively, there may be bi-directional relationship between SIRT1 and Hsf1. To address these possibilities, we tested the effects of Hsf1 depletion on the levels of SIRT1. Indeed, depletion of Hsf1 caused a decrease in SIRT1 protein levels by 60% and its mRNA levels by 50%, similar to the effects seen in the senescent cells (Fig. 5A,B). Furthermore, similar to its effects on SIRT1, Hsf1 depletion caused a dramatic decrease in both protein and mRNA levels of HuR (Fig. 5A,B). Thus, Hsf1 regulates the HuR–SIRT1 pathway (see Fig. 6I).

To investigate whether the discovered Hsf1 signaling occurs in vivo, we compared the levels of HuR and SIRT1 in wild-type and Hsf1 null mice. Twelve months old Hsf1 null mice and age-matched controls were sacrificed, and protein and mRNA levels of SIRT1 and HuR were assessed in various tissues. Indeed, the levels of both SIRT1 and HuR mRNA in brain and muscle were significantly decreased in the Hsf1 null mice compared to the wild-type animals (Fig. 5C,D). Consistent with the mRNA results, Hsf1 knockout strongly decreased the levels of HuR and SIRT1 protein in the muscle and to a lesser degree in the brain (Fig. 5E). These data demonstrate that feedback regulation of SIRT1 and HuR by Hsf1 occurs at the level of organisms.

Altogether these data demonstrate that decrease in HuR–SIRT1 leads to the suppression of Hsf1, while Hsf1 suppression in turn causes downregulation of HuR and SIRT1, thus creating a positive feedback loop.

Hsf1 plays a role in regulation of DNA-damage signaling-induced senescence

How does Hsf1 signal back to regulate HuR and SIRT1 levels? Because the effects of HSF1 depletion on SIRT were similar to those seen in DDS-induced senescence, we explored effects of Hsf1 on p53 and p38MAPK signaling pathways upstream of SIRT1 and on overall senescence development. We found that depletion of Hsf1 increased a fraction of SA-β-gal-positive cells, similar to the one observed after nultin-3 treatment (Fig. 6A). Moreover, Hsf1 depletion caused senescence associated heterochromatin foci formation, further evidencing senescence (Fig. S23). Importantly, depletion of p53 did not reverse SA-β-gal staining caused by Hsf1 depletion (Fig. 6A). Furthermore, even though we observed an upregulation of p53 and p21 in Hsf1-depleted cells, their levels were significantly lower than the levels seen in DDS-induced senescent cells (Fig. 6B). These data suggested that the development of the senescence phenotype in Hsf1 depleted cells was independent of p53 activity and that the alternative p38MAPK–SASP pathway may be an important contributor to the feedback loop. As an indicator of p38MAPK pathway activation, levels of p16 were elevated after Hsf1 depletion (Fig. 6B).

To test whether senescence in Hsf1 depletion was mediated by increased p38MAPK activity and SASP, we explored the role of Hsf1 in regulation of this pathway. Previously, it has been demonstrated that Hsf1 can regulate IL-6 and other chemokines and that LPS-stimulated levels of IL-6 were higher in Hsf1 knockout mice than in wild-type mice (Takii *et al.*, 2010). Here, we observed a strong increase in p38MAPK activity after Hsf1 depletion, measured by both phosphorylation of p38MAPK and phosphorylation of its substrate Hsp27 (Fig. 6C). This activation was comparable to the activation of p38MAPK by nutlin-3 (Fig. S24). Furthermore, the levels of IκB phosphorylation, which indicate activation of NFκB, as well as NFκB firefly luciferase reporter activity were increased significantly upon Hsf1 depletion (Fig. 6D; Fig. S25). As a corollary, we detected significantly increased transcription of NFκB target IL-6 (Fig. 6E). Interestingly, treatment with the p38MAPK inhibitor SB partially reversed SA-β-gal staining following Hsf1 depletion (Fig. S26). These data indicate that downregulation of Hsf1 following DNA damage signals back to the p38MAPK–SASP pathway to facilitate the maintenance of senescence (see Fig. 6I).

To further explore the role of Hsf1 in control of DDS-induced senescence pathways, we tested whether Hsf1 can suppress the activation of the p38MAPK–SASP pathway and the establishment of senescence phenotype after DNA damaging stress. Accordingly, we stably expressed FLAG-tagged Hsf1 in early passage TIG-1 cells using retroviral expression system and exposed them to IR. As a control, we used TIG-1 cells expressing an empty vector. Hsf1 strongly suppressed the IR-induced activation of p38MAPK, measured by reduced phosphorylation levels of both p38MAPK and Hsp27 (Fig. 6F). Similarly, it strongly inhibited production of IL-6 and IL-8 in the cells exposed to radiation (Fig. 6G; Fig. S27). These effects culminated in suppression of the DDS-induced senescence. In fact, we observed about 40% decrease in the fraction of SA-β-gal-positive cells in the irradiated samples (Fig. 6H). Taken together, these data demonstrate that Hsf1 plays an important role in DDS-induced senescence signaling via a positive feedback loop to regulate p38MAPK–SASP pathway (see Fig. 6I).

Discussion

This investigation provided an insight into the mechanisms of HSR suppression in DDS-induced senescence, including the multiple levels of Hsf1 regulation. Our data demonstrate

that in prematurely senescent cells from patients with WS, the suppression of HSR is caused by DDS. Moreover, the activation of DDR signaling that induces senescence in early passage cells leads to significant downregulation of Hsf1 levels and activity. Here, in the first endeavor to understand the effects of senescence signaling on HSR, we demonstrated that both p53–p21 and p38MAPK–SASP pathways significantly contribute to regulation of Hsf1. Both pathways affect Hsf1 activity and levels in large part via regulation of SIRT1 levels, which in turn are controlled by the translation regulator HuR (see Fig. 6I). These effects were seen both in normal fibroblasts exposed to DNA damage and in fibroblasts from patients with WS.

A significant advance in this work is the finding that SIRT1 and Hsf1 regulate each other in a positive feedback loop. In fact, depletion of SIRT1 or HuR caused downregulation of Hsf1, while depletion of Hsf1 caused downregulation of HuR and SIRT1. As SIRT1 and Hsf1 were implicated in aging, these feedback relations may explain multiple effects of both proteins on the aging process. Importantly, the discovery of the same regulation in tissues of Hsf1 knockout mice enables us to extrapolate the implications of DNA damage at the cellular level to an organismal level.

This study also established the role of Hsf1 in regulation of the senescence- associated secretory phenotype. SASP represents a chronic inflammatory response, some components of which, for example, IL-6 or IL-1, were reported to control cell senescence. Here, we indeed demonstrate that one of the endpoints of SASP is downregulation of HuR–SIRT1, and subsequent downregulation of Hsf1. In line with these data, chronic inflammation mediated by p38MAPK was reported to play an important role in cessation of growth of WS fibroblasts, and possibly the overall acceleration of aging of patients with WS (Davis *et al.*, 2005). Here we found that inhibition of p38MAPK partially restores Hsf1, as well as HuR and SIRT1. It is possible that such restoration may significantly improve patients' conditions.

Previously it was reported that the transcription factor C/EBPβ, which responds to SASP, can directly inhibit Hsf1 in human monocytes (Xie *et al.*, 2002). In our system, effects of SASP are certainly distinct, because we could not detect significant effects of C/EBPβ depletion on Hsf1 activity (not shown), even though SASP was clearly associated with downregulation of HuR–SIRT1 axis.

Importantly, the feedback regulation of HuR–SIRT1 upon Hsf1 depletion is mediated by the p38MAPK–SASP pathway. This finding is consistent with previous reports that suggest enhanced inflammatory response in Hsf1 knockout animals (McMillan *et al.*, 1998). In fact, such mice were more sensitive to LPS, and demonstrated stronger upregulation of TNF in response to LPS (Xiao *et al.*, 1999; Takii *et al.*, 2010). In this study, we have found the inextricable link between pro-inflammatory cytokines and Hsf1 activity. In addition to keeping the proteome in balance, Hsf1 prevents establishment of microenvironment that adversely affects the organism. This study introduces a novel perspective on the relations between proteostasis and aging. Previous reports indicated the necessity of functional Hsf1 in extension of lifespan or in decreasing onset of protein misfolding diseases, implicating the importance of proteostasis in these conditions. On the other hand, as pro-inflammatory cytokines, especially IL-6 has been linked to age-related diseases and certain types of cancer, our findings indicate that at least in part effects of Hsf1 could be associated with regulation of signaling pathways involved in chronic inflammation.

A significant finding in this work is that Hsf1 by the feedback signaling can suppress cell senescence. In fact, depletion of Hsf1 facilitated senescence, while overexpression of Hsf1 reduced DNA-damage-induced senescence. In other words, DDS via downregulation of

HuR–SIRT1 pathway suppresses Hsf1, which in turn enhances the p38MAPK–SASP pathway, thus supporting chronic inflammation and senescence phenotype (Fig. 6I). These findings uncover unexpected relations between the DNA damage and proteostasis; in normal cells, the proteostasis regulator Hsf1 controls cessation of proliferation of cells with damaged DNA. It is conceivable that failure of this control in cancer cells contribute to the loss of growth cessation of cells with DNA damage/instability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of abbreviations

HSR	heat shock response
DDS	DNA damage-induced signaling
SASP	senescence-associated secretion phenotype
WS	Werner Syndrome
Dox	doxorubicin
IR	γ-irradiation
SA-b-gal	senescence-associated b-galactosidase activity
HSE	heat shock elements
SB	SB203580 compound

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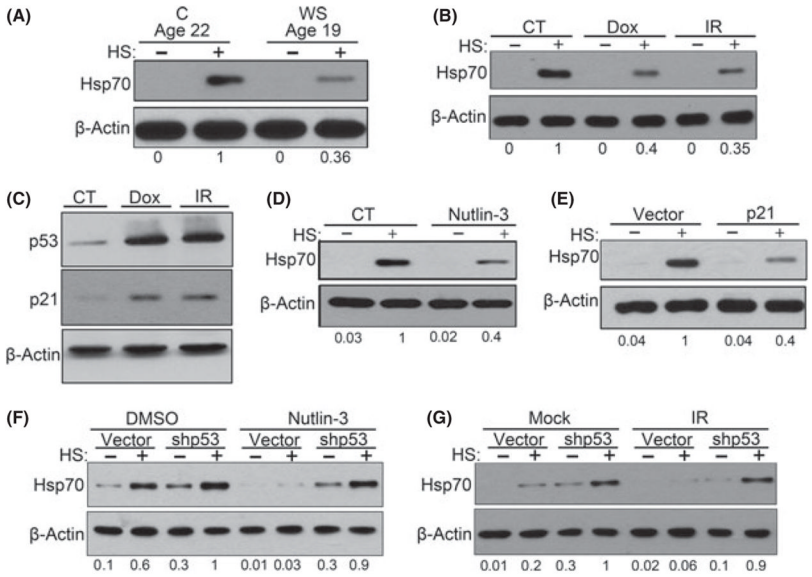

Fig. 1.

Activation of p53 pathway caused suppression of HSR. (A) WS fibroblasts (passage 4) and age-matched C fibroblasts (passage 15) were heat shocked and were collected for immunoblotting. (B) TIG-1 fibroblasts were treated with 100 nM of Dox overnight or 10 Gy IR. After 6 days, cells were heat shocked and incubated for 6 h before collection. (C) Same cells from (B) were immunoblotted for indicated proteins. (D) Cells that were induced senescent by 5 days of 10 μM nutlin-3 treatment were heat shocked, and Hsp70 levels were measured. (E) Hsp70 accumulation after heat shock in cells that overexpress p21. (F) TIG-1 cells were infected with empty or shp53 retroviruses, and nutlin-3 was added for additional 5 days. Cells were collected 6 h after heat shock and immunoblotted for Hsp70. Interestingly, we observed an increase in basal level and after heat shock level of Hsp70 upon p53 depletion. (G) Control or p53-depleted cells were treated with 6 Gy IR and cultured for 5 days. Cells were collected 6 h after heat shock and immunoblotted for Hsp70. Abbreviations: C, age-matched healthy control; CT, control.

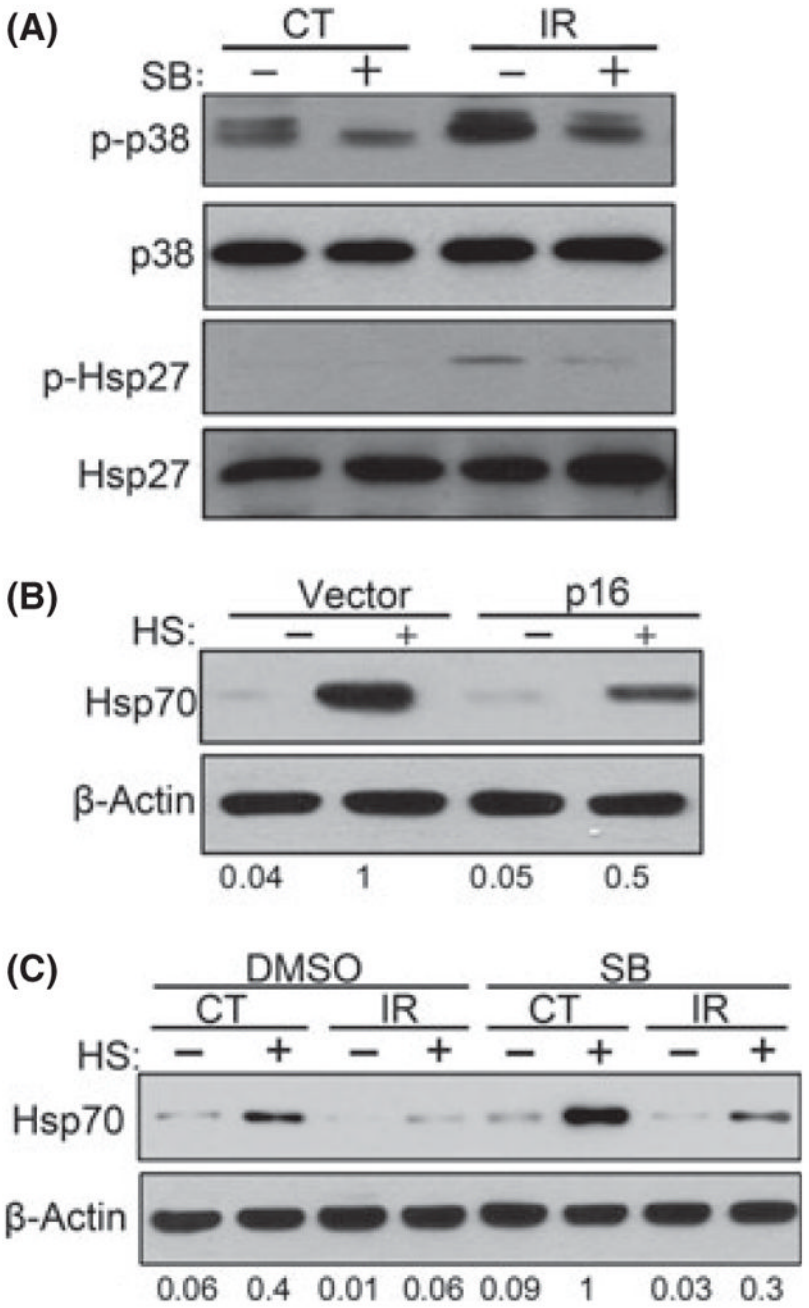

Fig. 2.

Activation of p38MAPK in DNA-damage-induced senescence contributes to suppression of HSR. (A) Early passage cells were pre-treated with p38MAPK inhibitor SB for 6 h prior to 10 Gy IR. After IR, cells were incubated for 6 days before collection. Levels of p–p38 and p-Hsp27 were measured by immunoblotting. (B) Hsp70 was measured after heat shock in cells with overexpression of p16. (C) Same cells as from (A) were heat shocked and incubated for 6 h before collection. Addition of SB led to partial restoration of Hsp70 induction level after heat shock.

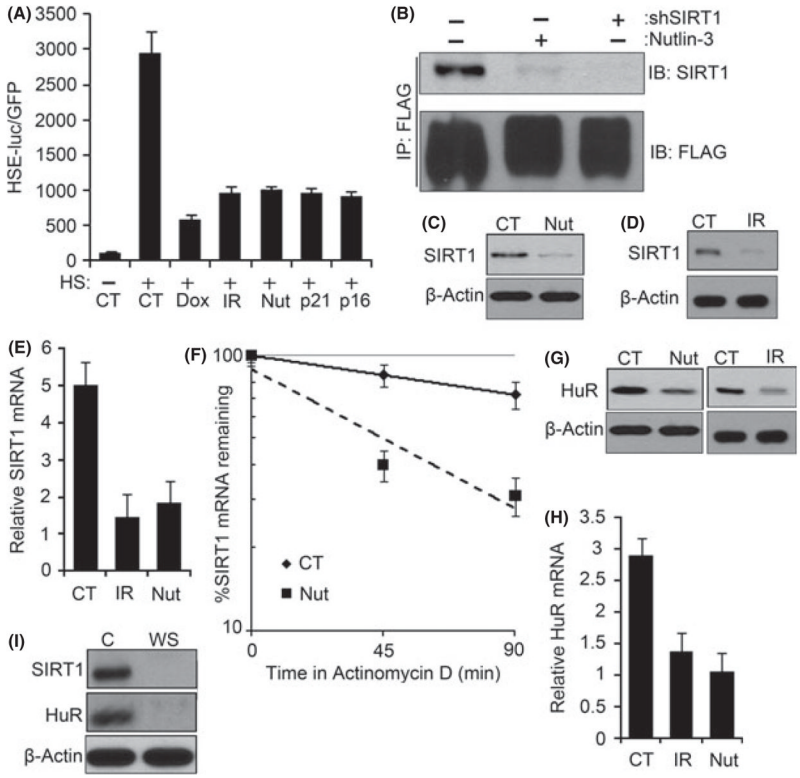

Fig. 3.

DNA-damage-induced senescence suppresses Hsf1 activity by decreasing SIRT1. (A) TIG-1 fibroblasts were infected with the HSE-luciferase and treated with 100 nM Dox overnight and cultured for 6 days; 10 Gy IR and cultured for 6 days; 10 μM nutlin-3 for 5 days or retroviral expression of p21 or p16 for 6 days. After heat shock, cells were incubated for 6 h before collection, and luciferase activity was measured. The means and ±SEM indicate three independent experiments. (B) FLAG-tagged wild-type Hsf1 was expressed with retrovirus in TIG-1 fibroblasts and treated with 10 μM of nutlin-3 for 5 days to induce senescence or depleted of SIRT1 by lentiviral shRNA. Cells were collected promptly after heat shock at 43 °C for 1 h, and immunoprecipitated with anti-FLAG antibody. The precipitates were immunoblotted for SIRT1. (C and D) Early passage TIG-1 fibroblasts were treated as in (A) and immunoblotted for SIRT1. (E) TIG-1 fibroblasts exposed to conditions mentioned (A) were collected for RNA, and qRT-PCR was performed using SIRT1 and GAPDH (housekeeping gene for control) mRNA-specific primers. The means and ±SEM are from three independent experiments. (F) SIRT1 mRNA half-life was measured in control (CT) and nutlin-3-induced senescent cells (Nut) by incubating with 5 μg mL^{-1} of actinomycin D and collecting RNA after 45 and 90 min. SIRT1 mRNA was normalized for GAPDH mRNA. The means and ±SEM were calculated from triplicates of two independent experiments. (G) Early passage TIG-1 fibroblasts were exposed to conditions mentioned in (A), and HuR protein levels were measured by immunoblotting. (H) HuR mRNA levels were measured by qRT-PCR, as in (F). The means and ±SEM are from three independent experiments. (I) Same set of cells as in Fig. 1(A) were immunoblotted for SIRT1 and HuR.

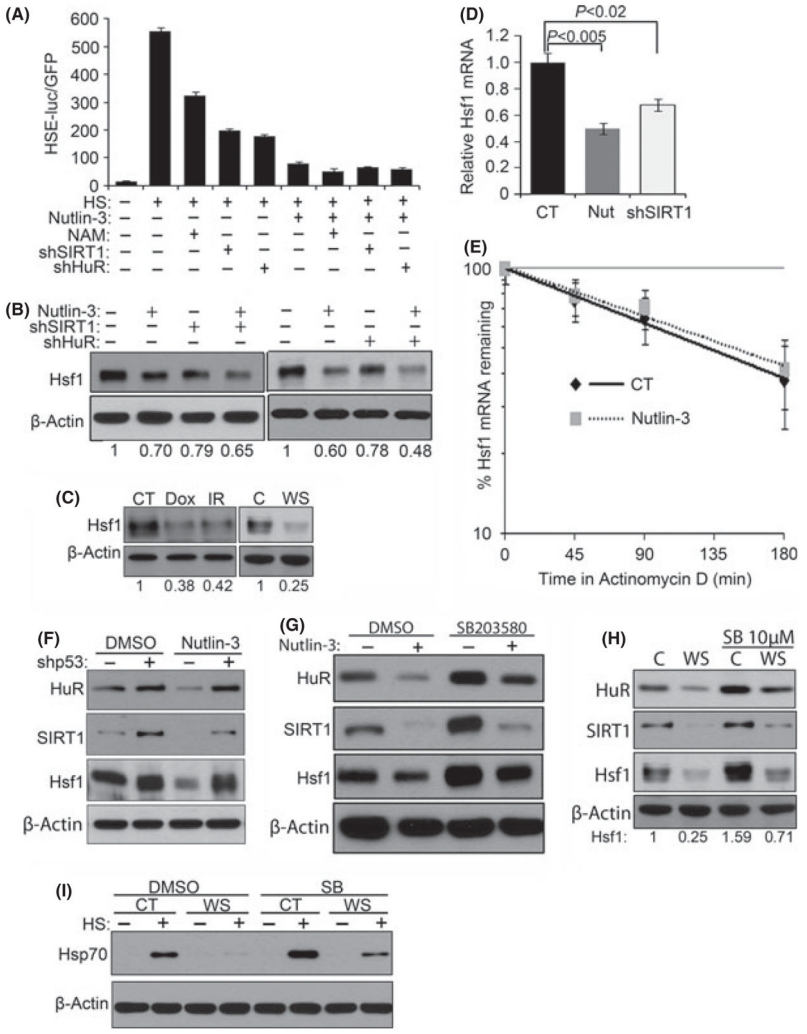

Fig. 4.

Decrease in Hsf1 level in senescence is related to SIRT1 downregulation. (A) HSE-luciferase lentiviral construct was expressed in early passage cells and treated with 5 mM NAM overnight, nutlin-3 for 5 days, or infected with shRNA for SIRT1 or HuR. After heat shock, cells were incubated for 6 h before collection, and luciferase activity was measured. The means and ±SEM indicate three independent experiments. (B) Control vector or lentiviral shRNA for either SIRT1 or HuR was expressed in early passage TIG-1 fibroblasts. Cells were selected with puromycin and treated with 10 μM nutlin-3 for 5 days. Levels of Hsf1 were measured by immunoblotting. (C) Lysates of cells described in Fig. 1(A,B) were immunoblotted for Hsf1. (D) Early passage TIG-1 fibroblasts were treated with nutlin-3 to induce senescence. Cells were collected and Hsf1 mRNA levels were measured by qRT-PCR. (E) Hsf1 mRNA half-life was analyzed as in Fig. 3E. The means and ±SEM were calculated from triplicates of two independent experiments. (F) Early passage cells were infected with retroviral empty vector or shRNA against p53 and then treated with 10 μM nutlin-3 for 5 additional days. Cells lysates were immunoblotted for HuR, SIRT1, and Hsf1. (G) Early passage cells were incubated with 10 μM SB and 10 μM nutlin-3 for 5 days, and levels of HuR, SIRT1, and Hsf1 were measured. (H) 10 μM of SB was added for 24 days to WS fibroblasts, and levels of HuR, SIRT1 and Hsf1 were measured. (I) 10 μM of SB was

added for 24 days to WS fibroblasts and heat shocked for 35 min at 43 °C. After 6 h incubation, cells were collected and immunoblotted for Hsp70.

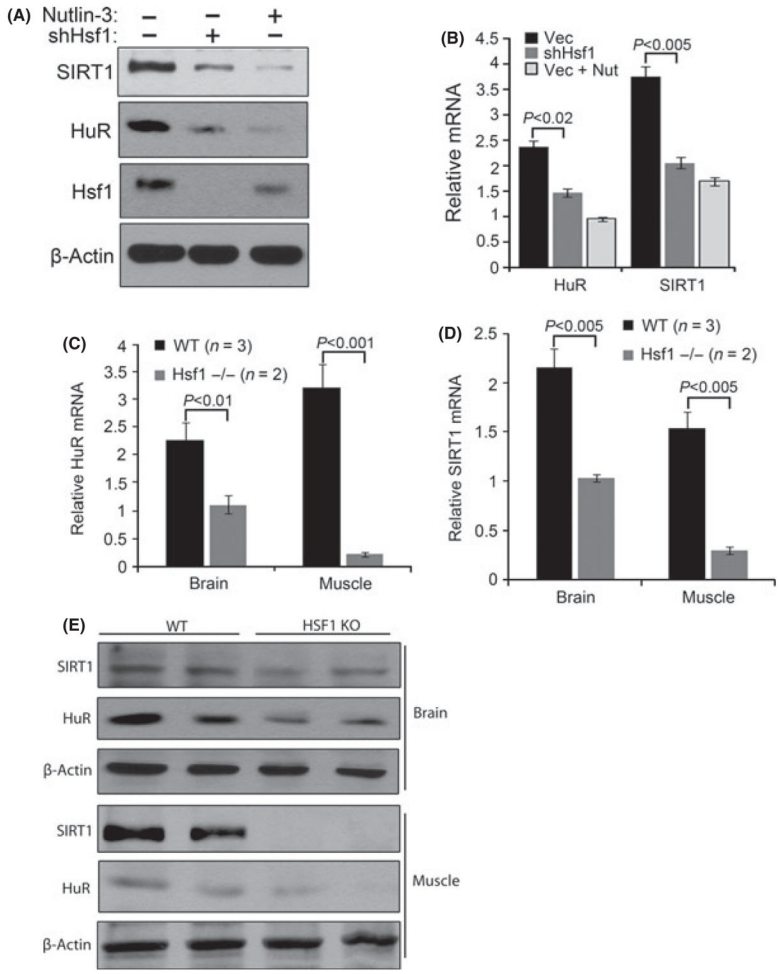

Fig. 5.

Hsf1 depletion downregulates SIRT1 and HuR. (A) Retroviral control or Hsf1 shRNA were expressed in early passage TIG-1 fibroblasts. Puromycin selected cells were treated with nutlin-3 for 5 days to induce senescence. Levels of SIRT1 and HuR were measured by immunoblotting. (B) Levels of SIRT1 and HuR mRNA were measured in cells described in (A). (C and D) Brain and soleus muscles were harvested from 12 months old Hsf1 null (−/−) mice ($n = 2$) and age-matched control mice ($n = 3$) and preserved in RNA stable buffer. RNA from whole brain and soleus muscle were isolated, and qRT-PCR was performed with mouse HuR-, SIRT1-, and GAPDH-specific primers. (E) SIRT1 and HuR protein levels were measured by immunoblotting in samples used in C and D.

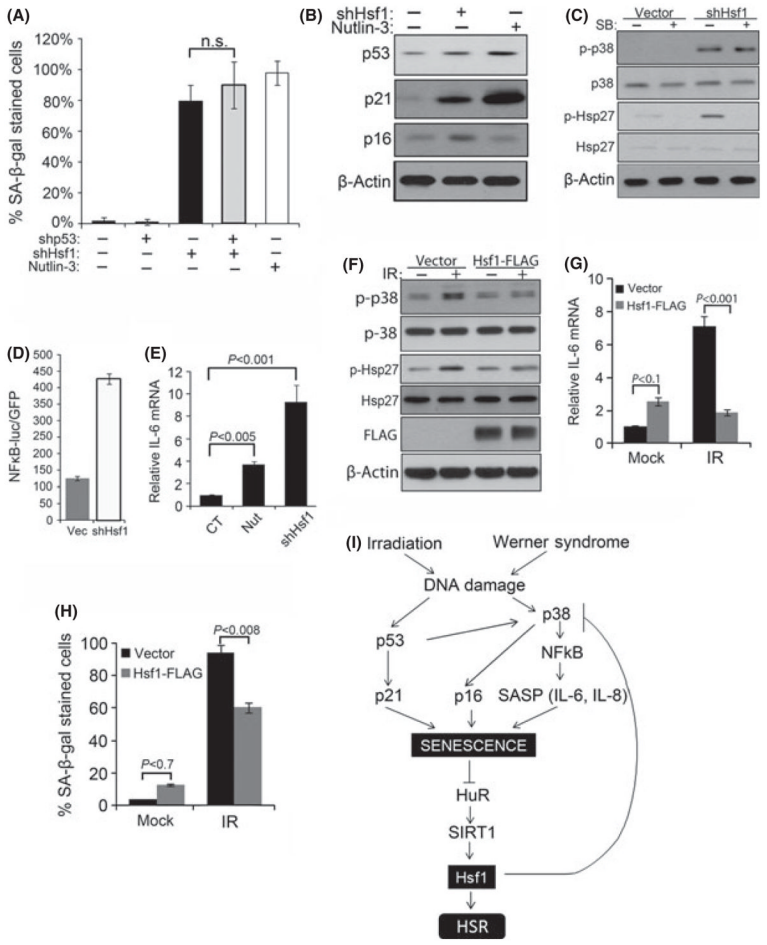

Fig. 6.

Changes in Hsf1 levels modulate senescence phenotype. Retroviral control or shRNA for Hsf1 and/or p53 were expressed in early passage TIG-1 fibroblasts and selected with puromycin. The 10 μM nutlin-3 was added for 5 days. (A) Cells were fixed and stained with SA-β-gal. The means and ±SD are from two independent experiments with 150 cell counts from four different fields. Cells were counted using images taken from bright phase microscope. (B) p53 and p21 were measured in Hsf1- depleted and nutlin-3-treated cells. (C) Control and Hsf1-depleted cells were treated with SB. The levels of p38MAPK and Hsp27 phosphorylation were measured by immunoblotting. (D) Effects of Hsf1 depletion on NF-κB. Cells were first infected with lentiviral NFκB luciferase reporter and then with retroviral empty vector or shRNA against Hsf1. First lane indicates basal luciferase activity reading without retroviral infection. (E) Control or Hsf1-depleted cells were treated with 10 μM nutlin-3, SB or vehicle for additional 5 days and IL-6 mRNA was measured. The mean and ±SEM were of three independent experiments. (F) Control of Hsf1-overexpressing cells were treated with 10 Gy IR and cultured for 6 days, and immunoblotted for indicated proteins. (G) IL-6 mRNA was measured in the same set of cells as in (F). The mean and ±SEM were from triplicates of two independent experiments. (H) Control of Hsf1-overexpressing cells was treated with 8 Gy IR and cultured for 6 days, then fixed and stained with SA-β-gal. The means and ±SD are from two independent experiments with 150 cell counts from four different fields. Cells were counted using images taken from bright phase microscope. (I) A model of relations between Hsf1 and senescence pathways. Radiation or DNA damage in WS causes activation of p53 and p38MAPK pathways. p53 induces p21

which activates senescence. Activation of p38MAPK stimulates NFκB pathway, which maintains senescence by increased production of SASP cytokines. Established senescence causes decrease in HuR, SIRT1, and Hsf1. Downregulation of Hsf1 in a feedback loop inhibits p38MAPK, which further promotes senescence and reduces levels of HuR and SIRT1.