

Heat shock factor-1 modulates p53 activity in the transcriptional response to DNA damage

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ABSTRACT

Here we define an important role for heat shock factor 1 (HSF1) in the cellular response to genotoxic agents. We demonstrate for the first time that HSF1 can complex with nuclear p53 and that both proteins are co-operatively recruited to p53-responsive genes such as p21. Analysis of natural and synthetic cis elements demonstrates that HSF1 can enhance p53-mediated transcription, whilst depletion of HSF1 reduces the expression of p53-responsive transcripts. We find that HSF1 is required for optimal p21 expression and p53-mediated cell-cycle arrest in response to genotoxins while loss of HSF1 attenuates apoptosis in response to these agents. To explain these novel properties of HSF1 we show that HSF1 can complex with DNA damage kinases ATR and Chk1 to effect p53 phosphorylation in response to DNA damage. Our data reveal HSF1 as a key transcriptional regulator in response to genotoxic compounds widely used in the clinical setting, and suggest that HSF1 will contribute to the efficacy of these agents.

INTRODUCTION

Functioning as a transcription factor p53, in response to various stresses including DNA damage, can control the expression of genes involved in cell-cycle arrest, apoptosis and DNA repair (1). For example, the cyclin-dependent kinase inhibitor p21^{Waf1/Cip1} is an important mediator of p53-dependent cell-cycle arrest (2,3), BH3-only members of the Bcl-2 family such as Noxa and PUMA are central to p53-mediated apoptosis (4,5) and gadd45 is involved in DNA repair (6). Over 50% of tumors carry inactivating mutations in the TP53 gene encoding p53 (7). Furthermore, many of these mutations occur within the DNA-binding domain suggesting transcriptional activities

are critical to p53-mediated tumor suppression (8). Other tumors may harbor aberrations that indirectly disrupt p53. For example excessive MDM2 activity which can result from gene amplification (found in 7% of solid tumors) could be a principal mechanism of p53 inactivation (9). MDM2 is an E3 ubiquitin ligase that interacts with the p53 N-terminus leading to p53 polyubiquitination and proteosomal destruction under normal conditions (10). Following genotoxic stress p53 can be phosphorylated on serine residues 15 and 20 (11,12) leading to MDM2 dissociation, thereby stabilizing p53 and releasing its transcriptional activity. Additional phosphorylation of multiple p53 residues (13) combined with C-terminal acetylation (14) enhances p53 transcriptional activities leading to upregulation of genes such as gadd45, p21 and PUMA. Recently, small molecule antagonists have been developed that can overcome the repressive effects of MDM2 on p53 thereby activating p53 in a non-genotoxic manner (15–18) and increasing the prospect of being able to reactivate p53 in tumors. For example, the MDM2 antagonist nutlin-3, is particularly effective in causing p53-dependent apoptosis and exhibits antitumor activity on human xenografts in nude mice (16,19,20).

Many different classes of proteins have been described that can increase p53-mediated transcription including p300/CBP (21), CARM1 (22), Set9 (23), JMY (24), ASPP (25), and more general transcription factors such as TBP, TAFs and Sp1 (26,27). Elegant *in vitro* work has demonstrated that posttranslational modification of histones by p53 coactivators is required for p53-mediated transcription from chromatin templates (22), while p53 itself is also a substrate for posttranslational modifications by coactivators such as p300 and Set9 (14,23). To add further complexity, new and unexpected p53-binding proteins are still being discovered that can influence the transcriptional activity of p53 (28–30). Our understanding of exactly how p53 uses these cofactors remains incomplete.

Heat shock factor 1 (HSF1) belongs to a family of four conserved transcription factors although only HSF1, HSF2 and HSF4 are characterized in humans (31).

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Upon activation by various types of stress including heat shock, osmotic imbalance and geldanamycin treatment (32,33), HSF1 is thought to trimerise and bind to heat shock response elements (HSEs) present in heat shock gene promoters, such as that of heat shock chaperone 70 (HSP70), which are composed of multiple adjacent and inverse copies of the pentanucleotide motif 5'-nGAA-3' (34). HSF1 is capable of increasing the transcriptional rate of target genes via a carboxy-terminal transactivation domain (35,36) and interaction with transcriptional co-regulators such as CHIP and Daxx (37,38), whereas interaction with the HSP70 chaperone inhibits HSF1 transcriptional activity through negative feedback (39). Various studies indicate that HSF1, and its associated factors such as CHIP and HSP70, protect cells from thermal stress and point towards a prosurvival role for the HSF1 pathway in response to hyperthermia (37,40,41). Specifically, mice lacking HSF1 fail to elicit heat shock protein expression in response to heat shock and do not acquire thermotolerance in response to sublethal doses of hyperthermia, resulting in accelerated heat shock-induced apoptosis (42). *Drosophila* harboring HSF mutants also fail to acquire thermotolerance (43). Interestingly, a recent study has shown that whilst loss of HSF1 in p53-deficient mice lead to suppression of lymphomas, the incidence of other tumors such as sarcomas and testicular tumors was increased (44). Additionally, loss of HSF1 contributed to genomic instability suggesting some overlap between p53 and HSF1 functions. Paradoxically, another study has demonstrated that HSF1 elimination protects mice from tumors induced by oncogenic RAS or p53^{R172H} and that a range of cancer cell lines harboring various mutations show some dependency on HSF1 for survival (45). For some time, p53 has been known to interact with some members of the HSF1 pathway including HSP70 and HSP90 (46,47) and HSP90 has been shown to play an important role in p53 transcriptional activities (48).

Here we describe a novel interaction between HSF1 and the tumor suppressor p53 that is enhanced upon DNA damage. We show that HSF1 is important for p53 function in cell-cycle arrest and apoptosis. These new HSF1 activities do not involve previously described properties of HSF1 in response to heat shock, such as increased chaperone expression, HSF1 phosphorylation and cellular relocalization.

MATERIALS AND METHODS

Cell culture and flow cytometry

Cell lines were maintained in RPMI 1640 as described (49). Doxorubicin, etoposide and actinomycin D and doxycycline were all from Sigma. For cell-cycle analysis cells were resuspended in PBS containing 2% FCS then permeabilized with 1% Triton-X-100 (Sigma), treated with 100 µg/ml RNase (Sigma) and stained with 500 µg/ml propidium iodide (Sigma) before analysis on a BD FACScan instrument. For active Caspase 3 measurements FITC-conjugated active caspase-3 antibody was used as recommended (BD Pharmingen) and annexinV was purchased from Sigma.

Immunoprecipitation, expression vectors and reporter gene assays, immunoblotting and protein purification

Immunoprecipitation was performed using cell extracts as described (49), with 1 µg of indicated antibody. Recovered material was analyzed by immunoblotting. For reporter gene assays the indicated reporter vectors were co-transfected with 50 ng of constitutively expressed β-galactosidase reporter gene and p53 or HSF1 in pcDNA3 (a gift from Richard Voellmy), where indicated, using Superfect (Qiagen). Post treatment, cell extracts were used in luciferase (Promega) and β-galactosidase reporter assays (50). Luciferase activities were calibrated to β-galactosidase values. For immunoblotting the following antibodies were used; p21 Ab-1, p53 Ab-6 (Oncogene research products), TBP Ab818 (Abcam), HSF1 Ab-1 (Neomarkers) or HSF1 SPA-901 (Stressgen), phospho p53 #9919 (Cell Signaling). GST-tagged p53 proteins were expressed in BL21 cells and purified according to standard procedures.

Chromatin immunoprecipitation (ChIP) and DNA-binding assay

ChIP was performed essentially as described (51), with minor modifications. Before immunoprecipitation, DNA concentrations from soluble chromatin fractions were quantified using a Nanodrop spectrophotometer. Fifty micrograms of this material were used per immunoprecipitation with specific antibodies against p53 (Ab-6, Oncogene research products) or HSF1 (Stressgen). Post-ChIP material and input samples were subject to quantitative PCR using an Applied Biosystems 7900 system with oligonucleotides GGATCTGTGGTAGGTGAGGGTCA GG and GGAATTAGTCACGGGAGGCAGTGCAG to amplify the gadd45 p53 response element (22), GGA GTTGAGTTGTCAGGAAAAGGG and GGTTGT GGTCTTTCAGGCCTCCACACC to amplify a gadd45 non-specific region 2 kb downstream (22) and GTGGCTC TGATTGGCTTTCTG and CTGAAAACAGGCAGCC CAAG to amplify the p53 response element of p21 (3). TransAM transcription factor assay (Active Motif) was used to measure p53-binding activity to a consensus sequence 5'-GGACATGCCCGGCATGTCC-3' immobilized onto a 96 well plate. Assays were performed as recommended by manufacturer.

RNAi

siRNAs against target sequences HSF1 GAGAAAGATC CCCCTGATGdTdT and p53 GACTCCAGTGGTAAT CTACdTdT were as described (52) and control (ns) siRNA was as described (51). Used at a final concentration of 5–20 nM, these were transfected with HiPerFect (Qiagen) according to manufacturers' protocols.

Proliferation assays

WST1 reagent (Roche) was used as described by the manufacturer to measure end point proliferation on cells seeded in 96-well plates. Six replicates were used per condition.

Quantitative RT-PCR

Measurements of PCNA and PUMA transcript levels were performed using Taqman products (Applied Biosystems). p21, gadd45 and GAPDH transcript levels were measured using SYBR Green as described (51) with oligonucleotide sequences GTGGTAGAAATCTGTCATGCTGGT and GACTCTCAGGGTTCGAAAA CGG for p21; CCCAAACTATGGCTGCACACT and CCATGCAGGAAGGAAAATATG for gadd45 and C GACCACTTTGTCAAGCTCA and GGGTCTTACTC CTTGGAGGC for GAPDH, respectively. Dissociation curves confirmed PCR product specificity. Transcript quantities were corrected to values for GAPDH.

RESULTS

HSF1 interacts with p53 during genotoxic stress

Given the potential link between p53 and HSF1 (44) we first investigated whether p53 and HSF1 might form protein-protein interactions. Human 293T cells that harbor high levels of wild-type p53 due to stabilization by SV40 DNA tumor virus large T antigen were initially examined. Immunoprecipitation of endogenous HSF1 with polyclonal HSF1 antibody resulted in specific co-immunoprecipitation of endogenous p53 suggesting the proteins can complex in cells (Figure 1A). In a reciprocal experiment, immunoprecipitation of endogenous p53 resulted in specific co-immunoprecipitation of endogenous HSF1 (Figure 1B). Additionally, immunoprecipitation using HSF1 antibody recovered p53 from wild-type MEFs, but not HSF1^{-/-} MEFs (Figure 1C). Finally, immunoprecipitation using an alternative HSF1 monoclonal antibody specifically recovered p53 from 293T cells (Figure 1D). We did not find evidence of interaction between HSF1 and p63 (Supplementary Figure 1). We next examined the p53-HSF1 interaction in non-virally immortalized U2OS osteosarcoma cells that express low p53 levels under normal growth conditions, thereby representing a more physiological setting. Under non-stressed conditions the level of p53 that co-immunoprecipitated with HSF1 varied from undetectable to very low in U2OS cells (Figure 1E and data not shown). However, after treatment of cells with the genotoxic compounds actinomycin D, doxorubicin or etoposide that stabilize p53, HSF1 could be efficiently co-immunoprecipitated with p53 (Figure 1E). Notably, exposure of U2OS cells to varying degrees of hyperthermia or geldanamycin did not increase either intracellular levels of p53 or the interaction between HSF1 and p53 (data not shown) suggesting that p53-HSF1 interactions are modulated by genotoxic stress but not by heat shock. In order to delineate the region of p53 that would interact with HSF1, we next assessed the ability of HSF1 to interact with p53 deletion mutants in a GST pull-down assay. HSF1 could interact with full-length p53 as well as residues 1-292, but not an N-terminal deletion mutant lacking residues 1-248 (Supplementary Figure 2). Additionally, HSF1 retained interactions with a proline-rich domain deletion mutant but did not interact with residues 1-42 that contain the

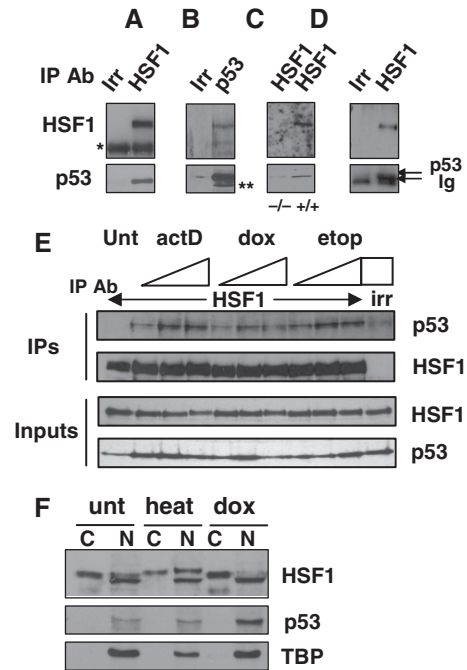


Figure 1. HSF1 interacts with p53. (A) Extracts from 5×10^5 293T cells were subject to immunoprecipitation with HSF1 polyclonal antisera or irrelevant FRS2 antibody (irr). Recovered material was immunoblotted with HSF1 polyclonal antisera or p53 monoclonal antibody. *Indicates IgG band. (B) Same as in (A) except that p53 polyclonal antisera was used for immunoprecipitation and monoclonal antibodies against HSF1 or p53 were used for immunoblotting. **Likely p53 degradation product. (C) HSF1^{+/+} or HSF1^{-/-} MEFs were used for immunoprecipitation as in (A) (D) 293T cells were used for immunoprecipitation with monoclonal HSF1 antibody as in (A) (E) U2OS cells were treated with actinomycin D (5, 50 or 500 nM) or doxorubicin (0.1, 0.5 or 5 μ M) or etoposide (1, 50 or 100 μ M) for 12 h. Cells lysates were then subject to immunoprecipitation and immunoblotting as in (A). Unt denotes untreated. (F) U2OS cells were heat shocked at 43°C for 2 h or treated with 0.5 μ M doxorubicin (dox). Cytoplasmic (C) and Nuclear (N) extracts were then prepared and analyzed by immunoblotting with antibodies as used in (E). TBP occurrence confirmed compartment separation.

p53 transactivation domain (Supplementary Figure 2). This suggests that the region on p53 that interacts with HSF1 lies between residues 42-248, which includes the DNA-binding domain of p53. We were unable to more finely delineate a single interaction site on p53; further experiments suggested that HSF1-p53 interactions might be multiple and complex. It is unknown how much HSF1 is interacting with p53 in these experiments.

We next wanted to examine whether HSF1 might be activated by DNA damaging agents in a similar manner to heat shock. Previous studies have demonstrated that HSF1 can undergo phosphorylation in response to hyperthermia which correlates with HSF1 accumulation in nuclear bodies and increased HSF1 target gene expression (31,53-55) and references therein. We assessed the nuclear:cytoplasmic ratio of HSF1 in response to doxorubicin or heat shock. In untreated U2OS cells HSF1 was present in both nuclear and cytoplasmic fractions and little evidence of HSF1 hyperphosphorylation was present (Figure 1F). As expected HSF1 hyperphosphorylation,

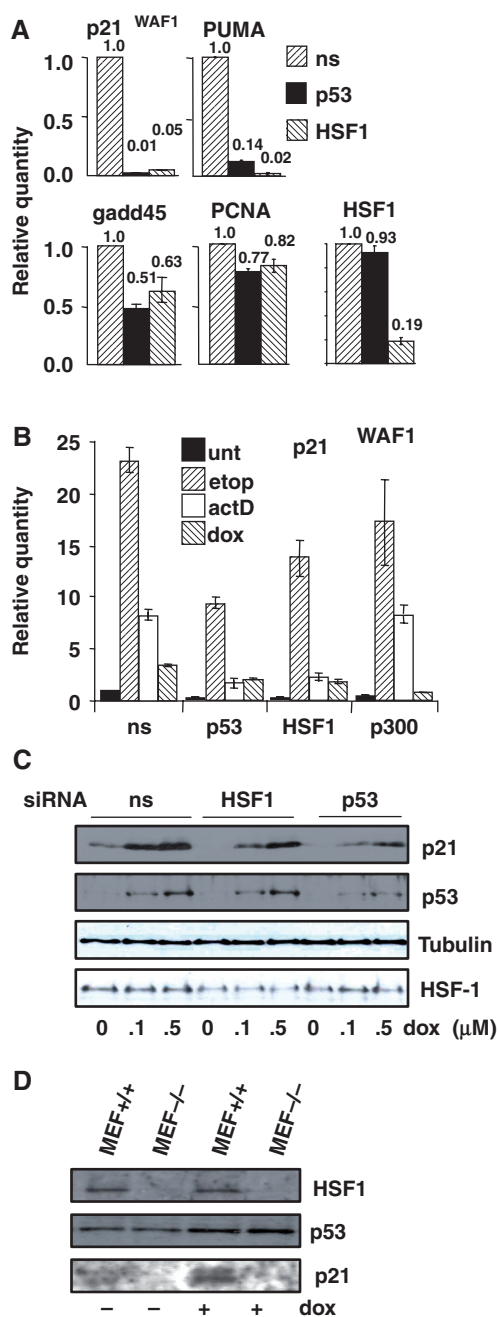


Figure 2. HSF1 regulates expression of p53 target genes. (A) siRNA targeting p53 or HSF1 or non-silencing (ns) siRNA were transfected into U2OS cells in 12-well plates. Seventy-two hours later cells were harvested and total RNA was purified and used to generate cDNA. Quantitative realtime PCR was performed on cDNA for the indicated gene transcripts. (B) U2OS cells in 12-well plates were transfected with indicated siRNAs then 48 h later treated with etoposide (100 μM), actinomycin D (1 nM) or doxorubicin (0.1 μM) overnight then used as in (A). (C) U2OS cells were transfected with the indicated siRNAs and treated with 0.1 μM or 0.5 μM doxorubicin as in (B). Equal quantities of whole-cell extracts were used for immunoblotting with the indicated antibodies. (D) Wild-type (+/+) or HSF1 null (-/-) MEFs were treated with 0.1 μM doxorubicin where indicated and used as in (C). Error bars represent standard deviation.

resulting in reduced electrophoretic mobility was observed upon heat shock (Figure 1F, lane 3 and 4), however no significant change in HSF1 phosphorylation or nuclear enrichment was detected after treatment of U2OS or HCT116 cells with doxorubicin or other genotoxins (Figure 1F and data not shown). We additionally examined the expression levels of HSF1 target chaperone genes in response to heat shock or genotoxic stress in U2OS cells. As expected, we detected large increases in Hsp70 and Hsp27 transcript levels by quantitative RT-PCR in response to heat shock (46-fold and 32-fold, respectively), but no significant changes were observed in response to doxorubicin or etoposide at the doses used (data not shown). This suggests that the well-established role of HSF1 in regulating chaperone expression, as part of the heat shock response, is probably not relevant in the response to chemotherapy. We cannot completely rule out this alternative mechanism, as even small changes in HSP transcript and/or protein levels may play a role in p53 activation.

HSF1 regulates p53-mediated transcription

Previous studies have shown that HSF1 can upregulate pro-survival genes in response to heat shock whereas p53 can regulate pro-apoptotic genes in response to genotoxic stress. However, no study has examined HSF1-mediated transcription in response to genotoxic stress. Given that p53 and HSF1 interact in a manner that can be influenced by genotoxic stress we specifically examined what role HSF1 might play in p53-regulated transcription.

First, to get an overview of whether HSF1 is involved in expression of p53 target genes, we depleted cells of HSF1 by means of RNAi then measured steady state levels of p53 target gene transcripts. Specific siRNA targeted towards HSF1 was used and cells transfected with p53 or non-silencing siRNAs served as controls. Depletion of p53 resulted in dramatic loss of p21 transcript expression to almost undetectable levels compared to control samples (Figure 2A) as measured by quantitative RT-PCR. Depletion of HSF1 also produced a striking decrease in p21 transcript levels suggesting p53 and HSF1 regulate p21 expression in a similar manner (Figure 2A). Analysis of the p53-responsive gadd45, PUMA and PCNA transcripts showed varied levels of reduced expression upon either p53 or HSF1 depletion (Figure 2A). We next wanted to determine whether HSF1 might be required for dynamic changes in p53 target gene expression that occur in response to genotoxins that activate p53. p21 transcript levels increased in a dose-dependent manner in response to etoposide or actinomycin D in U2OS cells (Supplementary Figure 3). Doxorubicin could also induce p21 expression, but only in the lower dose range (Supplementary Figure 3). siRNAs for p53 and HSF1 were again transfected and U2OS cells were then challenged with etoposide, doxorubicin or actinomycin D to induce p53 responsive genes. Silencing either p53 or HSF1 resulted in a blunted response to chemotherapy; cells could not increase p21 transcript levels to the same degree as control transfectants (Figure 2B). As expected,

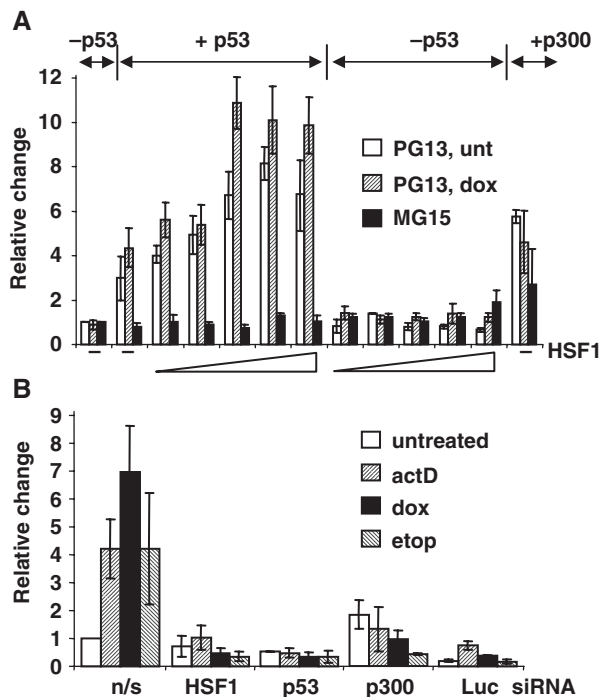


Figure 3. HSF1 enhances p53 transcriptional activities (A) HCT116^{-/-} cells were transfected in 48-well plates with 100 ng of the indicated reporter gene with or without 1 ng p53 and with either empty vector or increasing amounts of HSF1 (10, 20, 40, 80 or 160 ng) or 160 ng CMV-p300. Forty-eight hours later cells were treated for 12 h with doxorubicin (dox) where indicated then harvested for reporter gene assays. Transfections were performed in quadruplicate in three independent experiments. (B) U2OS cells in 24-well plates were transfected with the indicated siRNAs. After 24 h cells were split into 48-well plates then transfected with 200 ng of p21-luc reporter. Twenty-four hours later cells were treated with actinomycin D (1 nM), doxorubicin (0.5 μ M) or etoposide (50 μ M) for 12 h then harvested for reporter gene assays. Error bars represent standard deviation.

silencing p300 also produced some reduction in p21 transcript levels (Figure 2B).

To ascertain whether these effects could be detected at the protein level the expression of p21 protein was measured in extracts produced from cells transfected with the siRNAs. Depletion of HSF1 substantially reduced both steady-state levels of p21 and induction of p21 protein expression mediated by doxorubicin, without affecting p53 protein levels (Figure 2C). Similarly, p53 depletion partly abrogated the induction of p21 protein (Figure 2C). These data suggest that HSF1 is important for expression of p21 in response to chemotherapy. To validate this suggestion we examined p21 expression in MEFs that lack HSF1. In response to doxorubicin wild-type MEFs expressed a readily detectable amount of p21 protein yet HSF1^{-/-} MEFs did not (Figure 2D).

We wanted to delineate whether HSF1 could alter the expression of p53 targets by modulating p53 transcriptional activities or by affecting these genes via some other mechanism. To do this we firstly studied p53 *cis*-regulatory elements. Two reporter genes were initially used that contain either consensus p53-binding sequences (termed PG13) or mutated, non-functional,

sequence (termed MG15). These reporter genes were transfected into a p53-deficient background of HCT116 p53^{-/-} cells (2). As expected, co-transfection of increasing amounts of exogenous p53 resulted in increases in PG13 reporter gene activity, but no increase in MG15 reporter gene activity (Supplementary Figure 4) and 1 ng of exogenous p53 was sufficient to enhance PG13 2.5-fold. Interestingly, co-transfection of increasing amounts of HSF1 further enhanced p53-induced reporter gene activity (Figure 3A). This enhancement by HSF1 was not observed in the absence of co-transfected p53 (Figure 3A). Additionally, treatment with 0.5 μ M doxorubicin led to increases in reporter gene activity, which could be further enhanced by co-transfection of HSF1 (Figure 3A). As expected, p300 transfection also produced some increase in reporter gene expression (Figure 3A). No significant changes in reporter activity were observed with the MG15 reporter gene either in the presence of p53, HSF1 (Figure 3A) or doxorubicin (data not shown). These observations suggest that HSF1 overexpression can increase p53 transcriptional activity and that mutation of the p53-binding sequence is sufficient to abrogate the effects of HSF1 on p53-binding *cis* elements. We next assessed whether depletion of HSF1 would result in reduced p53 reporter gene expression. U2OS cells that harbor wild-type p53 were transfected with siRNA targeting either p53 or HSF1 or luciferase as an internal control, whereas non-silencing (ns) siRNA was used as a negative control. Additionally, cells were treated for 12 h with genotoxic agents to further induce reporter gene activity before harvesting. As expected, p53 or luciferase silencing followed by transfection of the PG13 or p21 reporter genes resulted in large reductions in reporter gene activity compared to control transfectants (Figure 3B). HSF1 silencing also resulted in a striking reduction in reporter gene activity in either untreated or genotoxin treated cells (Figure 3B).

Having shown an effect of HSF1 on p53 target gene expression and transfected reporter genes containing isolated p53 response elements, we next examined recruitment of HSF1 and p53 to natural gene promoters by quantitative ChIP. This would enable us to determine whether HSF1 is recruited to the promoter regions of native p53 target genes, thus affecting p53-dependent expression at the transcriptional level. As shown previously, treatment of cells with genotoxins resulted in increased recruitment of p53 to the p21 and gadd45 promoter (2,22,56). Interestingly, HSF1 recruitment to the p21 and gadd45 promoters could also be detected under basal conditions, which underwent a large increase in response to doxorubicin, actinomycin D or etoposide (Figure 4A and Supplementary Figure 5). To confirm specificity, we tested recruitment of p53 or HSF1 to a non-specific region of the gadd45 gene, 2 kb downstream from the gadd45 promoter. Only background levels of binding were found at this region, which did not show significant enrichment upon genotoxin treatment of cells (Figure 4A) suggesting that HSF1 and p53 binding to the p53 response elements was specific in our assay. Additionally, ChIP with irrelevant antibody (irr) recovered only background levels of DNA (Figure 4A). Next, we wanted to assess the

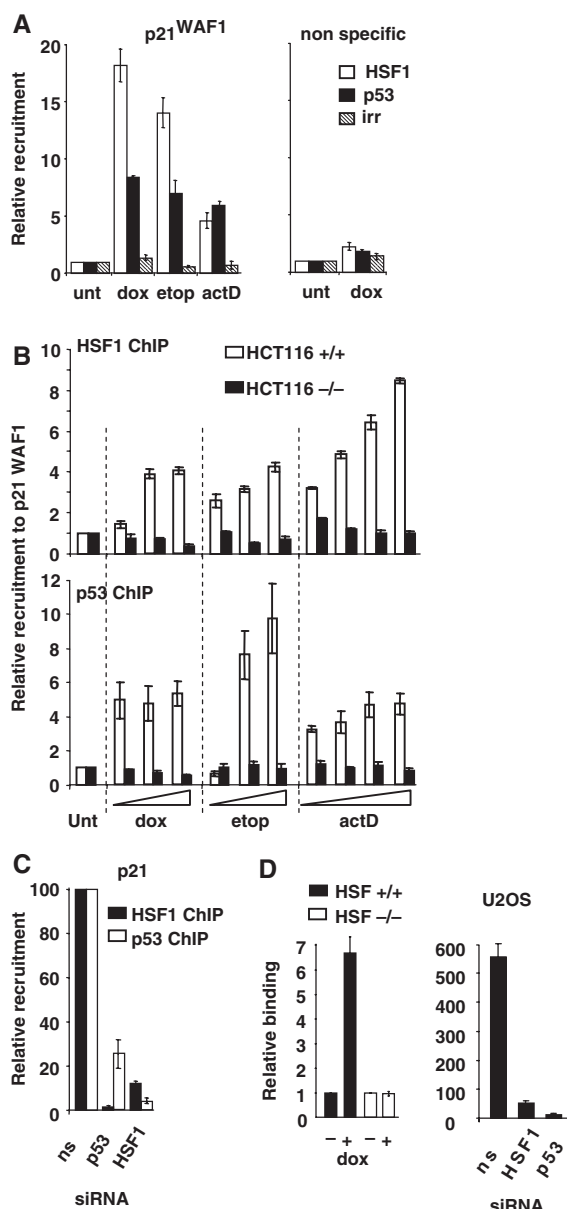


Figure 4. HSF1 is recruited to p53 target gene promoters (A) U2OS cells in 90 mm dishes were treated overnight with etoposide (1 μ M), doxorubicin (0.5 μ M), actinomycin D (50 nM) then used for ChIP with HSF1, p53 or irrelevant FRS2 antibody (irr). Subsequent quantitative real-time PCR was performed on the p21 promoter or a non-specific sequence downstream from the gadd45 gene promoter referred to as 'non-specific'. Data are expressed as fold change. (B) HCT116 cells lacking p53 (-/-) or harboring wild-type p53 (+/+) were treated as in (A) with increasing doses of doxorubicin (0.1, 0.5 or 5 μ M), etoposide (0.1, 1 or 100 μ M) or actinomycin D (1, 5, 50 or 500 nM) then subject to ChIP. Data are expressed as fold change for each cell line. (C) U2OS cells in 90 mm dishes were transfected with the indicated siRNAs. Forty-eight hours later cells were treated for 12 h with either 0.5 μ M doxorubicin then subject to ChIP with HSF1 or p53 antibodies. Data are expressed as a proportion of maximal observed recruitment. Experiments were performed three times. Data represent one experiment. Error bars represent standard deviation. (D) *Left panel:* Nuclear extracts from wild type or HSF1^{-/-} MEFs treated with 0.5 μ M doxorubicin or vehicle were used in p53 DNA-binding assay. Data are expressed as fold change. *Right panel:* Nuclear extracts from doxorubicin-treated U2OS cells transfected with the indicated siRNAs were used in the p53 DNA-binding assay and data are represented as fold increase above vehicle treated cells.

dependency of HSF1 and p53 on each other for their recruitment to p53 target gene promoters. Comparison of isogenic p53-proficient (HCT116^{+/+}) and p53-deficient (HCT116^{-/-}) cell lines showed that while a low level of HSF1 could be recruited to the p21 promoter in the absence of p53, drug induced increases in HSF1 binding occurred only in p53-proficient cells (Figure 4B). We also examined the interdependency of p53 and HSF1 for recruitment to the p21 and gadd45 promoters using siRNA (Figure 4C). Congruent with the data from HCT116 cells, p53 knockdown substantially reduced HSF1 binding to either of the endogenous p53 response elements within the p21 or gadd45 promoters in U2OS cells treated with doxorubicin (Figure 4C and Supplementary Figure 6). Conversely, HSF1 knockdown also resulted in a large reduction of p53 recruitment to the gadd45 or p21 p53 response elements (Figure 4C). We additionally examined the efficiency of p53 binding to a consensus DNA sequence by ELISA. First, p53 from wild-type MEF nuclear extracts showed a 6.7-fold increase in DNA binding in response to doxorubicin, whereas no increase was observed in HSF1^{-/-} MEFs (Figure 4D). Secondly, HSF1 or p53 siRNA-mediated knockdown in U2OS cells produced a striking reduction in p53 DNA-binding activity (Figure 4D) in response to doxorubicin.

HSF1 is involved in p53-mediated growth regulation

p53 can induce cell-cycle arrest and apoptosis in a manner dependent upon its competence as a transcription factor and murine HSF1 has previously been shown to be required for heat shock-mediated G2/M arrest (57). Having observed some dependency for HSF1 in p53-mediated transcriptional regulation we next assessed the contribution HSF1 might have to p53-mediated growth arrest.

We firstly assessed the effects of genotoxic compounds on MEF cell cycle. Strikingly, etoposide and doxorubicin could invoke a dramatic G2/M checkpoint cell-cycle arrest in wild-type MEFs but not in HSF1^{-/-} MEFs (Figure 5A). We next wanted to examine if human HSF1 would act similarly in cancer cells. First, U2OS cells were treated with the same compounds and showed a G2/M arrest, as expected (Figure 5B). Intriguingly, p53 or HSF1 depletion blunted this response to genotoxic stress; cells did not arrest as readily as judged by diminished increases in G2/M checkpoint populations in response to etoposide or doxorubicin (Figure 5C). In order to examine whether this effect was dependent upon p53, we next analyzed p53 deficient and proficient cells. In response to doxorubicin HCT116^{+/+} p53 proficient cells transfected with non-silencing siRNA demonstrated a G2/M arrest, however HSF1 knockdown completely abrogated this effect (Figure 5D). In agreement with previous studies (2,58) HCT116^{-/-} p53 deficient cells were still capable of efficient G2/M arrest in response to doxorubicin. Strikingly, HSF1 siRNA transfection did not affect G2/M arrest in HCT116^{-/-} p53 deficient cells (Figure 5D). This suggests that HSF1-mediated cell-cycle arrest in response to doxorubicin is p53-dependent. In order to examine the impact of HSF1 on cellular proliferation we compared MEFs

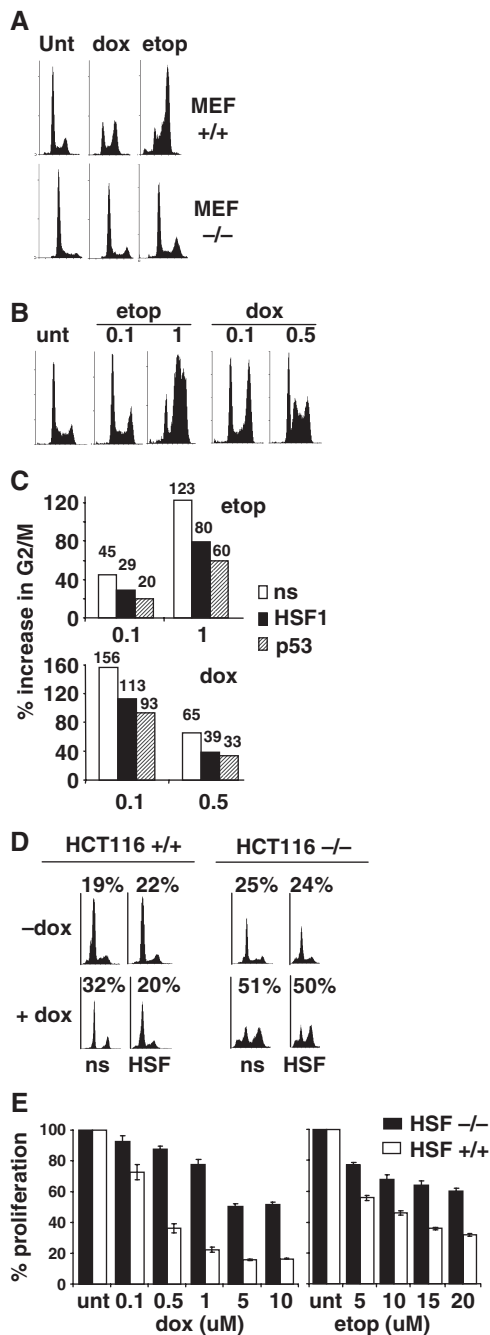


Figure 5. HSF1 is required for cell-cycle arrest in response to p53 activating agents (A) Wild-type or HSF^{-/-} MEFs were treated with 0.5 μM doxorubicin or 1 μM etoposide for 12 h then stained with propidium iodide and injected into flow cytometer. (B) U2OS cells in 12-well plates were treated overnight with increasing doses of genotoxic drugs or left untreated (Unt) then used as in (A). (C) U2OS cells were transfected with the indicated siRNAs then 48 h later treated with the indicated drugs overnight. Cells were then used as in (A). Percentage change in G2/M phase are presented from one of three experiments. Values are rounded to nearest integer. (D) HCT116 cells were used as in (C). (E) Wild-type or HSF^{-/-} MEFs were seeded in 96-well plates and treated with the indicated drugs for 48 h. WST-1 proliferation assays were then performed. Six replicates were used for each condition and error bars represent standard deviation.

lacking HSF1 to wild-type MEFs. The concentration of doxorubicin required to produce a 50% decrease in proliferation in wild-type MEFs was approximately 250 nM, compared to 5 μM in HSF1^{-/-} cells (Figure 5E). Wild-type MEFs were also more sensitive to etoposide and actinomycin D than HSF1^{-/-} MEFs (Figure 5E and data not shown).

To specifically study p53-mediated growth arrest and to be certain that HSF1 was required for p53-mediated changes in cell growth we utilized an alternative system. Rather than employ additional p53-activating drugs, that might have secondary effects on cells, we used SAOS2 osteosarcoma cells stably transfected with a doxycycline-inducible p53 vector (59), termed SAOS-tetWTp53 cells. First, we established that addition of 0.1 μg/ml doxycycline was sufficient to produce a 50% decrease in proliferation of SAOS-tetWTp53 cells (data not shown). Next we assessed the contribution of HSF1 to this growth arrest. Although very high transfection frequencies could not be achieved in these cells, transfection of p53 siRNA inhibited the reduction in proliferation from 53% to 70% and HSF1 depletion had the same effect (Figure 6A). We also examined apoptosis and found that addition of 0.1 μg/ml doxycycline to SAOS-tetWTp53 cells produced an 11-fold increase in apoptotic cell numbers from 5% to 55% (Figure 6B), measured by flow cytometry using the active caspase-3 marker. Inspection of cell morphology also revealed widespread membrane blebbing upon p53 induction (data not shown). Depletion of HSF1 or p53 partly diminished this response resulting in fewer apoptotic cells after doxycycline treatment (Figure 6B). These data indicate that loss of HSF1 can specifically interfere with p53-induced growth arrest and apoptosis. Lastly, we compared apoptosis in MEFs lacking HSF1 to wild-type MEFs using annexinV staining and flow cytometry. In response to etoposide we measured ~90% of wild-type MEFs to be annexinV positive compared to 55% in HSF1^{-/-} cells (Figure 6C). Addition of actinomycin D produced 80% annexinV positive wild type MEFs compared to 30% in HSF1^{-/-} cells (Figure 6C).

HSF1 interacts with DNA damage kinases and facilitates p53 phosphorylation

Finally, in order to delineate a precise mechanism by which HSF1 might confer sensitivity to chemotherapy we examined post-translational modification on p53. Doxorubicin is well known to induce phosphorylation of multiple serine residues on p53 which are likely to contribute to its efficacy as a transcription factor (13). First, we examined the phosphorylation status of p53 in HSF1 immunoprecipitated material. Using phospho-specific p53 antibodies we found that p53 modified on serine 6 and serine 15 exhibited strong increases in HSF1 interaction in response to doxorubicin, whilst other modified forms of p53 such as serine 9 modified p53 did not interact as markedly upon DNA damage (Figure 7A). We next examined whether DNA damage kinases that are known to mediate the phosphorylation of p53 could complex with HSF1. Although we found no evidence of an interaction with ATM or DNA-PK (data not shown) we could

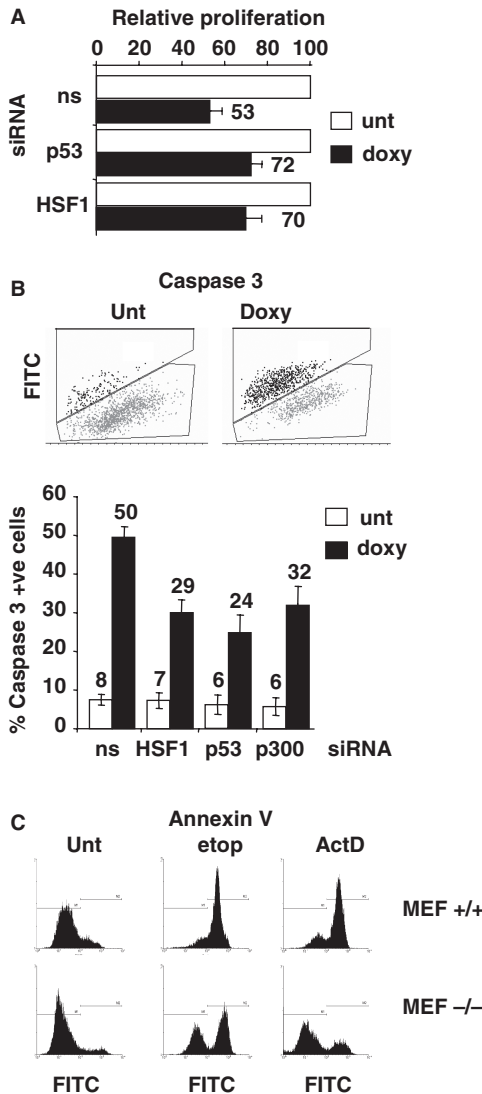


Figure 6. HSF1 is involved in p53-mediated apoptosis (A) SAOS2-tetWTp53 cells seeded into 48-well plates were transfected with indicated siRNAs. Twenty-four hours later cells were split into 96-well plates then treated with 0.1 μ g/ml doxycycline (doxy) for 48 h before proliferation was measured using WST-1 assay. (B) *Upper panel:* Saos2-p53 cells in 12-well plates were treated for 36 h with 0.1 μ g/ml doxy or left untreated (*left*) before analysis of active caspase-3 by flow cytometry. Events in upper region represent active caspase-3 staining. *Lower panel:* SAOS2-tetWTp53 cells were transfected with the indicated siRNAs. After 48 h cells were treated for a further 36 h with 0.1 μ g/ml doxy or left untreated then analyzed as before. Percentage of active Caspase-3 positive cells is shown from one of three experiments (lower panel). Error bars represent standard deviation. (C) Wild-type or HSF^{-/-} MEFs seeded in 6-well plates were treated with 50 μ M etoposide or 50 μ M actinomycin D for 48 h before harvesting and staining for FITC-conjugated AnnexinV.

reproducibly co-immunoprecipitate HSF1 with Chk1 or ATR (Figure 7B and C), both of which are known to mediate phosphorylation of serine 6 and serine 15 on p53 (60). The HSF1-Chk1/ATR complex was not altered upon treatment with doxorubicin whilst HSF1-p53 interaction was only readily detectable in the presence of doxorubicin (Figure 7B). We next tested whether loss of HSF1

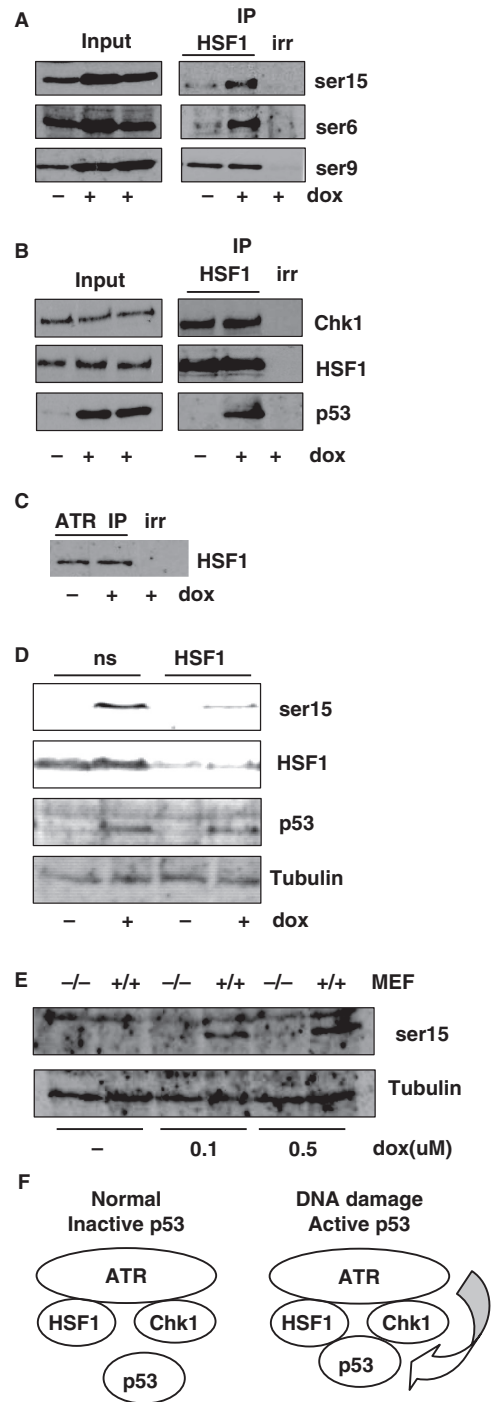


Figure 7. HSF1 affects p53 phosphorylation status. (A) HCT116 cells were treated with 0.5 μ M doxorubicin where indicated for 12h before immunoprecipitation (IP) with HSF1 monoclonal antibody or irrelevant antibody (irr). Material was then immunoblotted as indicated. (B) HCT116 cells were treated as in (A) and immunoprecipitated material was immunoblotted with the indicated antibodies. (C) HCT116 cells were treated as in (A) then subject to immunoprecipitation with ATR antibody. Material was then immunoblotted for HSF1. (D) HCT116 cells were transfected with indicated siRNA then after 48 h treated with 0.5 μ M doxorubicin for 12h. Equal quantities of whole-cell lysates were then immunoblotted as shown. (E) Wild-type or HSF^{-/-} MEFs seeded in 12-well plates were treated with the indicated dose of doxorubicin before equal quantities of cell lysate were immunoblotted as in (D). (F) Model of HSF1 modulation of p53 before and after DNA damage.

might influence the phosphorylation of p53. HCT116 cells were transfected with HSF1 siRNA and treated with doxorubicin. Compared to control cells transfected with non-silencing siRNA we found that phosphorylation of serine 15 was reduced upon depletion of HSF1 (Figure 7D). Additionally, we tested MEFs lacking HSF1. Whilst both wild-type and HSF1^{-/-} MEFs expressed p53 (Figure 2D) only in wild-type cells did p53 undergo phosphorylation on serine 15 (Figure 7E). Immunofluorescence using phospho-serine 15 also demonstrated a nuclear accumulation of serine 15 phosphorylation in wild-type MEFs which was absent in HSF1^{-/-} MEFs (Supplementary Figure 7).

DISCUSSION

Here we define a novel role for HSF1 in the regulation of the tumor suppressor p53. We propose that HSF1 is required for the steady-state expression of a number of p53-responsive genes, but is also required for dynamic changes in expression of at least one critical p53 target gene, p21, on which HSF1 appears to function as an obligate p53 cofactor.

p53 can be activated by a wide variety of different signals such as DNA double strand breaks, inhibition of RNA production and pharmacological inhibition of MDM2. Yet, p53 is somehow able to co-ordinately regulate the expression of distinct subsets of genes depending upon the activating signal. Exactly how p53 is able to perform this remarkable function remains to be fully explained, although elegant studies have shown gene-specific mechanisms to exist such as the requirement for P-TEFb and RNA polII phosphorylation (61) and signal-specific recruitment of basal transcriptional machinery including TAFII250 and TFIIB to p53 target genes (62). Here, we found that different classes of compound that stabilize and activate p53 via distinct mechanisms require HSF1 in order to fully elicit their effects. Actinomycin D inhibits RNA polymerase I, thereby blocking ribosomal biogenesis and activating p53 through a non-classical pathway (61) that does not alter phosphorylation of N-terminal p53 serine residues (63). Doxorubicin and etoposide act in a different manner; by inhibiting topoisomerase activities thereby causing DNA double strand breaks which subsequently results in phosphorylation of p53 N-terminal p53 serine residues. However, in response to actinomycin D, doxorubicin or etoposide the interaction between HSF1 and p53 was increased suggesting that simply increasing the intracellular concentration of p53 may be sufficient to enable HSF1-p53 complex formation. We also demonstrate that induction of p21 gene expression and associated cell-cycle changes generated by these agents are dependent, at least in part, on HSF1. This could imply that HSF1 is a universal regulator of p53 function, independent of the p53 activating signal. In a recent study, downregulation of HSF1 in HeLa cells did not result in altered sensitivity to the chemotherapeutic genotoxin cisplatin (64). These cells are functionally deficient in p53 due to expression of the papillomavirus E6 protein. Our reporter gene assay and ChIP data from the

HCT116-derived cell lines suggest that HSF1-mediated responses to chemotherapy are indeed dependent upon p53, which would explain the lack of altered drug sensitivity in HeLa cells.

Our ChIP studies, in keeping with other reports, showed that a basal level of p53 was present at the p21 promoter under non-stressed conditions but also that HSF1 was present under these conditions. This might provide a mechanism by which HSF1 and p53 can co-ordinately regulate inducible gene expression before further recruitment of the two factors in response to continued stress. Additionally, this would explain why HSF1 knockdown reduces p21 levels in unstressed cells. Strikingly, depletion of either HSF1 or p53 resulted in loss of recruitment of both factors to the p21 or gadd45 promoters suggesting that HSF1 and p53 are recruited co-operatively, in an interdependent manner. The mechanism by which HSF1 enhances p53 transcriptional activity could involve either increased recruitment of p53 to p53 response elements or activation/recruitment of additional factors such as HSPs that might facilitate p53 DNA binding. In keeping with the former model, our protein interaction data show that HSF1 binds near to, or in, the central DNA-binding domain of p53 which might facilitate p53-DNA interactions. However, we cannot dismiss the fact that HSF1 might also play an indirect role in p53 activation via HSPs which have previously been shown to be important for p53 folding, DNA binding and transcriptional activity (47,48).

In order to provide a more detailed mechanism by which HSF1 activates p53 we examined p53 phosphorylation in response to doxorubicin. p53 can be phosphorylated by multiple kinases including ATM (65,66), ATR (65,67), DNAPK (68) and CKII (69) on residues modified in response to DNA damage (11). HSF1 was required for phosphorylation of serine 15 on p53, but was not required for phosphorylation of other p53 serine residues. In some experiments we observed a reduction in serine 6 phosphorylation on p53 in response to HSF1 depletion however this was not always reproducible (data not shown). Our data show that HSF1 complexes with ATR and Chk1 which can phosphorylate p53 on residues 15 and 6 (60,70). Unlike the interaction with p53, the association of these kinases with HSF1 were not increased in response to doxorubicin. We therefore suggest that HSF1-Chk1-ATR form a steady-state complex that conditionally interacts with p53 in response to DNA damage to effect p53 phosphorylation (Figure 7F). We anticipate that there must also be other mechanisms by which HSF1 can activate p53 in response to signals such as actinomycin D which does not induce p53 phosphorylation. The indirect pathway referred to above, in which HSF1 could modulate HSP chaperone activities leading to p53 activation, is one putative mechanism.

Given previously published data derived from HSF1 deficient mice describing a pro-survival role for HSF1 in response to heat shock, we were surprised to discover that HSF1 can promote not only cell-cycle arrest but also p53-mediated apoptosis. Two recent studies have shown that HSF1 has a pro-apoptotic function, albeit in spermatogenesis. Transgenic mice expressing constitutively active

HSF1, either globally or restricted to spermatocytes, exhibit arrested spermatogenesis and abundant spermatocyte apoptosis, despite increased expression of HSPs (71,72). This effect was associated with increased levels of p53 and p53-responsive response genes of the Bcl-2 family (72) thereby providing another potential link between p53 and HSF1 pathways. Taken together with our data showing that HSF1 knockdown reduces PUMA levels this affords a transcriptional mechanism by which HSF1 could regulate p53-mediated apoptosis and suggests that a thorough assessment of the role of HSF1 in p53-mediated apoptosis should be undertaken. Intriguingly, HSF1 has recently been shown to be required for tumorigenesis in mice harboring activated oncogenes including RAS or p53^{R172H} (45) which points towards a putative oncogenic role for HSF1. Notably however HSF1 overexpression alone was insufficient to promote cellular transformation; potent oncogene activation was also required (45). Although some of these results may seem difficult to reconcile with our data, the findings are not contradictory to ours because we have examined the role of HSF1 in response to DNA damage rather than oncogene activation. Taken together, the data show that in response to DNA damage in wild-type p53 cells, HSF1 promotes cell cycle arrest and apoptosis whereas in cells containing powerful oncogenes HSF1 function is redirected to promote cell survival and tumorigenesis. Therefore, it would appear HSF1 can contribute to both pro- and anti-apoptotic pathways and what role HSF1 plays is dependent on the cellular context.

Since our data suggest that p53 and HSF1 have a common role in response to DNA damage, it would be useful to examine whether or not human cancers retain expression of wild-type HSF1 in the absence of p53 mutations. We speculate that mutations in HSF1 which inhibit the functions described herein would confer a growth advantage on tumor cells undergoing chemotherapy, a hypothesis yet to be explored. We have not yet investigated whether other members of the HSF family are capable of activating p53.

In summary, this study has uncovered a new role for HSF1 in the cellular response to stresses that activate p53. To define the precise mechanism(s) by which HSF1 can regulate p53-mediated transcription and apoptosis in response to multiple signals represents the next major challenge in understanding the complex functions of HSF1.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Zhou, B.B. and Elledge, S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, **408**, 433–439.
- Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W. and Vogelstein, B. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, **282**, 1497–1501.
- el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
- Nakano, K. and Vousden, K.H. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol. Cell*, **7**, 683–694.
- Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W. and Vogelstein, B. (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol. Cell*, **7**, 673–682.
- Smith, M.L., Chen, I.T., Zhan, Q., Bae, I., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M. and Fornace, A.J. Jr (1994) Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science*, **266**, 1376–1380.
- Olivier, M., Eeles, R., Hollstein, M., Khan, M.A., Harris, C.C. and Hainaut, P. (2002) The IARC TP53 database: new online mutation analysis and recommendations to users. *Hum. Mutat.*, **19**, 607–614.
- Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 mutations in human cancers. *Science*, **253**, 49–53.
- Momand, J., Jung, D., Wilczynski, S. and Niland, J. (1998) The MDM2 gene amplification database. *Nucleic Acids Res.*, **26**, 3453–3459.
- Honda, R., Tanaka, H. and Yasuda, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.*, **420**, 25–27.
- Shieh, S.Y., Ikeda, M., Taya, Y. and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, **91**, 325–334.
- Siliciano, J.D., Canman, C.E., Taya, Y., Sakaguchi, K., Appella, E. and Kastan, M.B. (1997) DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.*, **11**, 3471–3481.
- Meek, D.W. (2002) p53 Induction: phosphorylation sites cooperate in regulating. *Cancer Biol. Ther.*, **1**, 284–286.
- Gu, W. and Roeder, R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595–606.
- Hardcastle, I.R., Ahmed, S.U., Atkins, H., Calvert, A.H., Curtin, N.J., Farnie, G., Golding, B.T., Griffin, R.J., Guyenne, S., Hutton, C. *et al.* (2005) Isoindolinone-based inhibitors of the MDM2-p53 protein-protein interaction. *Bioorg. Med. Chem. Lett.*, **15**, 1515–1520.
- Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C. *et al.* (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*, **303**, 844–848.
- Wang, W., Kim, S.H. and El-Deiry, W.S. (2006) Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. *Proc. Natl Acad. Sci. USA*, **103**, 11003–11008.
- Yang, Y., Ludwig, R.L., Jensen, J.P., Pierre, S.A., Medaglia, M.V., Davydov, I.V., Safiran, Y.J., Oberoi, P., Kenten, J.H., Phillips, A.C. *et al.* (2005) Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer Cell*, **7**, 547–559.
- Tovar, C., Rosinski, J., Filipovic, Z., Higgins, B., Kolinsky, K., Hilton, H., Zhao, X., Vu, B.T., Qing, W., Packman, K. *et al.* (2006) Small-molecule MDM2 antagonists reveal aberrant p53 signaling in

- cancer: implications for therapy. *Proc. Natl Acad. Sci. USA*, **103**, 1888–1893.
20. Logan, I.R., McNeill, H.V., Cook, S., Lu, X., Lunec, J. and Robson, C.N. (2007) Analysis of the MDM2 antagonist nutlin-3 in human prostate cancer cells. *Prostate*, **67**, 900–906.
 21. Gu, W., Shi, X.L. and Roeder, R.G. (1997) Synergistic activation of transcription by CBP and p53. *Nature*, **387**, 819–823.
 22. An, W., Kim, J. and Roeder, R.G. (2004) Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. *Cell*, **117**, 735–748.
 23. Chuikov, S., Kurash, J.K., Wilson, J.R., Xiao, B., Justin, N., Ivanov, G.S., McKinney, K., Tempst, P., Prives, C., Gambelin, S.J. et al. (2004) Regulation of p53 activity through lysine methylation. *Nature*, **432**, 353–360.
 24. Shikama, N., Lee, C.W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M. and La Thangue, N.B. (1999) A novel cofactor for p300 that regulates the p53 response. *Mol. Cell*, **4**, 365–376.
 25. Samuels-Lev, Y., O'Connor, D.J., Bergamaschi, D., Trigiante, G., Hsieh, J.K., Zhong, S., Campargue, I., Naumovski, L., Crook, T. and Lu, X. (2001) ASPP proteins specifically stimulate the apoptotic function of p53. *Mol. Cell*, **8**, 781–794.
 26. Prives, C. and Hall, P.A. (1999) The p53 pathway. *J. Pathol.*, **187**, 112–126.
 27. Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.
 28. Enari, M., Ohmori, K., Kitabayashi, I. and Taya, Y. (2006) Requirement of clathrin heavy chain for p53-mediated transcription. *Genes Dev.*, **20**, 1087–1099.
 29. Bourdon, J.C., Laurenzi, V.D., Melino, G. and Lane, D. (2003) p53: 25 years of research and more questions to answer. *Cell Death Differ.*, **10**, 397–399.
 30. Zheng, H., You, H., Zhou, X.Z., Murray, S.A., Uchida, T., Wulf, G., Gu, L., Tang, X., Lu, K.P. and Xiao, Z.X. (2002) The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. *Nature*, **419**, 849–853.
 31. Morimoto, R.I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, **12**, 3788–3796.
 32. Jolly, C. and Morimoto, R.I. (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl Cancer Inst.*, **92**, 1564–1572.
 33. Zou, J., Guo, Y., Guettouche, T., Smith, D.F. and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell*, **94**, 471–480.
 34. Fernandes, M., Xiao, H. and Lis, J.T. (1995) Binding of heat shock factor to and transcriptional activation of heat shock genes in *Drosophila*. *Nucleic Acids Res.*, **23**, 4799–4804.
 35. Green, M., Schuetz, T.J., Sullivan, E.K. and Kingston, R.E. (1995) A heat shock-responsive domain of human HSF1 that regulates transcription activation domain function. *Mol. Cell Biol.*, **15**, 3354–3362.
 36. Chen, Y., Barlev, N.A., Westergaard, O. and Jakobsen, B.K. (1993) Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. *EMBO J.*, **12**, 5007–5018.
 37. Dai, Q., Zhang, C., Wu, Y., McDonough, H., Whaley, R.A., Godfrey, V., Li, H.H., Madamanchi, N., Xu, W., Neckers, L. et al. (2003) CHIP activates HSF1 and confers protection against apoptosis and cellular stress. *EMBO J.*, **22**, 5446–5458.
 38. Boellmann, F., Guettouche, T., Guo, Y., Fenna, M., Mnayer, L. and Voellmy, R. (2004) DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. *Proc. Natl Acad. Sci. USA*, **101**, 4100–4105.
 39. Abravaya, K., Myers, M.P., Murphy, S.P. and Morimoto, R.I. (1992) The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes Dev.*, **6**, 1153–1164.
 40. Nylandsted, J., Gyrd-Hansen, M., Danielewicz, A., Fehrenbacher, N., Lademann, U., Hoyer-Hansen, M., Weber, E., Multhoff, G., Rohde, M. and Jaattela, M. (2004) Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J. Exp. Med.*, **200**, 425–435.
 41. Westerheide, S.D., Kawahara, T.L., Orton, K. and Morimoto, R.I. (2006) Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. *J. Biol. Chem.*, **281**, 9616–9622.
 42. McMillan, D.R., Xiao, X., Shao, L., Graves, K. and Benjamin, I.J. (1998) Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J. Biol. Chem.*, **273**, 7523–7528.
 43. Jedlicka, P., Mortin, M.A. and Wu, C. (1997) Multiple functions of *Drosophila* heat shock transcription factor in vivo. *EMBO J.*, **16**, 2452–2462.
 44. Min, J.N., Huang, L., Zimonjic, D.B., Moskophidis, D. and Mivechi, N.F. (2007) Selective suppression of lymphomas by functional loss of Hsf1 in a p53-deficient mouse model for spontaneous tumors. *Oncogene*, **26**, 5086–5097.
 45. Dai, C., Whitesell, L., Rogers, A.B. and Lindquist, S. (2007) Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell*, **130**, 1005–1018.
 46. Zyllicz, M., King, F.W. and Wawrzynow, A. (2001) Hsp70 interactions with the p53 tumour suppressor protein. *EMBO J.*, **20**, 4634–4638.
 47. King, F.W., Wawrzynow, A., Hohfeld, J. and Zyllicz, M. (2001) Co-chaperones Bag-1, Hop and Hsp40 regulate Hsc70 and Hsp90 interactions with wild-type or mutant p53. *EMBO J.*, **20**, 6297–6305.
 48. Walerych, D., Kudla, G., Gutkowska, M., Wawrzynow, B., Muller, L., King, F.W., Helwak, A., Boros, J., Zyllicz, A. and Zyllicz, M. (2004) Hsp90 chaperones wild-type p53 tumor suppressor protein. *J. Biol. Chem.*, **279**, 48836–48845.
 49. Logan, I.R., Sapountzi, V., Gaughan, L., Neal, D.E. and Robson, C.N. (2004) Control of human PIRH2 protein stability: involvement of TIP60 and the proteasome. *J. Biol. Chem.*, **279**, 11696–11704.
 50. Gaughan, L., Logan, I.R., Neal, D.E. and Robson, C.N. (2005) Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation. *Nucleic Acids Res.*, **33**, 13–26.
 51. Logan, I.R., Gaughan, L., McCracken, S.R., Sapountzi, V., Leung, H.Y. and Robson, C.N. (2006) Human PIRH2 enhances androgen receptor signaling through inhibition of histone deacetylase 1 and is overexpressed in prostate cancer. *Mol. Cell Biol.*, **26**, 6502–6510.
 52. Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296**, 550–553.
 53. Pirkkala, L., Nykanen, P. and Sistonen, L. (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.*, **15**, 1118–1131.
 54. Vujanac, M., Fenaroli, A. and Zimarino, V. (2005) Constitutive nuclear import and stress-regulated nucleocytoplasmic shuttling of mammalian heat-shock factor 1. *Traffic*, **6**, 214–229.
 55. Yao, J., Munson, K.M., Webb, W.W. and Lis, J.T. Dynamics of heat shock factor association with native gene loci in living cells. *Nature*, **442**, 1050–1053.
 56. Kaeser, M.D. and Iggo, R.D. (2002) Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. *Proc. Natl Acad. Sci. USA*, **99**, 95–100.
 57. Luft, J.C., Benjamin, I.J., Mestrlil, R. and Dix, D.J. (2001) Heat shock factor 1-mediated thermotolerance prevents cell death and results in G2/M cell cycle arrest. *Cell Stress Chaperones*, **6**, 326–336.
 58. Bruno, T., De Nicola, F., Iezzi, S., Lecis, D., D'Angelo, C., Di Padova, M., Corbi, N., Dimiziani, L., Zannini, L., Jekimovs, C. et al. (2006) Che-1 phosphorylation by ATM/ATR and Chk2 kinases activates p53 transcription and the G2/M checkpoint. *Cancer Cell*, **10**, 473–486.
 59. Bates, G.J., Nicol, S.M., Wilson, B.J., Jacobs, A.M., Bourdon, J.C., Wardrop, J., Gregory, D.J., Lane, D.P., Perkins, N.D. and Fuller-Pace, F.V. (2005) The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. *EMBO J.*, **24**, 543–553.

60. Shieh, S.Y., Ahn, J., Tamai, K., Taya, Y. and Prives, C. (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev.*, **14**, 289–300.
61. Gomes, N.P., Bjerke, G., Llorente, B., Szostek, S.A., Emerson, B.M. and Espinosa, J.M. (2006) Gene-specific requirement for P-TEFb activity and RNA polymerase II phosphorylation within the p53 transcriptional program. *Genes Dev.*, **20**, 601–612.
62. Espinosa, J.M., Verdun, R.E. and Emerson, B.M. (2003) p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. *Mol. Cell*, **12**, 1015–1027.
63. Ashcroft, M., Taya, Y. and Vousden, K.H. (2000) Stress signals utilize multiple pathways to stabilize p53. *Mol. Cell Biol.*, **20**, 3224–3233.
64. Rossi, A., Ciafre, S., Balsamo, M., Pierimarchi, P. and Santoro, M.G. (2006) Targeting the heat shock factor 1 by RNA interference: a potent tool to enhance hyperthermochemotherapy efficacy in cervical cancer. *Cancer Res.*, **66**, 7678–7685.
65. Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. and Siliciano, J.D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, **281**, 1677–1679.
66. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y. *et al.* (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, **281**, 1674–1677.
67. Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C. and Abraham, R.T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev.*, **13**, 152–157.
68. Lees-Miller, S.P., Chen, Y.R. and Anderson, C.W. (1990) Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. *Mol. Cell Biol.*, **10**, 6472–6481.
69. Meek, D.W., Simon, S., Kikkawa, U. and Eckhart, W. (1990) The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. *EMBO J.*, **9**, 3253–3260.
70. Kohn, K.W. (1999) Molecular interaction map of the mammalian cell cycle control and DNA repair systems. *Mol. Biol. Cell*, **10**, 2703–2734.
71. Nakai, A., Suzuki, M. and Tanabe, M. (2000) Arrest of spermatogenesis in mice expressing an active heat shock transcription factor 1. *EMBO J.*, **19**, 1545–1554.
72. Vydra, N., Malusecka, E., Jarzab, M., Lisowska, K., Glowala-Kosinska, M., Benedyk, K., Widlak, P., Krawczyk, Z. and Widlak, W. (2006) Spermatocyte-specific expression of constitutively active heat shock factor 1 induces HSP70i-resistant apoptosis in male germ cells. *Cell Death Differ.*, **13**, 212–222.