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Heat Shock Factor 1 Deficiency Via its Downstream Target Gene αB-Crystallin (Hspb5) Impairs p53 Degradation

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ABSTRACT

Heat shock factor Hsf1 regulates the stress-inducibility of heat shock proteins (Hsps) or molecular chaperones. One of the functions attributed to Hsps is their participation in folding and degradation of proteins. We recently showed that *hsf1*$^{-/-}$ cells accumulate ubiquitinated proteins. However, a direct role for Hsf1 in stability of specific proteins such as p53 has not been elucidated. We present evidence that cells deficient in *hsf1* accumulate wild-type p53 protein. We further show that *hsf1*$^{-/-}$ cells express lower levels of αB-crystallin and cells deficient in αB-crystallin also accumulate p53 protein. Reports indicate that αB-crystallin binds to Fbx4 ubiquitin ligase, and they target cyclin D1 for degradation through a pathway involving the SCF (Skp1-Cul1-F-box) complex. Towards determining a mechanism for p53 degradation involving αB-crystallin and Hsf1, we have found that ectopic expression of Fbx4 in wild-type mouse embryo fibroblasts (MEFs) expressing mutant p53 (p53R175H) leads to increase in its degradation, while MEFs deficient in *hsf1* or *αBcry* are defective in degradation of this p53 protein. In addition, immunoprecipitated p53R175H from wild-type MEFs is able to pull-down both αB-crystallin and Fbx4. Finally, immunoprecipitated wild-type p53 from doxorubicin treated U2OS cells can pull-down endogenous αB-crystallin and Fbx4. These results indicate that *hsf1*- and *αBcry*-deficient cells accumulate p53 due to reduced levels of αB-crystallin in these cells. Elevated levels of p53 in *hsf1*- and *αBcry*-deficient cells lead to their increased sensitivity to DNA damaging agents. These data reveal a novel mechanism for protein degradation through Hsf1 and αB-crystallin. J. Cell. Biochem. 107: 504–515, 2009.

KEY WORDS: HSF1; αB-CRYSTALLIN; P53 DEGRADATION

The heat shock factor Hsf1 becomes transcriptionally activated upon exposure of cells to variety of environmental stresses and oncogenic stimulation, or to conditions that results in protein misfolding in the cells [Morimoto, 1998; Jolly and Morimoto, 2000; Min et al., 2007]. Increased Hsf1 activity leads to enhanced expression of heat shock proteins (Hsps) or molecular chaperones [Wu, 1995; Morimoto, 1998; Zhang et al., 2002; Ciocca and Calderwood, 2005]. Molecular chaperones play essential roles in protein folding and degradation of proteins [Hartl, 1996; Muchowski, 2002; Barral et al., 2004]. The function of molecular chaperones in protein folding varies among individual family members. The small Hsps, such as Hsp27 (mouse Hsp 25 or HspB1) and αB-crystallin (HspB5), known to prevent protein aggregation and enhance degradation of ubiquitinated proteins that are more evident in stressed cells [Haslbeck, 2002; Parcellier et al., 2003]. Hsp25/27 has been shown to interact with the ubiquitinated proteins and, in a yeast two-hybrid screening, αB-crystallin was found to interact with the 26S proteasome, and it is required for the degradation of phosphorylated IkBα [Parcellier et al., 2003]. Both Hsp25/27, IkBα and 26S proteasome have been found to be present in the same complex. In addition, αB-crystallin has been shown to interact with Fbx4, a component of E3 ligase SCF complex (Skp1-Cul1-F box protein) [den Engelsman et al., 2003]. The proteins ubiquitinated by Fbx4-ubiquitin ligase in combination with αB-crystallin remains unclear. However, recently αB-crystallin through its interaction with Fbx4 was shown to target Thr286 phosphorylated cyclin D1 and facilitate cyclin D1 ubiquitin-dependent degradation leading to cell cycle regulation [Lin et al., 2006].

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For the Hsp/Hsc70 family, binding of Hsp/Hsc70 to newly synthesized polypeptides or misfolded proteins facilitates proper folding in an ATP-dependent manner that requires co-chaperone Hsp40 [Hartl, 1996]. Hsp/Hsc70 and Hsp90 have been shown to interact with ubiquitin ligase, which is involved in ubiquitination and degradation of specific substrate proteins such as ErbB2, glucocorticoid hormone receptor, and aggregation-prone proteins such as the mutant form of cystic fibrosis transmembrane conductance regulator, hyperphosphorylated tau, and the mutant form of p53, through the UPS [Hohfeld et al., 2001; Whitesell and Lindquist, 2005]. The Hsp90, Hsc70, Hsp70, and their cochaperones have been shown to play a role in both intracellular localization and stabilization of wild-type and mutant p53 protein [Zylicz et al., 2001]. Recently, a p53 protein degradation pathway involving molecular chaperones Hsp/Hsc70 and Hsp90 and the Chip E3 ubiquitin ligase has also been uncovered. In this pathway, the carboxyl terminal of Hsp70 and Hsp90 bind to the tetracopeptide repeat (TPR) domain of Chip that also has a U-box domain facilitating ubiquitination of chaperone-bound proteins with the help of E2 enzymes of the Ubc4/5 family, inducing the degradation of proteins such as p53 by the 26S proteasome [Esser et al., 2005].

The wild-type p53 protein, which is involved in cell growth, apoptosis, and oncogenesis is normally turned over rapidly through the ubiquitin proteasome system (UPS). The p53 protein is stabilized and accumulates in the cells following exposure of the cells to stresses that results in DNA damage, leading to G1 cell-cycle arrest [Oren, 1999; Lavin and Gueven, 2006]. Cell-cycle check points are activated by X-irradiation or other DNA damaging agents in order to impose delay in progression from G1 to S phase and inhibit DNA synthesis and intra-S-phase check points in order to arrest cells and passage from G2 to M phase. The major pathway for regulation of p53 stability and activation is dependent on its interaction with and ubiquitination by Mdm2 ubiquitin ligase [Lavin and Gueven, 2006]. p53 is also targeted by other E3 ligases such as Cop1, Pirh2, Arf-BP1/mule, and p300 [Lavin and Gueven, 2006]. The post-transcriptional modifications of p53 by variety of stimuli are capable of stabilizing and activating p53 transcriptional activity. These p53 post-translational modifications are complex, but phosphorylation of p53 dominates.

In this study, we present evidence that *hsf1*-deficient cells accumulate p53 protein at significantly higher levels than the wild-type cells. The defect in *hsf1*-deficient cells that leads to p53 accumulation appears to be the lower levels of αB-crystallin expression in these cells. αB-crystallin participates in p53 degradation through its interaction with p53 and recruitment of Fbx4 ubiquitin liagse complex leading to p53 degradation.

EXPERIMENTAL PROCEDURES

PLASMIDS

Retroviral vectors containing E1A or p53R175H were as previously described [Crook et al., 1994; Samuelson and Lowe, 1997]. Plasmids encoding Flag-Fbx4 and dominant negative form of Fbx4 were as previously reported [Lin et al., 2006].

GENERATION OF MEFS DEFICIENT IN *HSF1*, *HSP25*, OR αB-CRY GENES

The generation of mice deficient in *hsf1*, *hsp25*, and *αB-crys* have previously been reported [Brady et al., 2001; Zhang et al., 2002; Huang et al., 2007]. MEFs were prepared from embryonic day E13.5 following timed pregnancies. MEFs were stably transformed using retroviral vectors containing E1A or E1A and p53R175H and were selected in puromycin (2 μg/ml) or blastidin (3 μg/ml) and cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS).

TRANSIENT TRANSFECTION ASSAYS

Transient transfection assays were performed using Mirus Trans IT-Lt1 (Mirus Corporation) [Hu and Mivechi, 2006]. Transfected DNA mixes included 4–8 μg of expression plasmid DNA and, when required, empty plasmid DNA was added to a total of 8 μg. The DNA mixes were added to 5×10^5 cells. The transfection efficiency varied between 60% and 70% in all experiments, as determined by immunofluorescence analysis.

FLOW CYTOMETRIC ANALYSES AND CELL SURVIVAL ASSAYS

Cells were treated as indicated in the figure legends and then stained with Annexin V-PE and propidium iodide and analyzed by flow cytometry. For cell cycle analyses, following the appropriate treatments, cells were rinsed with phosphate buffered saline (PBS) and re-suspended in 500 μl of PBS followed by the addition of 5 ml of methanol. The mixture was incubated for at least 2 h at 4°C. Cells were rinsed with PBS and re-suspended in 400 μl of PBS containing 20 μl propidium iodide (1 mg/ml) and 2 μl of RNase (50 μg/ml). Following 30 min incubation at 25°C, flow cytometric analyses were performed using CellQuest™ Pro with luminescence spectrophotometer (excitation at 480 nm and emission at 510 nm) [Min et al., 2007].

Cellular survival using colony formation assays were performed as previously described [Huang et al., 2007]. Briefly, untreated or cells treated with chemotherapeutic agents (doxorubicin at 0.5 μg/ml or etoposide 5 μg/ml concentrations for 16 h at 37°C). Cells were then counted and appropriate numbers of cells were plated for colony formation for 10 days. Colonies were stained with crystal violet and colonies containing more than 50 cells were counted. Plating efficiency of untreated cells was also determined. Surviving fraction was determined as number of colonies for treated cells divided by the number of cells plated, and divided by plating efficiency for each group.

IMMUNOFLUORESCENCE ANALYSES

For immunofluorescence analyses, MEFs were fixed in 4% paraformaldehyde and incubated with primary antibodies to p53 (Santa Cruz, Santa Cruz, CA) for 1 h at 25°C and then with fluorescent-labeled secondary antibody. Fixed cells were stained with 4,6-diamidino-2-phenylindole (DAPI) before analyses.

IMMUNOBLOT AND IMMUNOPRECIPITATION ANALYSES

Whole cell extracts were subjected to SDS–PAGE (30 μg of total protein) and immunoblotting as previously described [Hu and Mivechi, 2006]. The primary antibodies to: p53, Mdm2, Cyclin D,

and αB-crystallin were purchased from Santa Cruz; Hsps, Bax, Bcl2, and Bad were purchased from assaydesigns/Stressgen (Ann Arbor, MI); β-actin was purchased from Calbiochem (San Diego, CA); Fbx4 was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). For immunoprecipitation analyses, cells (6×10^5) were cotransfected with the appropriate plasmids, allowed to recover for 48 h, rinsed with PBS, and appropriately treated and harvested. Cells were lysed with lyses buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 7.5 containing 1× cocktail of protease inhibitors (Roche)). The protein concentration of the supernatant was estimated using a BCA protein assay kit (Pierce, Rockford, IL). One milligram of each cell lysate was mixed with 40 μl of a 50% solution of protein A-agarose and incubated at 4°C for 1 h. The protein A-agarose was then centrifuged, and the pre-cleared supernatant was incubated with 2.5 μg of primary antibody and incubated at 4°C for 2 h or overnight. Forty micoliters of 50% solution of protein A-agarose was then added at 4°C for 2 h. The protein A complexes were centrifuged at 10,000*g* for 1 min, and the pellet was washed with lysis buffer three times [Hu and Mivechi, 2006]. One hundred microliters of 2 × SDS sample buffer was added, and samples were heated at 100°C for 5 min. Samples were fractionated on SDS–PAGE and analyzed by immunoblotting using appropriate antibodies. The corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were used, and signals were developed using the enhanced chemiluminescence method (ECL kit, GE Healthcare, Piscataway, NJ). The quantitation of the immunoblots were performed using densitometer.

STATISTICAL CONSIDERATION

All experiments were performed at least three times. Data are expressed as mean ± SD. Differences between groups were analyzed by Student's *t* test. *P* values less or equal to 0.05 were considered significant.

RESULTS

HSF1-DEFICIENT CELLS ACCUMULATE WILD-TYPE P53 PROTEIN

We have recently reported that $hsf1^{-/-}$ SV-40 transformed MEFs accumulate elevated levels of short-lived ubiquitinated (Ub)-EGFP when compared to wild-type MEFs [Homma et al., 2007]. To investigate whether $hsf1^{-/-}$ primary MEFs also accumulate short-lived proteins critical for cellular proliferation, cell death, and oncogenesis, for example, p53 protein, we performed immunoblot analyses and expression of p53 was detected following treatment of cells with doxorubicin and then with cycloheximide to prevent new protein synthesis. The results indicate that p53 expression was enhanced in both wild-type and $hsf1^{-/-}$ cells following treatment of cells with doxorubicin but decayed slower in $hsf1^{-/-}$ cells (Fig. 1). In wild-type cells, p53 decayed with time ½ = 8 h, while in $hsf1^{-/-}$ cells, p53 decayed with a time ½ = 14 h post-cycloheximide treatment.

Introduction of proliferative oncogenes such as E1A, E2F, or c-myc into primary MEFs activates a p53 response [Lowe and Ruley, 1993]. Therefore, to investigate the mechanisms underlying Hsf1 regulation of p53 stabilization, we used adenoviral E1A-transformed MEFs originating from $hsf1^{-/-}$ embryos and performed immunoblotting experiments to detect the expression level of wild-type p53 protein. We found that $hsf1^{-/-}$ MEFs harbor 10-fold higher levels of wild-type p53 than the wild-type MEFs (Fig. 2A). Following E1A expression in MEFs, p53 has been found in the nuclei and transcriptionally active [Lowe and Ruley, 1993]. To determine whether elevated levels of p53 in $hsf1^{-/-}$ cells results in its increased transcriptional activity and lead to elevated expression of its downstream target genes such as p21, immunoblotting experiments were performed. The results indicate that p21 expression was elevated in $hsf1^{-/-}$ cells compared to wild-type cells, suggesting that these cells harbor elevated levels of transcriptionally active p53 (Fig. 2A). To visualize the intracellular distribution of p53 protein in $hsf1^{-/-}$ cells, immunofluorescent analyses were performed and the results demonstrate that the wild-type p53 protein is located almost exclusively in the nuclei (Fig. 2B). Since p53 protein normally undergoing rapid degradation (time ½ = 10–20 min [Giaccia and Kastan, 1998]), we investigated whether the increased accumulation of p53 in $hsf1^{-/-}$ cells is a result of increased stability of p53 protein. The decay rate of p53 protein following treatment of cells with cycloheximide was determined by immunoblotting and quantitation of the results indicate that the time ½ of wild-type p53 protein degradation in $hsf1^{-/-}$ cells was >3 h, while the p53

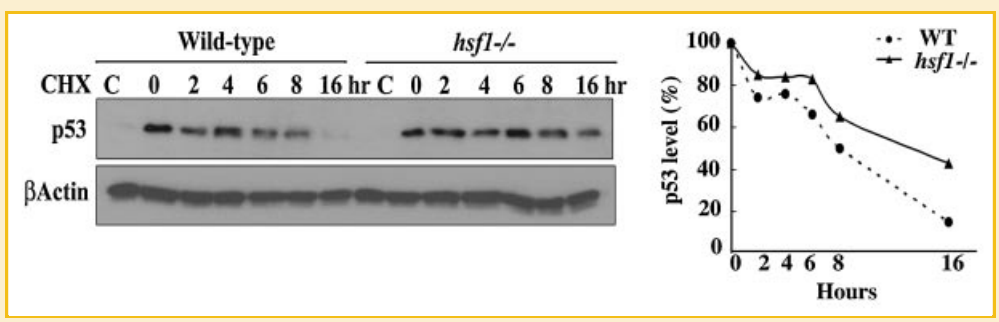

Fig. 1. $Hsf1^{-/-}$ primary MEF express elevated levels of wild-type p53 protein. MEF prepared from wild-type or $hsf1^{-/-}$ mice were treated with doxorubicin (0.5 μg/ml) for 12 h. Cells were then incubated in the presence of cycloheximide (CHX) (25 μg/ml) for the indicated times (h) and immunoblotting experiments were performed with 30 μg of protein from each sample to detect p53. Quantitation of the data is presented in the right panel. C is untreated control. β-actin is loading control.

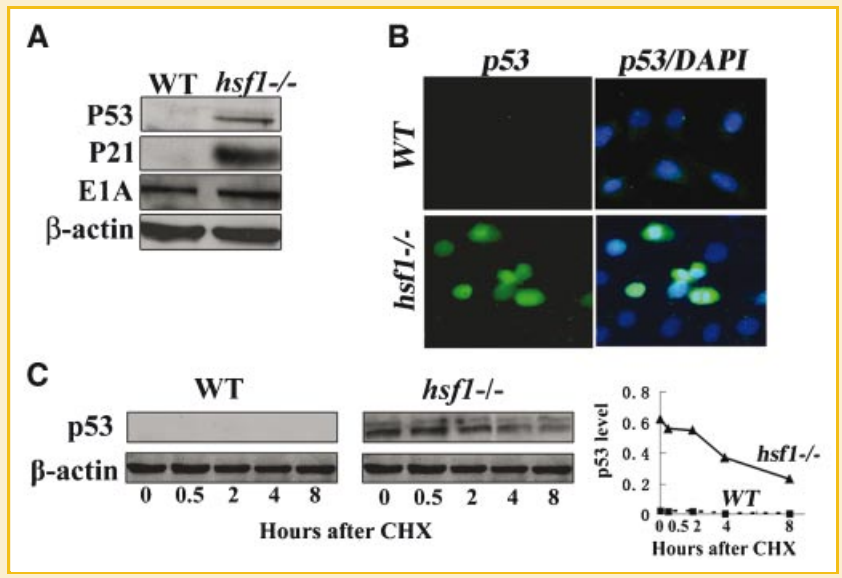

Fig. 2. *Hsf1*$^{-/-}$ cells express elevated levels of wild-type p53 protein. A: Wild-type and *hsf1*$^{-/-}$ MEFs were stably transformed with retroviral vector expressing adenoviral E1A, and the expression of p53 protein was determined by immunoblotting. The expression of E1A, p21^{Cip1}, and β-actin are presented as control. B: Immunofluorescene analyses of endogenous wild-type p53 protein. Wild-type and *hsf1*$^{-/-}$ cells were fixed and immunostained with antibody specific to p53 protein (green) or DAPI (blue) which stains the nuclei. C: *hsf1*$^{-/-}$ cells exhibit reduced p53 protein degradation. Cells were treated with 25 μg/ml of cycloheximide, and p53 levels were detected by immunoblotting at the indicated times. β-actin is loading control. Right panel shows the quantitation of p53 levels (relative unit) in wild-type and *hsf1*$^{-/-}$ cells after treatment with cycloheximide.

protein was undetectable under comparable exposure conditions in the wild-type cells (Fig. 2C). Quantitation of the data from three different experiments is presented in the right panel of Figure 2C.

HSF1$^{-/-}$ CELLS EXPRESS REDUCED LEVELS OF SMALL HSPS

Previous data indicate that the chaperone-mediated degradation of wild-type and mutant p53 protein through the UPS involves the participation of Hsp/Hsc70 and Hsp90 and their co-chaperones

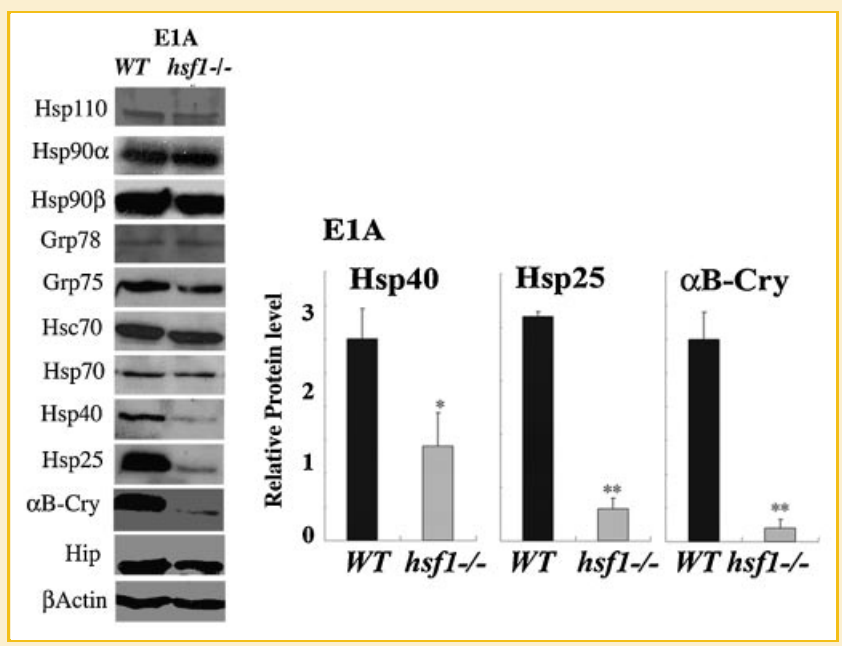

Fig. 3. *Hsf1*$^{-/-}$ cells exhibit reduced expression of specific heat shock proteins (Hsps). Wild-type or *hsf1*$^{-/-}$ MEFs expressing E1A were analyzed by immunoblotting to detect the expression of the indicated Hsps. β-actin is loading control. The levels of Hsps with significant expression changes in the left panel were quantitated using densitometry. All immunoblots contained equal amounts of protein (30 μg). Statistical significance is indicated by asterisks; $^{*}P < 0.05$, $^{**}P < 0.01$.

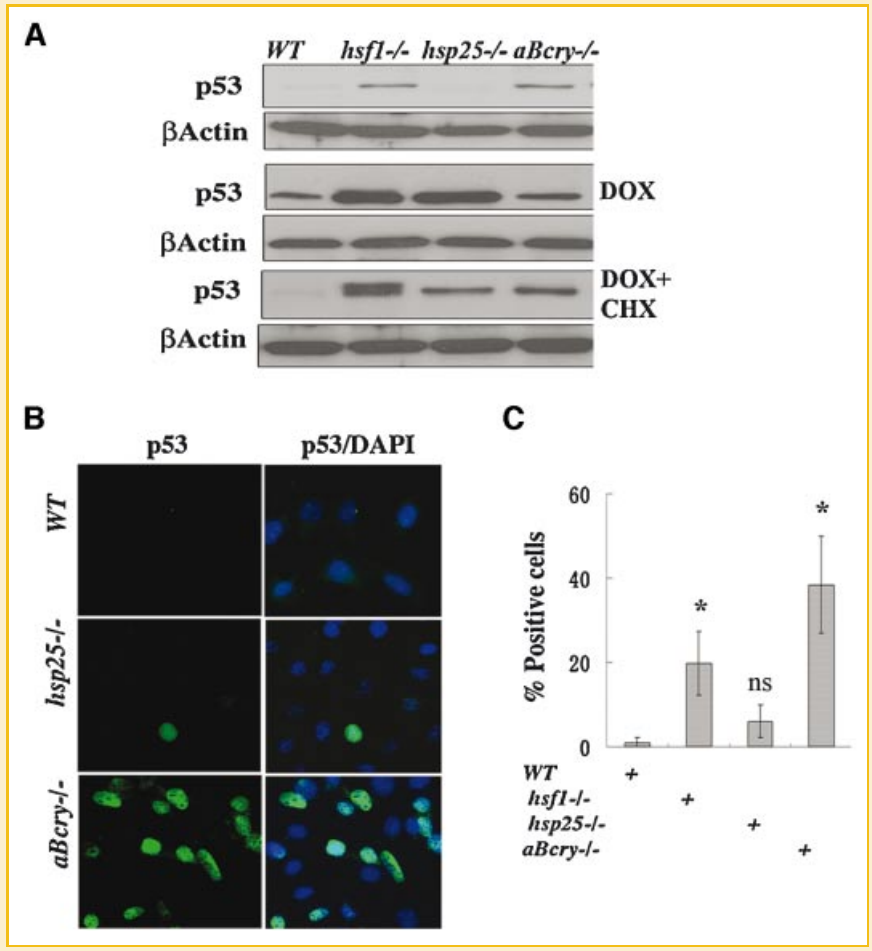

Fig. 4. Cells deficient in small Hsps express elevated levels of p53. A: Wild-type, *hsf1*$^{-/-}$, *hsp25*$^{-/-}$, and *aBcry*$^{-/-}$ MEFs were stably transformed with E1A, and either left untreated (first panel), or were treated with doxorubicin (DOX) for 16 h (0.5 μg/ml) or doxorubicin (DOX) (0.5 μg/ml) for 16 h plus cycloheximide (CHX) (25 μg/ml) for 8 h. Cell lysates were prepared and used in immunoblotting to detect p53 expression. β-actin is loading control. B: Immunofluorescence analyses were performed to detect endogenous wild-type p53 protein in E1A transformed MEFs of the indicated cell lines. Cells were fixed and immunostained with antibody specific to p53 protein (green) or DAPI (blue) that stains the nuclei. C: Quantitation of cells positively staining for p53 is presented for the indicated cell lines. Percentages of *hsf1*$^{-/-}$ cells expressing p53 are presented for comparison. $^{*}P < 0.001$; n.s., not significant.

[Whitesell et al., 1998; Zylicz et al., 2001]. To determine whether accumulation of wild-type p53 protein in *hsf1*$^{-/-}$ cells is due to the reduced levels of specific Hsps, wild-type or *hsf1*$^{-/-}$ cells were subjected to immunoblotting using antibody to the indicated Hsps (Fig. 3). The quantitation of the immunoblotting experiments indicate that *hsf1*$^{-/-}$ cells show significantly reduced expression levels of αB-crystallin, Hsp 25, and Hsp 40 (Fig. 3). The expression levels of Hsp90α Hsp90β, Hsc70, Hsp70, and their co-chaperones (Hop), as well as the expression of the glucose regulated proteins (Grp) 75 and Grp78 appeared comparable between *hsf1*$^{-/-}$ and wild-type cells (Fig. 3, and data not shown).

CELLS DEFICIENT IN αB-CRYSTALLIN ACCUMULATE WILD-TYPE P53 PROTEIN

Immunoblotting experiments presented in Figure 3 suggest that *hsf1*$^{-/-}$ cells express reduced levels of αB-crystallin and Hsp 25 compared to wild-type cells. To determine whether lower levels of αB-crystallin or Hsp 25 expression contribute to the accumulation of wild-type p53 protein in *hsf1*$^{-/-}$ cells, we performed immunoblotting experiments to determine the wild-type p53 level in E1A transformed wild-type cells, or cells deficient in Hsf1, αB-crystallin, or Hsp25. The results show that similar to *hsf1*$^{-/-}$, the *aBcry*$^{-/-}$ cells also accumulate elevated levels of wild-type p53 protein when compared to wild-type cells (Fig. 4A). Accumulation of wild-type p53 protein in *hsp25*$^{-/-}$ cells appeared not to be significantly different than in the wild-type cells.

Mammalian cells exposed to DNA damaging agents accumulate p53 protein [Lakin and Jackson, 1999; Lavin and Gueven, 2006]. Therefore, to test further whether exposure of mutant cells to DNA damaging agents leads to comparable accumulation of wild-type p53 protein levels as in wild-type cells, cells were exposed to doxorubicin or doxorubicin plus cycloheximide and p53 protein levels were determined by immunoblotting (Fig. 4A). The results indicate that all cell lines responded to doxorubicin treatment and accumulate p53 protein. Interestingly, we found that while p53 in doxorubicin-treated wild-type cells was degraded entirely after 8 h

in the presence of cycloheximide, significant levels of p53 protein remained undegraded in doxorubicin-treated *hsf1*$^{-/-}$, *hsp25*$^{-/-}$, and *aBcry*$^{-/-}$ cells. These data indicate that the degradation of p53 following treatment of cells with DNA damaging agents require the activity of Hsf1 and αB-crystallin. Furthermore, although the constitutive levels of wild-type p53 levels in *hsp25*$^{-/-}$ cells appear not to be significantly elevated compared to wild-type cells, doxorubicin-treated *hsp25*$^{-/-}$ cells exhibit some defects in fully degrading the drug-induced wild-type p53 compared to wild-type cells after 8 h.

To visualize the intracellular location of wild-type p53 protein in cells deficient in small Hsps, we performed immunofluorescent analyses. Figure 4B shows that as expected wild-type p53 is undetectable in wild-type cells, but cells deficient in *hsp25* or *aBcry* exhibit p53 nuclear staining. The quantitation of the number of cells expressing elevated levels of p53 protein in wild-type cells, or in cells deficient in small *hsps* is presented in Figure 4C. Thus, in the absence of αB-crystallin, p53 levels are elevated suggesting that expression of αB-crystallin is critical for p53 protein degradation. The quantitation of the number of *hsf1*$^{-/-}$ cells expressing wild-type p53 under comparable culture conditions is presented in comparison with *aBcry*$^{-/-}$ cells.

Since the elevated expression level of wild-type p53 protein normally reduces the progression of cells from G1 to S, we determined cell cycle distribution of wild-type, *hsf1*$^{-/-}$, and *αBcry*$^{-/-}$ cells. The data in supplementary Figure S1 shows that as predicted, both *hsf1*$^{-/-}$ and *αBcry*$^{-/-}$ cells exhibit accumulation of cells in the G1 phase compared to wild-type cells.

ELEVATED LEVELS OF WILD-TYPE P53 IN *HSF1*$^{-/-}$ CELLS LEAD TO THEIR INCREASED SENSITIVITY TO DNA DAMAGING AGENTS

The increased expression of wild-type p53 is associated with increased apoptotic cell death [Lakin and Jackson, 1999; Lavin and Gueven, 2006]. To determine whether *hsf1*$^{-/-}$ cells exhibit increased cell death in response to DNA damaging agents, we exposed wild-type, *hsf1*$^{-/-}$, *hsp25*$^{-/-}$, and *aBcry*$^{-/-}$ cells to different concentrations of doxorubicin or etoposide and determined cellular survival by colony formation assays. Results indicate that *hsf1*$^{-/-}$ cells exhibit highest levels of sensitivity to these chemotherapeutic agents compared to other cell lines (Fig. 5A). However, both *hsp25*$^{-/-}$ and *aBcry*$^{-/-}$ cells also exhibit significant increase in cellular sensitivity to drug treatment compared to wild-type cells (Fig. 5A). These results indicate that *hsf1*$^{-/-}$, *aBcry*$^{-/-}$, and *hsp25*$^{-/-}$ cells exhibit increased sensitivity to the DNA damaging agents compared to wild-type cells.

One of the downstream target genes of p53 that is activated following exposure of the cells to DNA damaging agents is the p21Cip1 protein [Lakin and Jackson, 1999; Lavin and Gueven, 2006]. To determine whether wild-type p53 expression in all knockout cell lines leads to elevated levels of p21Cip1 following exposure of the cells to drug treatment, we determined p21Cip1 expression levels by immunoblot analyses. The data indicate that p21Cip1 expresses in untreated *hsf1*$^{-/-}$ and *αBcry*$^{-/-}$ cells. The levels of p21Cip1 expression increased following exposure of the cells to the DNA damaging agents (Fig. 5B).

One of the hallmark of apoptotic response is the activation of caspase 3 that leads to degradation of key cellular proteins. The data presented in Figure 5B shows the expression of activated caspase 3 following drug treatment in all cell lines. Enhanced expression of activated caspase 3 is evident in *hsf1*$^{-/-}$, *hsp25*$^{-/-}$, and *aBcry*$^{-/-}$ cells compared to wild-type cells.

Since *hsf1*$^{-/-}$ cells exhibited higher than wild-type levels of p53 protein and exhibit cellular sensitivity once exposed to doxorubicin and etoposide, we also determined whether the apoptotic response of these cells differed compared to wild-type cells using Annexin V staining. We thus exposed the wild-type or *hsf1*$^{-/-}$ cells to reduced levels of serum, or to X-irradiation, doxorubicin, or etoposide, and the apoptotic response was determined. The results indicate that apoptotic cell death was significantly increased in *hsf1*$^{-/-}$ cells compared to wild-type cells (Fig. 5C). The increased apoptotic cell death in *hsf1*$^{-/-}$ cells following exposure of the cells to the above cytotoxic agents correlated with twofold increase in basal expression of the pro-apoptotic protein Bax (also a p53 target gene), while there were reduced levels of the anti-apoptotic proteins Bcl2 (fivefold) and Bcl-XL (fourfold) (Fig. 5D). The level of the pro-apoptotic protein Bad remained unchanged.

P53 PROTEIN INTERACTS WITH αB CRYSTALLIN

As we presented data in Figures 2 and 4, wild-type p53 protein accumulates in E1A transformed *hsf1*$^{-/-}$ and *aBcry*$^{-/-}$ cells under normal physiological growth conditions. In addition, *hsf1*$^{-/-}$ and *aBcry*$^{-/-}$ cells have a reduced ability to degrade p53 protein compared to wild-type cells once cells are exposed to the DNA damaging agents. Previous reports indicate that αB-crystallin binds to Fbx4 [den Engelsman et al., 2003] and that αB crystallin binds cyclin D1, enhancing its degradation through the Fbx4 ubiquitin ligase complex [Barbash et al., 2007]. To determine whether αB-crystallin bind p53 thereby facilitating its degradation through the Fbx4 ubiquitin ligase complex (SCF complex), we performed co-immunoprecipitation experiments. For these experiments we used U2OS cells since these cells express both wild-type p53 protein and αB-crystallin (Fig. 6). Immunoprecipitation experiments show that wild-type p53 protein can pull-down αB-crystallin following exposure of the cells to DNA damaging agents to increase p53 levels (Fig. 6). These results indicate for the first time that wild-type p53 interacts with αB-crystallin.

FBX4 UBIQUITIN LIGASE COMPLEX INTERACTS WITH P53 AND FACILITATES DEGRADATION OF BOTH WILD-TYPE AND MUTANT P53 PROTEINS

There are two ubiquitin E3 ligase complexes that have been identified to facilitate ubiquitination of cell cycle proteins [Nakayama and Nakayama, 2006; Barbash et al., 2007]: The Skp1-Cul1-F-box protein (SCF complex) ligases and anaphase promoting complex/cyclosome or APC/C. The SCF ligases are known to control the G1 to S transition, and their substrates include cyclin D1, cyclin E, p27kip1, Myc, and other proteins [Nakayama and Nakayama, 2006]. Since *hsf1*$^{-/-}$ and *aBcry*$^{-/-}$ cells exhibited a general increase in ubiquitinated proteins compared to wild-type cells and also accumulate p53 protein (Figs. 1, 2, and 4 [Homma et al., 2007] and data not shown), we speculated that other

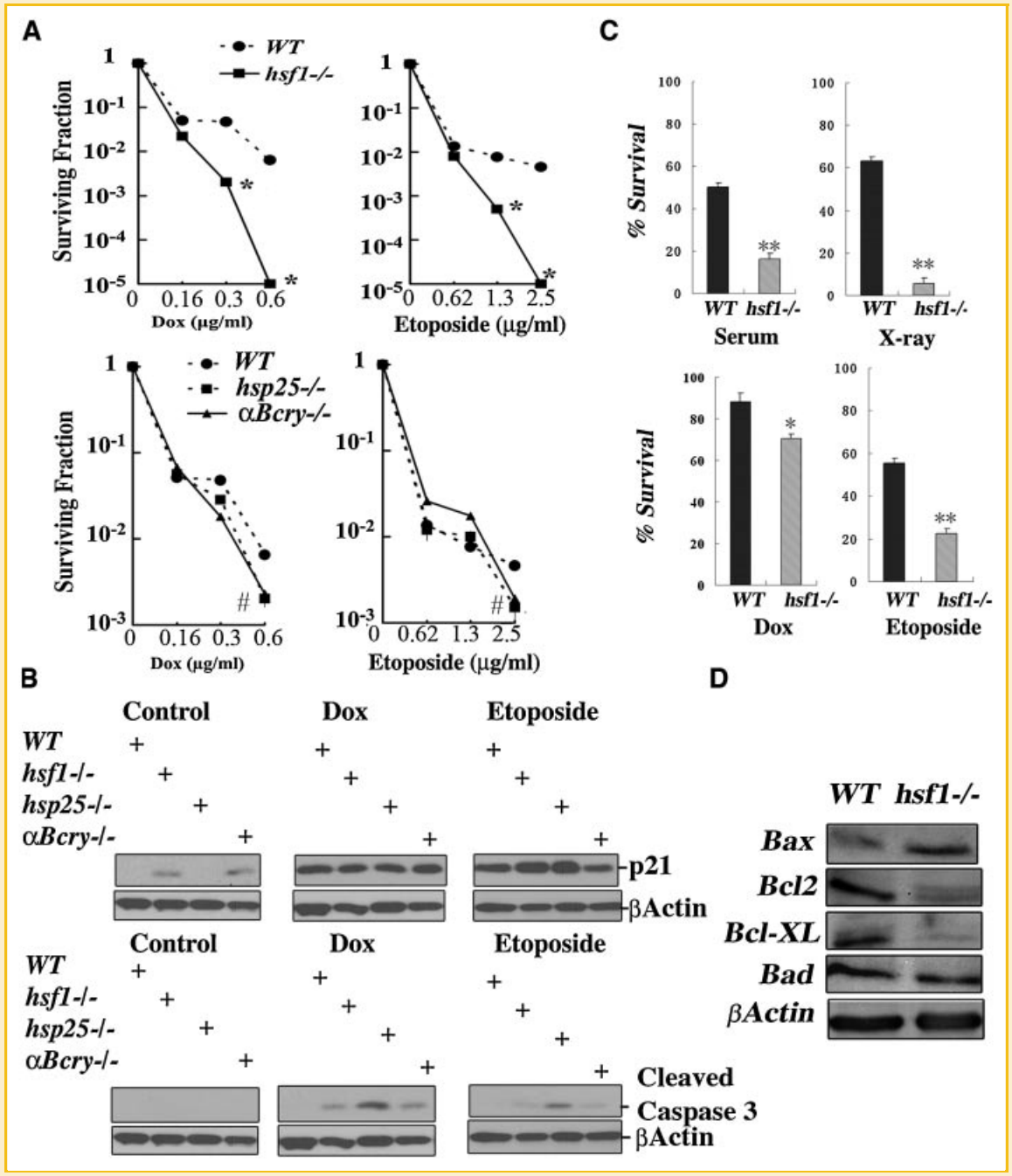

Fig. 5. Cells deficient in Hsf1 or αB crystallin exhibit enhanced sensitivity to DNA damaging agents. A: Wild-type, *hsf1*$^{-/-}$, *aBcry*$^{-/-}$, or *hsp25*$^{-/-}$ cells were exposed to different concentrations of doxorybicin or etoposide for 16 h. Cells were then rinsed with PBS and cellular survival was determined by colony formation assay. $^{*}P$-value <0.001 for the indicated concentrations. $^{\#}P$-value <0.05 for the indicated concentration and refers to both *aBcry*$^{-/-}$ and *hsp25*$^{-/-}$ cells compared to wild-type cells. B: Cells were left untreated, or exposed to DNA damaging agents (0.5 μg/ml of Dox or 5 μg/ml of etoposide for 16 h) and the expression of p21Cip1 and cleaved caspase 3 were determined by immunoblotting. β-actin is loading control. C: Wild-type and *hsf1*$^{-/-}$ cells were exposed to medium supplemented with low level of serum, 20 Gy of X-irradiation, 0.5 μg/ml of doxorubicin for 16 h, or 5 μg/ml of etoposide for 16 h. Cells were then stained with Annexin V and propidium iodide and the proportion of cell survival was determined. Statistical significance is indicated by asterisks; $^{*}P<0.05$, $^{**}P<0.01$. D: Immunoblot analyses of proteins with pro- and anti-apoptotic function in untreated wild-type, or *hsf1*$^{-/-}$ cells have been presented. All immunoblots contained equal amounts of protein (30 μg). β-actin is loading control.

ubiquitinated proteins (e.g., Cyclin D1) could also accumulate in these cells. As noted before, previous studies indicate that cyclin D1 degradation is linked to αB-crystallin since this protein, together with Fbx4, binds the phosphorylated Thr286 of cyclin D1 and promotes its degradation [Lin et al., 2006]. To determine cyclin D1 and p53 levels in the presence or absence of Fbx4, we used E1A transformed wild-type, *hsf1*$^{-/-}$, and *aBcry*$^{-/-}$ cells stably expressing mutant p53R175H. We stably overexpressed p53R175H in

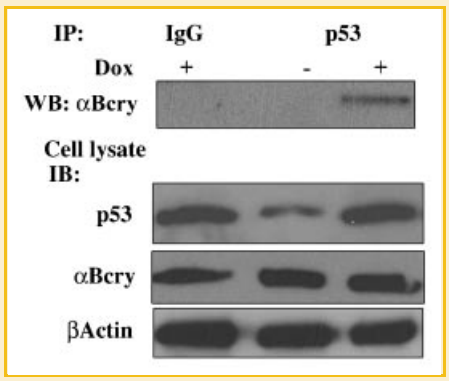

Fig. 6. p53 interacts with αB-crystallin. U2OS cells were left untreated or were treated with doxorubicin (0.5 μg/ml for 16 h). Cell extracts were then used to immunoprecipitate p53 followed by immunoblotitng to detect αB-crystallin. IgG is non-specific antibody used in immunoprecipitation experiments. Lower panels show the levels of p53 and αB-crystallin in U2OS cells before and after treatment with doxorubicin. β-actin is loading control.

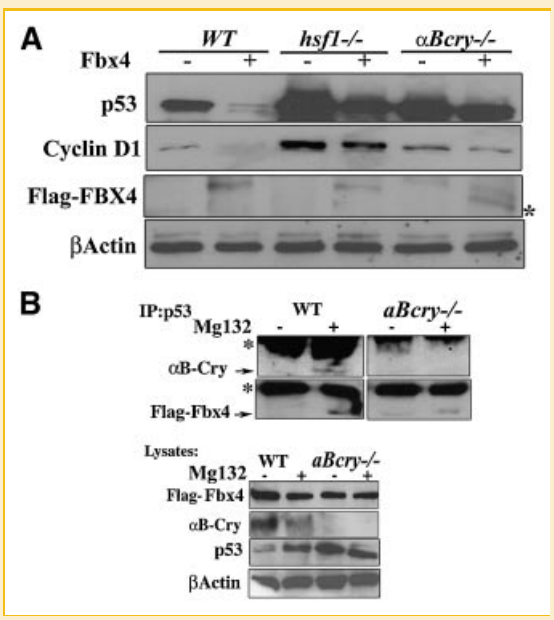

Fig. 7. Fbx4-dependent degradation of cyclin D1 and p53R175H. A: Wild-type, $hsf1^{-/-}$, or $aBcry^{-/-}$ cells expressing E1A and p53R175H were transiently transfected with Flag-Fbx4 and were analyzed by immunoblotting using antibody to p53, cyclin D1, or Flag-Fbx4. β-actin is loading control. B: Wild-type or $\alpha Bcry^{-/-}$ cells stably expressing E1A and p53R175H were transiently transfected with expression vector containing Flag-Fbx4. Forty eight hours post-transfection, equal amounts of cell lysates were subjected to immunoprecipitation (IP) using antibody to p53. The products were analyzed by immunoblotting using antibody to αB-crystallin or Flag to detect expression of αB-crystallin and Fbx4. * Represents IgG. The − and + Mg132 (1 μM for 8 h) indicate transiently transfected cells were left untreated, or treated with Mg132 to inhibit UPS. Lower panels show same cell lysates were subjected to immunoblotting using antibody to Flag-Fbx4, αB-crystallin, and p53. β-actin is loading control.

MEFs because these cells express very low levels of wild-type p53 as expected. Thus, we determined the level of the cell cycle regulator cyclin D1 in wild-type, $hsf1^{-/-}$, and $aBcry^{-/-}$ cells in the presence or absence of exogenous Fbx4 (Fig. 7). We found that not only cyclin D1 expression was higher in $aBcry^{-/-}$ and $hsf1^{-/-}$ cells compared to wild-type cells, exogenous expression of Fbx4 lead to increased degradation of cyclin D1 in wild-type cells (Fig. 7A). Perhaps not surprisingly, we found that Fbx4 expression in cells also lead to increase in p53 (and p53R175H) degradation in the same pattern as cyclin D1 in above cell lines, suggesting that p53 is also targeted by Fbx4, and that this degradation appears to be dependent on αB-crystallin levels in the cells (Fig. 7A). This is because the ectopic expression of Fbx4 only partially reduced p53 expression levels in $hsf1^{-/-}$ and $aBcry^{-/-}$ cells that express less αB-crystallin, or no αB-crystallin, respectively, compared to wild-type cells. The expression levels of other cyclins were less affected in these mutant cells compared to wild-type cells (data not shown). Lower panel of Figure 7B shows the expression of Flag-Fbx4, αB-crystallin, and p53 in wild-type and $\alpha B\text{-}cry^{-/-}$ cells.

We then tested whether p53 interacts with Fbx4 and αB-crystallin complexes. Thus, wild-type and $\alpha Bcry^{-/-}$ cells expressing p53R175H were transiently transfected with Fbx4, and p53 was immunoprecipitated following treatment of cells with Mg132 (to inhibit protein degradation through UPS). The data indicate that p53 interacts with both αB-crystallin and Fbx4 in wild-type cells treated with Mg132 (Fig. 7B). In cells expressing no αB-crystallin there was a weak interaction between p53 and Fbx4.

Since Fbx4 has not previously been shown to be involved in p53 protein degradation, we thus determined whether wild-type or mutant p53R175H that can be degraded through the UPS, can be detected in Fbx4 containing complexes, perhaps suggesting that Fbx4 ubiquitin ligase complex, can bind and degrade both wild-type and mutant p53 proteins. Thus, immunoprecipitation experiments were performed with wild-type or $hsf1^{-/-}$ MEFs expressing p53R175H and ectopically expressing Fbx4. The results indicate that using antibody to p53 we were able to immunoprecipitate Fbx4 from $hsf1^{-/-}$ cells that accumulate more p53R175H than the wild-type cells, and from both wild-type and $hsf1^{-/-}$ cell lysates when cells were treated with Mg132. These results suggest that p53R175H can be targeted and degraded by the Fbx4 complex (Fig. 8A). As a positive control, lower panel of Figure 8A shows the interaction of cyclin D1 and Fbx4 in wild-type and $hsf1^{-/-}$ cells in the presence of Mg132 [Lin et al., 2006]. We also performed experiments to detect the interaction of p53R175H with endogenous Fbx4 in wild-type and $hsf1^{-/-}$ cells (Fig. 8B). The results indicate that antibody to p53 could immunoprecipitate endogenous Fbx4 in both wild-type and $hsf1^{-/-}$ cells. Additionally, we also performed experiments to determine whether endogenous wild-type p53 interact with endogenous Fbx4 protein (Fig. 8C). The data indicate that immunoprecipitated wild-type p53 can pull-down Fbx4 from U2OS cells treated with doxorubicin (to increase wild-type p53 levels). There was no interaction between p53 and Fbx4 in cells not pretreated with doxorubicin that expressed very low levels of p53 protein.

Taken together data presented in Figures 7 and 8 provide strong evidence that both wild-type and mutant p53 are targets of

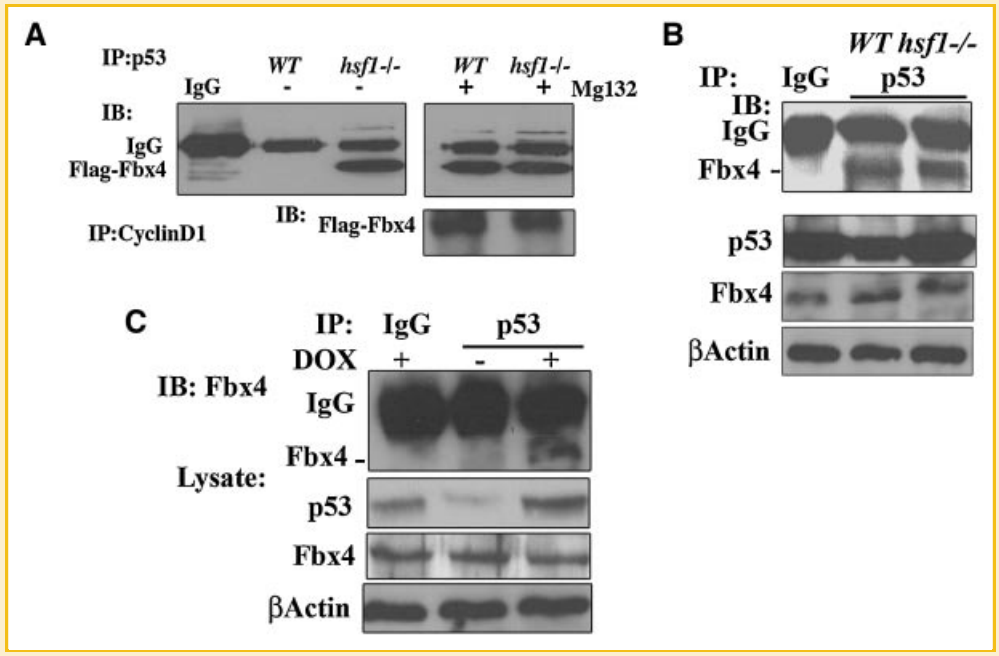

Fig. 8. p53 interacts with Fbx4. A: Wild-type or *hsf1*$^{-/-}$ MEFs expressing E1A and p53R175H were transiently cotransfected with plasmids containing Flag-Fbx4. After 48 h, cells were left untreated (−) or were treated (+) with Mg132 (1 μM for 8 h) and cell lysates were subjected to immunoprecipitation (IP) using antibody to p53 and immunoblotting were performed to detect Flag-Fbx4. Lower panel: same cell lysate was used in immunoprecipitation experiment using antibody to cyclin D1 and immunoblotting were performed using antibody to Flag-Fbx4. B: Wild-type and *hsf1*$^{-/-}$ cells expressing p53R175H were subjected to immunoprecipitation using antibody to p53. The pull-down materials were used in immunoblotting experiments using antibody to detect endogenous Fbx4. Cell lysates were also immunoblotted to detect p53 and Fbx4. C: U2OS cells were treated with doxorubicin (0.5 μg/ml for 16 h) (+) to enhance endogenous wild-type p53 levels. Cell lysates were subjected to immunoprecipitation using antibody to p53 and pull-down fractions were analyzed by immunoblotting using antibody to detect endogenous Fbx4. +DOX is doxorubicin treated groups. Lower panels show expression of p53 and Fbx4 in the cell lysates. In all panels, IB: IgG represent the antibody heavy chain. IP: IgG represents a non-specific antibody used as control for immunoprecipitation. β-actin is loading control.

Fbx4 and at least in part this interaction requires αB-crystallin in the complex.

In many instances the proteins targeted for degradation by the Fbx4 complex possess post-translational modifications [Nakayama and Nakayama, 2006]. Therefore, the next question was to determine whether Fbx4 requires the phosphorylated form of p53 and target it for degradation through the UPS. Thus, we performed immunoblotting experiments where vectors containing wild-type p53, p53 with N-terminal serine/threonine (S6, S9, S15, T18, S20, S33, S37) residues mutated to alanines, or C-terminal serine (S315, S371, S376, S378, S392) residues mutated to alanines [Ashcroft et al., 1999] were ectopically expressed in H1299 cells that do not express wild-type p53 to determine whether these phosphorylation mutant p53 proteins can be degraded by the ectopically expressed Fbx4. Results indicate that the wild-type p53, and p53 with the above N-terminal, or the C-terminal phosphorylation mutants were degraded in the presence of Fbx4 complexes (Fig. S2). These results indicate that Fbx4 is a new F-box protein that is capable of degrading p53, and the above phosphorylation sites are not required for p53 degradation.

We also performed experiments to determine whether expression of dominant negative form of Fbx4 would lead to increased stability of wild-type p53 protein. Thus, U2OS cells were transiently transfected with Fbx4 or dominant negative form of Fbx4 (F-box deletion that leads to abolishment of SKP1 binding, but not αB-crystallin binding or substrate recognition) [Lin et al., 2006]. Forty-eight hours post-transfection, cells were treated with doxorubicin to enhance expression of endogenous wild-type p53 protein and then with cycloheximide to determine the level of p53 remaining in the cells by immunoblotting. The data is presented in Figure 9 show that, U2OS cells expressing dominant negative form of Fbx4 express elevated levels of p53 at 0 h post-doxorubicin treatment and at 4 h post-cycloheximide treatment. At 4 h post-cycloheximide treatment, the expression of p53 is threefold higher in cells expressing dominant negative form of Fbx4, than the wild-type Fbx4. These results indicate that while Fbx4 facilitates p53 degradation, dominant negative form of Fbx4 reduces p53 degradation.

DISCUSSION

Molecular chaperones expression and activity are critical for protein folding, transport, and higher order assembly of multi-protein complexes, and their expression is in part controlled by Hsf1 transcription factor [Hartl, 1996; Morimoto, 1998; Haslbeck, 2002; Muchowski, 2002]. Molecular chaperones are also involved in protein degradation via the UPS by specific recognition of substrates or phosphorylated substrates, targeting these proteins for degradation [Arndt et al., 2007]. The UPS is involved in timely degradation

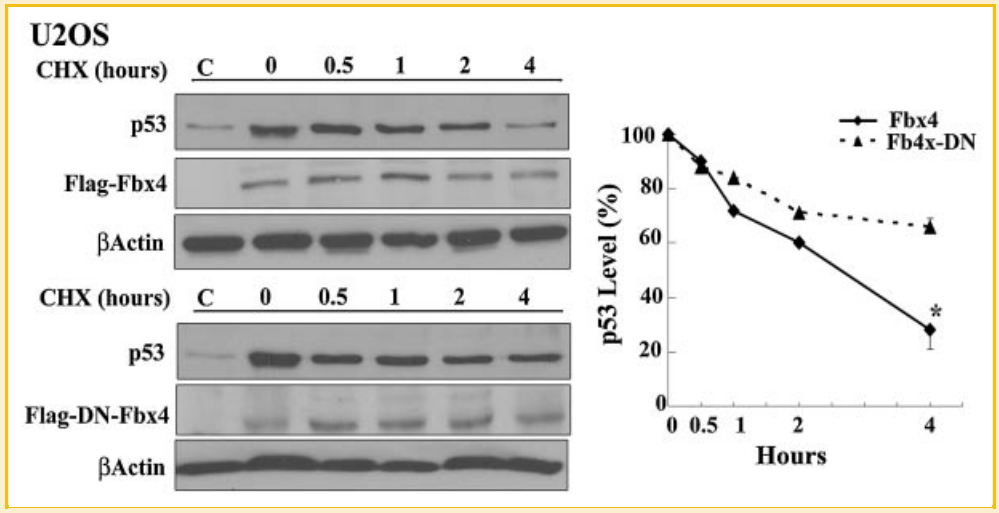

Fig. 9. Dominant negative form of Fbx4 inhibits p53 degradation. U2OS cells expressing wild-type p53 were transiently transfected with expression vector containing Flag-Fbx4 or dominant negative form of Fbx4 (Flag-DN-Fbx4). Forty-eight hours following transfection, cells were exposed to doxorubicin (0.5 μg/ml for 16 h) to enhance p53 levels. Cells were then exposed to cycloheximide (CHX) (25 μg/ml) for 4 h. Samples were collected at indicated times and immunoblotting experiments were performed to detect expression of p53 and Flag-Fbx4. "C" represent p53 expression in cells without doxorubicin treatment. β-actin is loading control. Quantitation of the data is presented in the right panel. $^{*}P$-value < 0.05 for the indicated time point.

of critical proteins essential during cell cycle progression, recognition, and degradation of misfolded proteins [Nakayama and Nakayama, 2006]. Accumulation of aggregated proteins is cytotoxic to the cells, specifically to neuronal cells, and this is the hallmark of neurodegenerative diseases [Muchowski, 2002]. The failure or inefficiency of quality control mechanisms, including pathways that affect protein degradation and generation of misfolded proteins, leads to cell death. However, the mechanism of how protein misfolding and aggregation may affect cell growth or tumorigenesis remains elusive.

In this study, we investigated the underlying mechanisms for p53 protein accumulation in the cells that lack the *hsf1* gene. We found that both Hsf1 and its downstream target gene αB-crystallin are essential for degradation of p53 protein following oncogenic transformation and/or exposure of the cells to DNA damaging agents. Our findings can be summarized as follows: $hsf1^{-/-}$ cells accumulate p53 as well as other ubiquitinated proteins (such as cyclin D1 or short-lived ubiquitinated GFP [Homma et al., 2007]). Oncogene E1A transformed $hsf1^{-/-}$ cells exhibit lower basal expression levels of αB-crystallin, Hsp25, and Hsp40. While $\alpha Bcry^{-/-}$ cells also accumulate p53, $hsp25^{-/-}$ cells do not accumulate p53 under comparable conditions. Although we did not find elevated expression levels of p53 protein in E1A transformed *hsp70.1/hsp70.3*-deficient cells (data not shown), we have not tested the p53 expression levels following reduction in Hsp40. As noted before, Hsp25 has been shown to interact with the 26S proteasome and facilitate IkBα protein degradation [Parcellier et al., 2003]. Additionally, αB-crystallin binds to Fbx4 ubiquitin ligase and facilitate protein degradation of specific substrates [den Engelsman et al., 2003]. We also have found that endogenous wild-type p53 interacts with αB-crystallin. Since αB-crystallin was shown to interact with cyclin D1 leading to recruitment of Fbx4 followed by cyclin D1 degradation [Lin et al., 2006], we tested the possibility of a complex containing p53-αB-crystallin-Fbx4 and our data indicate that indeed wild-type p53 protein (and mutant p53R175H protein) is present in the same complex with αB-crystallin and Fbx4. In addition, p53 degradation is stimulated by ectopic expression of Fbx4 into the cells. In contrast, the expression of a dominant negative form of Fbx4 did not lead to degradation of wild-type p53 protein.

F-box proteins often facilitate degradation of phosphorylated proteins [Nakayama and Nakayama, 2005, 2006]. Therefore, we determined whether phosphorylation of p53 is required for p53 degradation by αB-crystallin and Fbx4. We found that none of the phosphorylation sites tested on p53 protein were required for p53 degradation by Fbx4 ubiquitin ligase complex. The p53 protein undergoes multiple modifications that control its stability [Lavin and Gueven, 2006]. Phosphorylation of p53 dominates the changes and occurs through multiple protein kinases such as ATM, ATR, Chk1 and Chk2, JNK, and p38 [Lavin and Gueven, 2006]. ATM mediates phosphorylation of serines 6, 9, 15, 20, and 46 and threonine 18 following exposure of the cells to X-irradiation. Some of these sites are also phosphorylated following exposure of the cells to other DNA damaging agents. The modifications in the N-terminal domain appear to prevent p53-Mdm2 interaction, while C-terminal domain may increase conformational changes that prevent interactions with the C-terminal and DNA binding domain that is required for stabilizing the p53 protein [Bruins et al., 2004]. However, the p53 protein is also phosphorylated in number of C-terminal residues, namely serines 315, 371, 376, 378 and 392 and threonines 377 and 387 [Lavin and Gueven, 2006]. Thus far, phosphorylation of p53 has not been directly correlated with an increase in its interaction with any ubiquitin E3 ligases [Lavin and Gueven, 2006]. The main proteins that appear to be so far involved in p53 stability are the Mdm2 and MdmX, and any alterations that interfere with those interactions lead to p53 stabilization [Lavin and

Gueven, 2006]. Our results show that p53 phosphorylation sites namely serines 6, 9, 15, 20, 33, 37, 315, 371, 376, 378 and 392 and threonine 18 [Ashcroft et al., 1999] are not potentially required for αB-crystallin and Fbx4 recognition of p53 and its degradation. However, p53 contains other phosphorylation sites such as threonines 91, 377, or 387, and the serines 46, 366, and 392, and the latter two threonine residues are the potential Chk1 phosphorylation sites, and the latter two serine residues are the potential Chk2 phosphorylation sites [Lavin and Gueven, 2006]. We therefore, envision that αB-crystallin and Fbx4 either recognize the other p53 phosphorylation sites that we have not tested, or they may require no p53 modifications, or p53 modifications other than phosphorylation for recognition. In a separate experiment, we also tested whether ectopic expression of Mdm2 [Lavin and Gueven, 2006] or Chip [Esser et al., 2005] could lead to increase degradation of p53 in wild-type cells expressing mutant p53. We found that although in wild-type cells expression of above ubiquitin ligases leads to complete degradation of p53R175H, the level of p53 in *hsf1*$^{-/-}$ cells was reduced, but did not totally degraded (data not shown). These results indicate that *hsf1*$^{-/-}$ cells do not have defects in Mdm2 or Chip mediated degradation of proteins, however, there is other defects (e.g., lower αB-crystallin expression) that lead to accumulation of p53 protein in these cells.

The p53R175H expressed in *hsf1*$^{-/-}$ cells also accumulate higher than the wild-type cells, suggesting that *hsf1*$^{-/-}$ cells have a reduced ability to degrade both wild-type and mutant p53R175H (Fig. 7 and data not shown). Experiments indicate that the $^1/_2$ time for degradation of p53R175H in wild-type cells was <4 h, while in *hsf1*$^{-/-}$ cells was >8 h. The p53R175H mutation results in change of p53 conformation, loss of DNA binding ability, and an extended half-life of p53 protein [Zambetti and Levine, 1993; Cadwell and Zambetti, 2001]. The p53R175H mutant protein is however degraded through the UPS similar to wild-type p53 [Cadwell and Zambetti, 2001; Terzian et al., 2008]. Molecular chaperones such as Hsp70/Hsc70 and Hsp90 and their cochaperones have been shown to be involved in wild-type or mutant p53 protein degradation [Zylicz et al., 2001]. These molecular chaperones are involved in p53 degradation at least in part through the Chip ubiquitin ligase [Esser et al., 2005]. Chip preferably ubiquitinates the substrates that are bound to molecular chaperones leading to their degradation. Hsp70/Hsc70 and Hsp40 have been found in complexes with conformational p53 mutants [Zylicz et al., 2001]. Both Hsp70/Hsc70 and Hsp90 also have been found in complexes with mutant p53 protein [Zylicz et al., 2001]. Treatment of cells overexpressing mutant p53 protein with geldanamycin that inhibit Hsp90 client proteins increases the degradation of Hsp90 client proteins (e.g., p53 mutant protein) [Xu et al., 2002; Esser et al., 2005; Germano et al., 2006]. These data indicate a role for Hsp70/Hsc70 and Hsp90 in mutant p53 protein degradation. E1A transformed *hsf1*$^{-/-}$ cells have wild-type levels of Hsp70/Hsc70, Hsp90α and β expression. However, in addition to lower levels of Hsp25 and αB-crystallin expression, *hsf1*$^{-/-}$ cells also possess lower levels of Hsp40 expression. Since Hsp40 cochaperone is required for Hsp70/Hsc70 chaperone activity [Zylicz et al., 2001], a fraction of the wild-type and mutant p53 protein accumulation in *hsf1*$^{-/-}$ cells could be due to inefficient Hsc70/Hsp70 and Hsp40 chaperone activity as well. Indeed, our data indicate a higher level of ubiquitinated mutant p53 protein accumulation in *hsf1*$^{-/-}$ cells than in *αBcry*$^{-/-}$ cells (data not presented).

Our results indicate that alterations in the level or function of molecular chaperones such as small heat shock proteins αB-crystallin is important in maintaining wild-type levels of p53 protein which could affect the apoptotic response of cells. The level of these small Hsps can also affect expression of mutant p53 protein which could reduce intracellular wild-type p53 levels in cancer cells leading to reduction in apoptotic response. Lower levels of αB-crystallin not only can affect degradation of mutant p53 protein as shown here, but also can lead to increased levels of cyclin D1 and therefore, acceleration of cellular proliferation [Lin et al., 2006]. Indeed, positive correlations have been made between reduced αB-crystallin expression and an increase in cyclin D1 expression in human tumors.

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