Published in final edited form as:

Breast Cancer Res Treat. 2010 July ; 122(1): 159–168. doi:10.1007/s10549-009-0542-7.

Induction of the Small Heat Shock Protein α B-Crystallin by Genotoxic Stress is Mediated by p53 and p73

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Abstract

The small heat shock protein α B-crystallin is a molecular chaperone that is induced by stress and protects cells by inhibiting protein aggregation and apoptosis. To identify novel transcriptional regulators of the α B-crystallin gene, we examined the α B-crystallin promoter for conserved transcription factor DNA binding elements and identified a putative response element (RE) for the p53 tumor suppressor protein. Ectopic expression of wild-type p53 induced α B-crystallin mRNA and protein with delayed kinetics compared to p21. Additionally, the induction of α B-crystallin by genotoxic stress was inhibited by siRNAs targeting p53. Although the p53-dependent transactivation of an α B-crystallin promoter luciferase reporter required the putative p53RE, chromatin immunoprecipitation (ChIP) failed to detect p53 binding to the α B-crystallin promoter. These results suggested an indirect mechanism of transactivation involving p53 family members p63 or p73. Δ Np73 was dramatically induced by p53 in a TAp73-dependent manner, and silencing p73 suppressed the transcriptional activation of α B-crystallin by p53. Moreover, ectopic expression of Δ Np73 α (but not other p73 isoforms) increased α B-crystallin mRNA levels in the absence of p53. Collectively, our results link the molecular chaperone α B-crystallin to the cellular genotoxic stress response via a novel mechanism of transcriptional regulation by p53 and p73.

Keywords

p53; p73; Δ Np73 α ; TAp73; α B-crystallin; DNA damage

Introduction

α B-crystallin/HspB5 is a widely expressed member of the small heat shock protein (sHSP) family, which also includes Hsp27/HspB1 and HspB2/MKBP, defined by the presence of a conserved α -crystallin domain [1]. α B-crystallin promotes cell survival following its induction by cellular stressors, such as heat and reactive oxygen species (ROS), by inhibiting the aggregation of denatured or misfolded proteins and by increasing intracellular glutathione levels [2-5]. More recently, α B-crystallin has been shown to inhibit apoptosis by suppressing caspase-3 activation and by sequestering pro-apoptotic effectors, such as Bax and Bcl-X_s, in the cytoplasm, thereby conferring additional cytoprotection against cellular stress [6-9].

HSF-1, lens epithelial derived growth factor (LEDGF)/p75 and the glucocorticoid receptor have been demonstrated to regulate the induction of α B-crystallin in response to hyperthermia, ROS and glucocorticoids [10-12].

We postulated that additional transcriptional regulators of the mammalian stress response might regulate α B-crystallin gene expression. Here, we report that the α B-crystallin gene is regulated by a novel mechanism involving multiple p53 family members. The p53 protein is a critical mediator of the cellular response to genotoxic stress and plays a fundamental role in tumor suppression, as evidenced by the frequent inactivation of the p53 pathway in a broad spectrum of tumors [13]. Classically, p53 inhibits tumor initiation by transcriptionally coordinating several cellular programs that prevent propagation of damaged genomes: cell cycle arrest, DNA repair, senescence and apoptosis. p53 biology has been dramatically revised with the discovery of additional p53 family members, p63 and p73. TAp63 and TAp73 have been shown to transactivate many p53 target genes [14-16]. Moreover, p63 and p73 are required for p53-dependent apoptosis and/or cell cycle arrest in some systems, and p73 can mediate these responses in the absence of p53 [17-19]. Through an alternative promoter, the p63 and p73 genes also express Δ N isoforms which lack the N-terminal transactivation domain. Δ Np63 and Δ Np73 act in a dominant negative fashion on p53 activity by competing for p53RE binding, and on TAp63 and TAp73 activity through the formation of inactive heterotetramers [20,21]. In light of their ability to inhibit p53, Δ N isoforms likely play an important role in tumor biology. Indeed, Δ Np73 expression enhances Ras-mediated transformation, counters p53-mediated apoptosis in development, and is expressed in several clinically aggressive tumor types [22-27]. In addition to this antagonistic function, Δ Np63 and Δ Np73 appear to independently transactivate genes and induce cell cycle arrest or apoptosis [28-31]. Alternative 3' splicing generates 3 p63 (α , β , γ) and 8 p73 (α , β , γ , ζ , δ , θ , η , η 1) C-terminal isoforms [32, 33]. Additionally, p53 family members transcriptionally regulate each other to form overlapping positive and negative feedback loops [20,34,35]. The complexity of the p53 family transcriptional regulation network and its role in cancer are topics of intense investigation and have yet to be fully resolved.

In this manuscript, we demonstrate that genotoxic stress induces α B-crystallin by a p53-dependent mechanism. Intriguingly, p53 activates α B-crystallin gene expression by an indirect mechanism that results in delayed induction of α B-crystallin compared to p21. Instead, p53 robustly induces Δ Np73, which in turn activates α B-crystallin gene expression. Taken together, our findings point to a novel link between α B-crystallin and genotoxic stress that is regulated by the cooperative actions of multiple p53 family members.

Materials and methods

Cell culture

EJ-p53 cells (kind gift of Sam W. Lee, Harvard Medical School) were grown in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 300 μ g/mL G418 (Mediatech), 100 μ g/mL hygromycin B (Mediatech), and 1% penicillin/streptomycin/L-glutamine (Invitrogen). Tetracycline (1 μ g/mL, Sigma-Aldrich) was added fresh to the media every 3-4 days to maintain repression of p53 [36]. MCF-10A cells (ATCC) were grown in DMEM/F-12 (1:1 mixture, Invitrogen) supplemented with 5% horse serum (Invitrogen), 0.5 μ g/mL hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), 10 μ g/mL insulin (Sigma-Aldrich), 20 ng/mL EGF (Sigma-Aldrich), and 1% penicillin/streptomycin/L-glutamine. Phoenix cells (ATCC) were grown in DMEM supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin/L-glutamine. Doxorubicin (Sigma-Aldrich) was dissolved in DMSO.

Retrovirus production and infection

Retrovirus was produced in Phoenix cells and used to infect MCF-10A as previously described [37,38]. Briefly, 2.5×10^6 Phoenix cells were transfected with 5.45 μ g pLXSN retroviral expression plasmid and 0.55 μ g pMD2-VSV-G (a kind gift of Ronald DePinho, Harvard Medical School) using 50 μ L 2.5 M CaCl_2 and 500 μ L 2X HBS, pH 7.10 with 25 μ M chloroquine (Sigma-Aldrich) in the media. Retrovirus production was carried out at 32°C, and EGF was added to 20 ng/mL in addition to polybrene after 0.45 μ m-filtering. Retroviral supernatant was used to infect 300,000 MCF-10A cells in 6 cm dishes for 3-5 h, then fresh media was added.

Site-directed mutagenesis

Point mutations were made using the QuikChange Site Directed Mutagenesis kit (Stratagene) per the manufacturer's protocol with the primers listed in Supplementary Table 1. The coding sequence of wild-type p53 (GenBank NM_000546) in the pLXSN retroviral expression vector was mutated to R175H and R273H. The α B-crystallin/HspB2 promoter sequence in the pGL3 luciferase reporter vector was mutated at the 5' and 3' p53RE half-sites (the sequences of the putative wild-type and mutated p53 REs are shown in Fig. 2A). Mutations were confirmed by direct sequencing.

Luciferase experiments

EJ-p53 cells (80,000 cells per well in a 24-well plate) were transfected with 300 ng pcDNA3 empty vector, 100 ng pGL3 firefly luciferase reporter construct, and 100 pg pRL-CMV *Renilla* luciferase expression vector (Promega) using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured with the Dual-Luciferase Reporter Assay kit reagents (Promega) per the manufacturer's protocol. Briefly, cells were lysed with 1X Promega Passive Lysis Buffer and transferred to a Lumitrac 200 96-well plate (Greiner Bio-One). Firefly and *Renilla* luciferase activities were measured for 30 s following LAR II and Stop-n-Glo injection, respectively, in a Clarity Luminescence Microplate Reader (BioTek). Firefly luciferase activity was normalized to *Renilla* activity.

Protein isolation and immunoblotting

Cells were lysed with RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA pH 8.0), supplemented with 1% each: Protease Inhibitor Cocktail (100X, Sigma-Aldrich), Phosphatase Inhibitor Cocktail Set II (Calbiochem), and 100 mM PMSF (Sigma). Lysate protein concentration was measured by BCA colorimetry (BCA Protein Assay Kit, Pierce). DTT was added to 100 mM, 5X sample buffer (250 mM Tris-HCl, pH 6.8, 10% w/v SDS, 50% (v/v) glycerol, ~0.4% w/v bromophenol blue) added, and lysates were boiled.

Proteins lysates were separated by size using SDS-PAGE, and transferred to a PVDF membrane (Immobilon P, Millipore) using a semi-dry transfer apparatus. The membranes were blocked for 1 h at RT in TBS with 0.05% Tween-20 and 5% non-fat milk, incubated overnight at 4°C with the primary antibodies listed in Supplementary Table 2, and then incubated with anti-mouse HRP-conjugated secondary antibody (Southern Biotech) for 1 h at RT. Proteins were detected by enhanced chemiluminescence (ECL) assay.

RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated with the RNeasy Plus Mini Kit (QIAGEN). cDNA was made from 0.5 μ g total RNA with the ReactionReady First Strand Synthesis Kit (SABiosciences) per manufacturer's protocol. cDNA was used as template for real-time PCR with 0.4 μ M each primer using the RT² SYBR Green/ROX PCR Master Mix (SABiosciences). Commercially

available primer sets listed in Supplementary Table 3 (RT² qPCR Primer Set for Human genes, SABiosciences) were used to amplify the following genes: CDKN1A (p21), CRYAB (α B-crystallin), GAPDH, HSPB1 (Hsp27), HSPB2 and TP53. Primer sequences obtained from the literature (Supplementary Table 4) were used to amplify TAp63, Δ Np63, TAp73, and Δ Np73 [39].

Reactions were carried out in triplicate in 384-Well Clear Optical Reaction Plates (Applied Biosystems) using a 7900HT Fast Real-Time PCR Machine (Applied Biosystems) with the following thermal cycling profile: 50°C 2 min, 95°C 10 min, 40 $\times$ (95°C 15 sec, 60°C 1 min with fluorescence detection). SDS 2.3 software (Applied Biosystems) generated cycle threshold (C_t) values, which were converted to L values by the standard curve method, followed by internal normalization to the GAPDH L value. Each fold-induction value reported is the mean of 3-6 independent experiments.

siRNA oligo duplex transfection

MCF-10A cells (878,000 cells in 6 cm dishes) were transfected with 100 nM ON-TARGET^{plus} siRNA oligo duplexes targeting TP53 (Dharmacon, J-003329-14 and J-003329-15) or Non-Targeting siRNA Control #2 (Dharmacon, D-001810-02) with Oligofectamine transfection reagent (Invitrogen) per the manufacturer's protocol. EJ-p53 cells (100,000 cells per well in 6-well plates) were transfected with 5 pmol siRNA oligo duplexes using 7.5 μ L Lipofectamine RNAiMAX (Invitrogen) per the manufacturer's protocol. Targeting sequences for TAp73, Δ Np73, and pan-p73 isoforms (Supplementary Table 5) were obtained from the literature [35, 40].

EJ-p53 transient transfection

EJ-p53 cells (630,000 cells in 6 cm dishes) were transfected with 2.21 μ g pcDNA3 expression plasmid DNA using Lipofectamine 2000.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed according to the EZChIP kit (Upstate) protocol. Briefly, EJ-p53 cells were plated at 4.5×10^6 cells per 15 cm dish. The cells were then lysed at a final concentration of 2.5×10^7 cells/mL. ChIP lysates were sonicated on ice with 15 cycles of 10 s ON/20 s OFF using a microtip probe with a sonicator (Fisher Sonic Dismembrator). For each IP, 5 μ g antibody was added to $\sim 1 \times 10^7$ cells of pre-cleared input lysate. The DO-1 p53 mouse monoclonal antibody was used for p53 ChIP, and normal mouse IgG (Santa Cruz Biotechnologies) was used as a negative control. The DNA was eluted into 50 μ L Elution Buffer with the PCR Purification kit (QIAGEN). One μ L DNA was assayed by real-time PCR as described above. The primer sequences used are listed in Supplementary Table 6. Dilutions of the 2% input DNA were used to generate a standard curve.

Results

The human α B-crystallin gene is juxtaposed head-to-head with the related sHSP, HspB2 on chromosome 11q22.3-q23.1. The two genes share an 1111 base pair intergenic promoter that is transcribed in an orientation-dependent fashion to allow for differential gene regulation [41,42]. In searching the α B-crystallin/HspB2 promoter for DNA binding elements that might be utilized by stress-induced transcription factors, we found a sequence at -199/-169 (relative to the α B-crystallin transcription start site) that closely matches the consensus sequence for a p53 response element (p53RE) (Fig. 1a) [43,44]. This putative p53RE is highly conserved across mammals and features two p53RE half-sites separated by 11 base pairs which match the consensus sequence at 17/20 bases, including the required cytosines and guanines at positions 4 and 7 in each half-site, respectively. The presence of this putative p53RE in the

shared α B-crystallin/HspB2 promoter suggests that α B-crystallin or HspB2 might be transcriptionally regulated by p53.

To determine whether α B-crystallin or HspB2 is induced by p53, we utilized an inducible p53 expression system (EJ-p53 cells) in which wild-type p53 expression is induced by tetracycline removal in p53-null human EJ bladder carcinoma cells [36]. Upon removal of tetracycline from the media, p53 and p21 mRNA levels increased rapidly within 12 h (Fig. 1b). Moreover, α B-crystallin mRNA levels were dramatically increased, although the induction of α B-crystallin mRNA, which was first apparent 24 h after tetracycline removal, was delayed compared to that of p53 or p21. Importantly, mRNA levels of the related sHSP Hsp27 were unchanged (Fig. 1b), and HspB2 mRNA levels were at the lower limit of detection or undetectable at all time points examined (data not shown). These results indicate that the α B-crystallin gene is specifically and robustly induced by p53. Immunoblotting of whole cell lysates collected in parallel confirmed rapid p53-dependent induction of p21 protein levels within 12 h and delayed induction of α B-crystallin protein levels within 48 h (Fig. 1c). Similar results were obtained when wild-type p53 was expressed by retroviral transduction in immortalized human MCF-10A breast epithelial cells (Fig. 1d), indicating that the observed induction of α B-crystallin protein by p53 is not cell type-specific. In contrast, retroviral delivery of p53 DNA binding mutants (R175H and R273H) into MCF-10A cells did not induce α B-crystallin protein (Fig. 1e), thereby confirming that the induction of α B-crystallin by p53 is mediated by a transcriptional mechanism. To determine whether genotoxic stress induces α B-crystallin by a p53-dependent mechanism, we transfected MCF-10A cells with two different p53 siRNAs (sip53-14 and sip53-15) or control non-silencing siRNA (NS) targeting luciferase and then treated the cells with the topoisomerase II inhibitor doxorubicin (Fig. 1f). Forty-eight h later, p53, p21 and α B-crystallin protein levels were greatly increased in NS-transfected MCF-10A cells treated with doxorubicin (lane 9) compared to NS-transfected cells treated with DMSO vehicle (lane 6). In contrast, siRNAs targeting p53 (sip53-14 and to a lesser extent sip53-15) partly inhibited p53 induction and dramatically suppressed p21 and α B-crystallin induction by doxorubicin (lanes 10 and 11 vs lane 9). Collectively, these data indicate that p53 is required for α B-crystallin induction in response to genotoxic stress.

We next investigated whether the putative p53RE in the α B-crystallin promoter is transactivated by p53. To this end, EJ-p53 cells were transiently transfected with luciferase reporters containing the full-length α B-crystallin promoter (−1081/+30) or progressive 5' truncations which contained (−289/+30) or lacked (−171/+30) the putative p53RE (Fig. 2a). In addition, the conserved cytosine and guanine residues in both half-sites of the putative p53RE were mutated in the full-length and −289/+30 truncated promoter constructs; mutation of these sites in p53REs abrogates p53 binding [43]. When p53 was induced in the EJ-p53 cells by tetracycline removal, the full-length and −289/+30 α B-crystallin promoter reporters mediated 10.5- and 7-fold increased luciferase activity, respectively, compared to the promoter-less pGL3-Basic luciferase vector. Mutation of the putative p53RE in the full-length and −289/+30 promoter constructs attenuated p53 transactivation of these reporters. Similarly, deletion of the putative p53RE in the −171/+30 α B-crystallin promoter reporter completely abrogated p53 activation of the promoter. These data demonstrate that p53 activates the α B-crystallin promoter through the putative p53RE.

In order to determine whether p53 binds to the putative p53RE *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed. ChIP with anti-p53 antibodies enriched for p21 5' p53RE genomic DNA as expected (Fig. 2b), confirming that the ChIP assay worked. In contrast, no enrichment of α B-crystallin promoter genomic DNA was seen. While it is formally possible that p53 may be binding to the α B-crystallin promoter in a manner not detectable by ChIP, this data strongly suggests that p53 does not directly bind to the putative p53RE in the α B-crystallin promoter. Together with the delayed kinetics of α B-crystallin

induction by p53 compared to p21 (Figs. 1b and 1c), these results suggest an indirect mechanism of transactivation whereby p53 induces the expression of other target genes, such as p63 or p73, which also bind to p53REs to regulate gene expression.

In order to investigate the potential role of the p53 family network in α B-crystallin transactivation, we first determined p63 and p73 mRNA levels following wild-type p53 induction in EJ-p53 cells. TAp63 mRNA levels were initially low and unaffected by p53 induction, while Δ Np63 mRNA levels were undetectable at all time points (data not shown). TAp73 mRNA levels showed a modest transient increase at 6 h with and without p53 induction (Fig. 3a). In contrast, Δ Np73 mRNA levels increased dramatically upon p53 expression (Fig. 3b), consistent with previous reports showing that Δ Np73 is a p53 target gene [20,45,46]. The observed induction of Δ Np73 suggests that Δ Np73 may serve as an intermediary factor between p53 expression and α B-crystallin transactivation. To test this hypothesis, we examined the requirement for p73 in the p53-dependent induction of α B-crystallin. Pan p73, TAp73, and Δ Np73 siRNAs inhibited p53 induction of α B-crystallin mRNA levels, indicating that both TAp73 and Δ Np73 are required for p53-dependent transactivation of α B-crystallin (Fig. 3c). Intriguingly, the TAp73 siRNA suppressed Δ Np73 mRNA induction by p53, suggesting that TAp73 is required for p53-mediated Δ Np73 transactivation. Importantly, the p73 siRNAs had modest or no effect on p53, p21, and Mdm2 mRNA levels following p53 induction, indicating that p73 RNAi does not globally disrupt p53-mediated transcription (Fig. 3d). Collectively, these results implicate one or more p73 isoforms in the observed p53-dependent transactivation of α B-crystallin.

In order to specifically determine which p73 isoform(s) transactivate α B-crystallin, EJ-p53 cells were transiently transfected with N-terminal HA-tagged TAp73 α , TAp73 β , Δ Np73 α , Δ Np73 β , and untagged Δ Np73 β -13 expression constructs. Δ Np73 β -13 lacks the N-terminal 13 amino acids unique to Δ Np73 that are required for transactivation by Δ Np73 β [30]. Immunoblotting confirmed that the p73 isoforms are expressed 24 and 48 h after transfection (Fig. 4a). Although ectopic expression of most of the p73 isoforms had little effect on α B-crystallin mRNA levels, Δ Np73 α increased α B-crystallin mRNA 11- and 17-fold at 24 and 48 h, respectively (Fig. 4b). Taken together, these data strongly support a model whereby p53 transactivates Δ Np73 α which, in turn, induces α B-crystallin expression.

Discussion

We have demonstrated for the first time that the molecular chaperone α B-crystallin is induced by a novel collaborative relationship among p53 and multiple p73 isoforms. Specifically, p53 leads to the robust transactivation of Δ Np73, which subsequently induces α B-crystallin gene expression by a p53-independent mechanism. Although Δ Np73 α is a well-established dominant negative inhibitor of p53 and TAp73 [20,45,47], it has also been implicated in p53-dependent and -independent transcriptional regulation of a variety of target genes, including EGR1 and BTG2 [29,31]. As is the case with α B-crystallin, the mechanisms by which Δ Np73 α transactivates these genes is unclear but may involve a direct interaction (*e.g.*, as a transcriptional co-activator) or indirect mechanism (*e.g.*, by repressing a negative transcriptional regulator or activating a positive regulator of the target gene). Interestingly, ectopic expression of Δ Np73 α has been reported to induce HSF-1 expression, thereby providing a potential indirect mechanism for α B-crystallin transactivation by Δ Np73 α [48]. Our observation that silencing TAp73 inhibits the induction of Δ Np73 by p53 adds another layer of complexity. These results suggest that TAp73 cooperates with p53 to transactivate Δ Np73 in this system, a plausible scenario given that TAp73 itself has been demonstrated to transactivate Δ Np73 [49]. Collectively, our data point to an intricate and tightly coordinated cooperation between p53, TAp73 and Δ Np73 in the transcriptional regulation of α B-crystallin in response to genotoxic stress.

The induction of α B-crystallin, an anti-apoptotic and anti-oxidant protein [4,6-8], by genotoxic stress may also serve a homeostatic function to suppress apoptosis and reduce ROS levels in the setting of DNA damage. Indeed, recent studies indicate that p53 induces the expression of several pro-survival and anti-oxidant genes, including COX-2, the sestrins, TIGAR, glutathione peroxidase 1, aldehyde dehydrogenase 4 family member A1, and the receptor tyrosine kinase DDR1 [50-55]. Induction of anti-oxidant genes by p53 inhibits tumor initiation by reducing ROS levels, which can cause oxidative DNA damage and promote genomic instability [56]. Transactivation of α B-crystallin downstream of p53 represents a potentially novel mechanism of countering pro-apoptotic and pro-oxidant signaling initiated by genotoxic stress, an hypothesis we will explore in future studies. Intriguingly, α B-crystallin was also recently reported to bind p53 and sequester it in the cytoplasm, suggesting an additional mechanism by which α B-crystallin may inhibit p53 signaling [57].

Our results also provide potentially new insights into the mechanisms of deregulated expression of α B-crystallin in cancer. The cytoprotective function of α B-crystallin has been exploited by diverse cancers, which constitutively express α B-crystallin to overcome apoptosis. For instance, α B-crystallin is aberrantly expressed in apoptosis-resistant triple negative (estrogen receptor, progesterone receptor, and HER2/ErbB2-negative) breast tumors and glioblastoma multiforme and contributes to their aggressive tumor biology [8,37,58]. Furthermore, expression of α B-crystallin correlates with poor survival in several malignancies, including breast cancer, head and neck cancer and hepatocellular carcinoma [37,59-61]. Although p53 is frequently inactivated in cancer, Δ Np73 is commonly expressed in a wide range of clinically aggressive human tumors and is associated with poor outcomes [24-27]. Given the novel link between Δ Np73 and α B-crystallin we have demonstrated, it will be important in future studies to determine whether Δ Np73 and α B-crystallin are co-expressed in human tumors and whether they cooperate in tumor initiation and/or progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are indebted to Dr. Sam Lee (Harvard Medical School) for providing the EJ-p53 cells and Dr. Xinbin Chen (University of California-Davis) for providing p73 plasmids and advice. These studies were supported by NIH grants R01CA097198 (VLC), R21CA125181 (VLC) and T32CA09560 (JRE), and by the Breast Cancer Research Foundation (VLC).

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Fig. 1. The shared α B-crystallin and HspB2 promoter contains a putative p53 response element (p53RE) and p53 induces α B-crystallin expression

(a) Schematic representation of the shared intergenic α B-crystallin and HspB2 promoter. The sequence -199/-169 relative to the α B-crystallin transcriptional start site is shown compared to the p53RE consensus sequence. Vertical lines indicate the base pairs in the human sequence half-sites that match the consensus sequence, and bases conserved among human, mouse, and rat are highlighted in gray. R, purine; Y, pyrimidine; W, A or T. (b) and (c) EJ-p53 cells were grown in the presence or absence of tetracycline, and mRNA and protein were collected at the indicated times. (b) p53 (triangles), p21 (inverted triangles), α B-crystallin (squares), and Hsp27 (diamonds) mRNA levels were measured by real-time RT-PCR, and are shown as fold induction relative to the level before induction. Statistical significance was determined by two-way ANOVA with a Bonferroni post-test and is noted for the fold induction value compared to the initial value. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (c) p53, p21, α B-crystallin and tubulin protein levels were assayed by immunoblotting. (d) MCF-10A cells were infected with wild-type p53 or empty pLXSN retrovirus, and protein was collected at the indicated times. p53, p21, α B-crystallin and tubulin levels were determined by immunoblotting. (e) MCF-10A cells

were mock infected or infected with empty vector, N-FLAG-tagged wild-type p53, untagged wild-type p53, R175H p53, or R273H p53 pLXSN retrovirus. Forty-eight h later, p53, α B-crystallin and actin protein levels were assayed by immunoblotting. (f) MCF-10A cells were transfected with siRNAs targeting p53 (sip53-14 or sip53-15) or luciferase (NS). Sixteen h later, the cells were treated with 500 nM doxorubicin or DMSO vehicle. Forty-eight h later, p53, p21, α B-crystallin, HspB2 and tubulin expression was determined by immunoblotting. Positive control lysates (C) were used for immunoblotting.

Fig. 2. The putative p53RE mediates p53-dependent activation of the α B-crystallin promoter without binding p53

(a) The top line schematically depicts the shared α B-crystallin/HspB2 promoter. The transcriptional start sites of α B-crystallin and HspB2 are indicated by bent arrows; open-ended boxes depict coding sequences. Below, the portion of the promoter upstream of the firefly luciferase coding sequence in each of the pGL3 reporter constructs is displayed. The indicated point mutations in the putative p53RE in the α B-crystallin promoter (highlighted in gray) were introduced to abrogate wild-type p53 protein binding. EJ-p53 cells transfected with 100ng of wild-type, truncated, or p53RE-mutated α B-crystallin promoter luciferase reporter and 100 pg of a *Renilla* luciferase reporter were induced to express p53 for 24 h. Firefly luciferase activity

was normalized to *Renilla* luciferase activity and is indicated relative to promoter-less pGL3-Basic sample activity. For wild-type p53RE samples, statistical significance (two-way ANOVA with Bonferroni post-test) is noted for the difference compared to the promoter-less pGL3-Basic construct (labeled '0'). For the mutated p53RE samples, statistical significance is noted for differences compared to wild-type p53RE. *** $P < 0.001$. **(b)** EJ-p53 cells after 24 h with or without p53 induction were assayed by p53 ChIP for binding of p53 to the α B-crystallin promoter. ChIP-isolated DNA was assayed by real-time RT-PCR with primers flanking the putative p53REs in the α B-crystallin or p21 promoters. The fraction of input chromatin (% INPUT) immunoprecipitated was calculated by the standard curve method. Statistical significance (two-way ANOVA with Bonferroni post-test) is noted for %INPUT differences between p53 and IgG ChIP under each condition. * $P < 0.05$.

Fig. 3. p73 is required for αB-crystallin induction by p53

(a) and (b) EJ-p53 cells were grown with (filled boxes) or without p53 induction (open boxes), and RNA was collected at the indicated times. TAp73 (a) and ΔNp73 (b) mRNA levels were assayed by real-time RT-PCR, and the mRNA levels are shown normalized to the initial (0 h) level. Statistical significance (two-way ANOVA with Bonferroni post-test) is noted at each time point compared to the 0 h mRNA level. (c) and (d) EJ-p53 cells transfected with control non-silencing (NS) siRNA targeting luciferase or siRNAs targeting all p73 isoforms (siPANp73), ΔNp73 (siΔNp73) [35] or TAp73 [40] were re-transfected 24 h later and induced to express p53. RNA was collected 48 h later and mRNA levels were assayed by real-time RT-PCR: (c) TAp73 (open bars), ΔNp73 (filled bars), and αB-crystallin (vertically-striped bars); (d) p53 (open bars), p21 (filled bars), and Mdm2 (vertically-striped bars). The mRNA levels are shown relative to the levels after 48 h of p53 induction in cells transfected with non-silencing siRNA. Statistical significance (two-way ANOVA with Bonferroni post-test) is noted for mRNA level differences compared to the level in the non-silencing sample. In (a)-(d), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4. Ectopic expression of ΔNp73α induces αB-crystallin mRNA

EJ-p53 cells were transfected with empty vector, HA-TAp73α, HA-TAp73β, HA-ΔNp73α, HA-ΔNp73β, or untagged ΔNp73β-13 (13 amino acid N-terminal truncation) pcDNA3 plasmids. RNA and protein were collected at 0, 24, and 48 h post-transfection. **(a)** p73 protein levels were assayed by immunoblotting. **(b)** αB-crystallin mRNA levels 24 h (open bars) and 48 h (filled bars) after transfection were assayed by real-time RT-PCR and normalized to the level in the pcDNA3 empty vector sample at each time point. Statistical significance (two-way ANOVA with Bonferroni post-test) is noted for differences compared to pcDNA3 transfection. *** $P < 0.001$.