BID regulation by p53 contributes to chemosensitivity

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The role of the p53 protein (encoded by *TP53*) in tumour suppression relies partly on the ability of p53 to regulate the transcription of genes that are important in cell-cycle arrest and in apoptosis. But the apoptotic pathway mediated by p53 is not fully understood. Here we show that *BID*, a member of the pro-apoptotic Bcl-2 family of proteins, is regulated by p53. *BID* mRNA is increased in a p53-dependent manner *in vitro* and *in vivo*, with strong expression in the splenic red pulp and colonic epithelium of γ-irradiated mice. Both the human and the mouse *BID* genomic loci contain p53-binding DNA response elements that bind p53 and mediate p53-dependent transactivation of a reporter gene. In addition, *BID*-null mouse embryonic fibroblasts are more resistant than are wild-type fibroblasts to the DNA damaging agent adriamycin and the nucleotide analogue 5-fluorouracil, both of which stabilize endogenous p53. Our results indicate that *BID* is a p53-responsive 'chemosensitivity gene' that may enhance the cell death response to chemotherapy.

Activation of p53 in response to cellular or genotoxic stress induces several responses, including DNA repair, senescence, differentiation, cell-cycle arrest and apoptotic cell death[1]. The best understood p53 responses — transient or irreversible cell-cycle arrest and apoptosis — are regulated in part by the transcriptional activation of target-genes[1,2]. Many p53 target-genes have been identified. For example, the cyclin-dependent kinase inhibitor, p21, is transcriptionally activated by p53 and required for p53-dependent arrest in G1 (ref. 3). Several p53 target-genes are involved in p53-dependent apoptosis, including *BAX*, *NOXA*, *PUMA*, *PIDD*, *p53AIP1*, *PERP*, *FAS/APO1* and *KILLER/DR5*; however, no single target gene has been found to be required for the full response[4–12]. In addition to transcriptional activation, the expression of some genes is repressed by p53 and may also contribute to apoptosis[13,14].

The Bcl-2 family consists of both pro- and anti-apoptotic members that seem to be involved in regulating apoptosis[15]. All members of the Bcl-2 family contain one of four conserved motifs termed BH1 to BH4. In general, the members involved in pro-survival responses are structurally most similar to Bcl-2, whereas the more distant relatives of Bcl-2 are involved in pro-apoptotic responses. The BH3 domain is required for the function of the pro-apoptotic genes, and some Bcl-2 family members contain only a BH3 domain and are otherwise unrelated to any known proteins[15].

The pro-apoptotic Bcl-2 family member BID is a 'BH3-only' protein[16–18]. In a process that acts as a molecular switch, the cytoplasmic BID protein is cleaved within an unstructured loop by active caspase-8 to expose a new amino-terminal glycine that undergoes post-translational myristoylation. The myristoylated BID translocates to the mitochondria where truncated p15tBID inserts into the membrane[19]. tBID activates and requires the multidomain pro-apoptotic members BAX and BAK to initiate mitochondrial dysfunction and apoptotic death[20,21]. In essence, BID is a protein that connects activation of the death receptor pathway to activation of the mitochondrial pathway[17,22].

BID$^{-/-}$ deficient mice have been shown to develop normally. But whereas most *BID*$^{-/-}$ mice survive the injection of an antibody against Fas, wild-type mice die by hepatic apoptosis and haemorrhagic necrosis within 4 h of injection[23]. These results provide evidence that BID is required in the death receptor and caspase pathway, as well as in mitochondrial events, for the efficient amplification of the death response in selected types of cells known as type II cells[23,24]. *BID*$^{-/-}$ murine embryonic fibroblasts (MEFs) treated with TNF-α or an antibody against Fas show delayed and diminished caspase activity and less cleavage of downstream apoptotic substrates[23]. Studies have also shown that tBID is involved in both the redistribution and release of cytochrome *c* from the mitochondria during apoptosis[25].

Here we report that the *BID* gene is transcriptionally regulated by p53. *BID* mRNA is induced after temperature-sensitive expression of p53, overexpression of p53 through an adenovirus and stabilization of p53 after DNA damage. A marked increase in *BID* expression, particularly in colonic epithelium, occurs *in vivo* in a p53-dependent manner after γ-irradiation. Analysis of the mouse and human *BID* genomic loci reveals several potential p53-binding elements. There is a functional p53 site upstream of the promoter in murine *BID*, and a functional p53 site in the first large intron in human *BID*. Analysis of the response of primary wild-type and *BID*$^{-/-}$ MEFs to the chemotherapeutic agents adriamycin and 5-fluorouracil (5-FU) shows that *BID*-deficient MEFs are much more resistant to adriamycin and 5-FU than are wild-type MEFs.

Together, our results show that *BID* is transcriptionally regulated by p53 and may be functionally important in the cellular response to treatment with the chemotherapeutic agents adriamycin and 5-FU. Thus, *BID* may be a 'chemosensitivity gene' — one of a subset of p53-upregulated targets whose induction and subsequent processing or activation contributes to chemosensitivity.

Results

Overexpression of p53 increases *BID* mRNA. We used a microarray chip hybridized with cDNA from the p53-expressing temperature-sensitive cell line Vm10 to screen for p53 transcriptional targets. The generation of this cell line has been described previously[26,27].

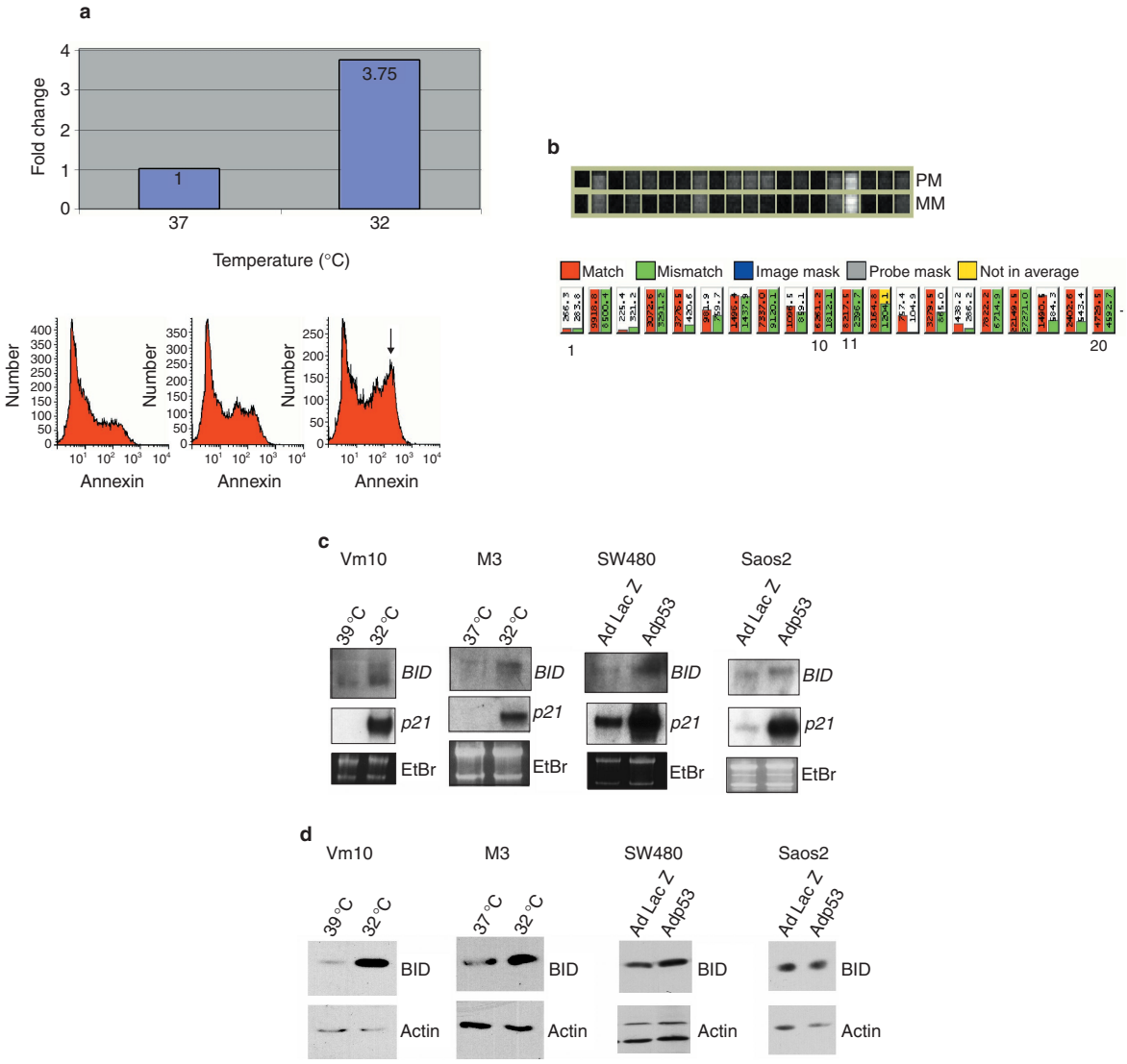

Figure 1 **Identification of *BID* as a p53 transcriptionally regulated gene.** **a**, Characterization of Vm10 cells at the permissive and restrictive temperature by flow cytometric analysis. Top, graph shows the fold change in annexin-V-positive cells after the temperature shift. Bottom, primary data of the cells before the temperature shift and 24 h after the cells had been divided and cultured at the restrictive or the permissive temperature. Arrow indicates the increase in annexin-V-positive apoptotic cells. **b**, Microarray analysis of *BID* cDNA from the Murine 11K Affymetrix GeneChip. The Vm10 sample cultured at 32 °C was normalized to that cultured at 39 °C, and the probe pair matches of the *BID* gene are shown using this comparison. The perfect match probe pair set (top) is compared against the mismatch probe pair set (bottom). **c**, Northern blot analysis confirms *BID* as a p53 transcriptionally regulated gene in two murine temperature-sensitive cell lines, Vm10 and M3, and two human cell lines that overexpress p53 after infection with an adenovirus vector. Northern blots were hybridized with *BID* and *p21* probes as a positive control. **d**, Western blot analysis of BID expression in the cell lines described in **c**.

Briefly, a temperature-sensitive p53 mutant, Val 135, was introduced into an immortalized mouse fibroblast cell line that has no endogenous expression of p53. This cell line, Val5, undergoes arrest in G1 when shifted to the permissive temperature (32 °C), in other words, when p53 is in a wild-type conformation. To establish the Vm10 cell line, a murine c-*myc* oncogene plasmid was introduced into the Val5 cell line at the restrictive temperature. The Vm10 cells are healthy at the restrictive temperature (that is, they overexpress c-*myc* but p53 is in a mutant conformation); however, when the cells are shifted to 32 °C, p53 changes to its wild-type conformation and the Vm10 cells undergo p53-dependent apoptosis (ref. 27 and Fig. 1a).

On the murine 11K Affymetrix GeneChip, levels of *BID* mRNA were 3.2-fold higher when Vm10 cRNA from cells incubated at 32 °C for 24 h was used as a probe than when that from cells incubated at 39 °C for 24 h was used (Fig. 1b). The induction of *BID* mRNA by the temperature shift in Vm10 cells was confirmed by northern blotting (Fig. 1c).

We also tested the induction of endogenous *BID* in other cell lines. M3 is a murine lymphoma cell line containing a temperature-sensitive p53 that causes massive apoptosis at the permissive temperature[28]. Expression of *BID* mRNA was induced when M3 cells were shifted to the permissive temperature (Fig. 1c).

The induction of a *BID* transcript (2.4 kilobases (kb), corresponding to GenBank accession number AK094795) in human cell lines was established by overexpressing wild-type p53 by adenoviral expression in SW480 cells, which express mutant p53, and in Saos2 cells, which are null for p53. As compared with cells infected

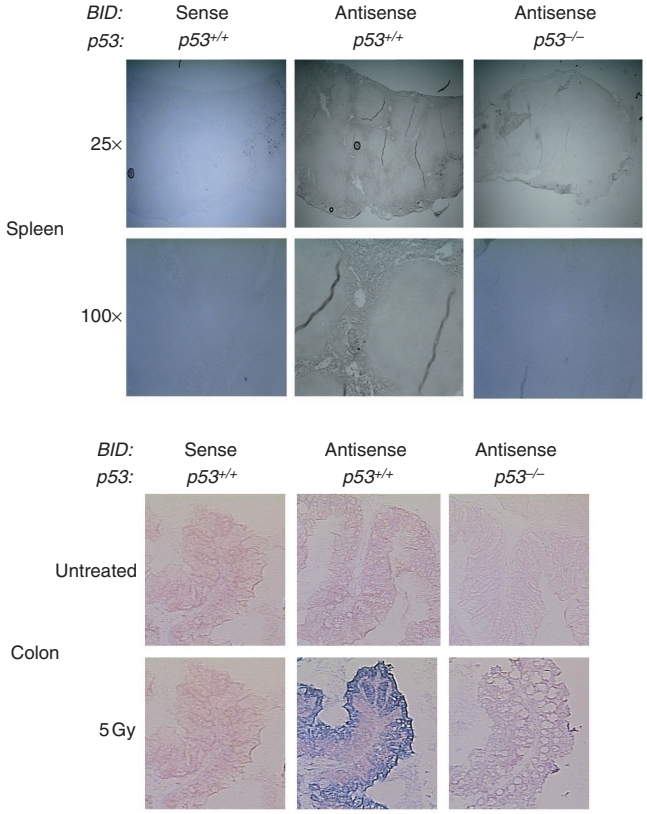

Figure 2 **Induction of *BID* mRNA in response to DNA damage.** *In situ* hybridization of irradiated spleen and transverse colon in wild-type and $p53^{-/-}$ mice. Light microscopy images at the indicated magnifications are shown. Sense and antisense *BID* probes were hybridized to the spleen (top) or colon (bottom) of mice irradiated with 5 Gy of γ-radiation. *BID* mRNA is induced only in the wild-type spleen (red pulp) and colon (epithelium) and not in the $p53^{-/-}$ spleen and colon tissues. Negative controls included the hybridization of unirradiated or irradiated tissues with a sense *BID* probe, and the hybridization of p53-null tissues with an antisense *BID* probe.

with the control adenovirus AdLacZ, cells transfected with the adenovirus expressing p53 showed an increase in *BID* mRNA (Fig. 1c). Quantities of BID protein were also upregulated in response to the conformational change of p53 in the temperature-sensitive cell lines, as well as in response to overexpression of p53 through infection with adenovirus for 20–24 h (Fig. 1d). Upregulation of BID protein would be expected to occur only transiently in cells that are undergoing apoptosis.

***BID* mRNA is increased *in vivo* after DNA damage.** As some tissues undergo p53-dependent apoptosis in response to ionizing radiation[29–32], we examined the p53-dependent expression of *BID* mRNA in irradiated mice. We treated wild-type and $p53^{-/-}$ mice with 5 Gy of γ-irradiation and analysed the spleen, thymus and colon by *in situ* hybridization for the induction of *BID* (Fig. 2). Six hours after irradiation, the *BID* antisense probe revealed a distinct pattern of *BID* expression in the wild-type irradiated spleen and transverse colon but not in the $p53^{-/-}$ irradiated spleen and colon (Fig. 2). In the spleen, expression of *BID* was induced in the red pulp, whereas induction of *PUMA* is restricted to the white pulp (P.F. *et al.*, unpublished). Induction of *BID* mRNA in the thymus after irradiation was minimal (data not shown).

These results indicate that *BID* may be upregulated in response to expression of p53 after DNA damage. We did not observe substantial induction of *BID* mRNA expression after exposure to adriamycin or etoposide in human cancer lines (data not shown), although there was weak p53-dependent DNA binding of *BID* locus sequences in these cells (Supplementary Information Fig. S1b, c, and see below).

***BID* genomic loci contain p53-binding elements.** The coding sequence for *BID* comprises five exons[33]. Upstream of the first coding exon, which contains the start codon ATG, there is a large intron and a small noncoding exon in both the human and the mouse *BID* genomic regions (Fig. 3a). Although structurally similar, the first intron in human *BID* is roughly 23.5 kb, whereas the first intron in mouse *BID* is about 15 kb. The noncoding exon in both human and mouse *BID* is similar in size.

Analysis of the genomic sequence in both human and mouse *BID* identified potential sequence-specific p53-binding sites. p53 binds to DNA in a sequence-specific manner through a repeat of the consensus sequence 5′-RRRCWWGYYY-3′ (where R denotes A or G, W denotes A or T and Y denotes C or T) separated by 0–13 base pairs[34]. Although the precise locations of the potential p53-binding sites in the human and the mouse sequence are not conserved across species, both human and mouse genomic *BID* loci seem to contain putative p53-binding sites (Fig. 3a). It is well known that p53-regulated genes may contain p53 response elements in their upstream regulatory regions as well as in their intronic regions.

To determine whether p53 can bind to the candidate response elements in the human and the mouse *BID* genomic loci, we used a nonisotopic electrophoretic mobility-shift assay ($EMSA^{NI}$) with double-stranded oligonucleotides containing the potential p53-binding sites. Nuclear extracts were prepared from several cell lines under different conditions for inducing p53 expression. Saos2 cells, which do not express endogenous p53, were infected with an adenovirus expressing either β-galactosidase (AdLacZ) or p53 (Adp53). The human *BID* oligonucleotide was shifted only in the extracts infected with Adp53 (Fig. 3b). In addition, as found with a p53-binding element from the $p21^{WAF1}$ promoter, a shift could be observed by incubation with an antibody specific to the carboxy-terminal region of p53 (Fig. 3b). The shift of the protein–DNA complex was not observed with a oligonucleotide encoding a mutated human p53-binding site (Fig. 3b). Formation of the protein–DNA complex could be reduced by including an untagged competitor oligonucleotide (Supplementary Information, Fig. S1a). Taken together, these results indicate that the candidate p53-binding site located roughly 6.6 kb upstream of the ATG codon in the human *BID* genomic locus is specifically bound by p53.

We prepared additional nuclear extracts to test the specificity of the p53-binding site. Calu-6 cells, which do not express p53, were either infected with AdLacZ or Adp53 or treated with adriamycin. The human *BID* oligonucleotide was shifted only in extracts from cells infected with Adp53 (Supplementary Information, Fig. S1b). SW480 cells, which express a mutant p53, were infected with either AdLacZ or Adp53, or were treated with adriamycin. As expected, only extracts infected with adenovirus expressing wild-type p53 produced a shift in the $EMSA^{NI}$ (Supplementary Information, Fig. S1b). H460-neo and H460-E6 stable cell lines were treated with adriamycin. The formation of a complex that was electrophoretically retarded occurred only in H460-neo cells treated with adriamycin (Supplementary Information, Fig. S1b).

We tested the murine *BID* p53 DNA-binding element in similar assays. Like the human *BID* p53-binding element, the mouse element could form a complex with DNA only in extracts from Saos2 cells infected with Adp53 (Fig. 3c). In addition, the mouse *BID* oligonucleotide was electrophoretically retarded only when p53 was overexpressed or when p53 was stabilized in H460-neo cells in response to adriamycin (Supplementary Information, Fig. S1c).

Taken together, both the human and the mouse *BID* genomic loci contain potential p53-binding sites. The human site is located in the first large intron, whereas the mouse p53-binding element is located upstream of the first noncoding exon. These p53-binding elements specifically form protein–DNA complexes only in the

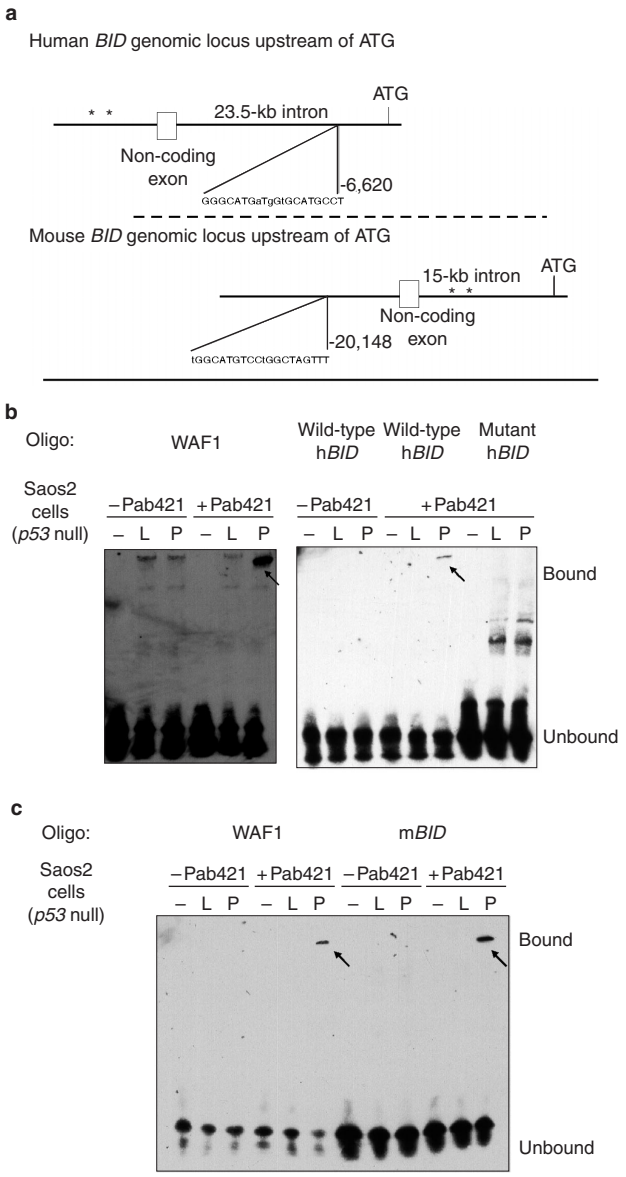

Figure 3 **EMSA^{NI} of potential p53-binding elements in *BID* genomic loci.** **a**, Human and mouse *BID* genomic loci. Both genomic regions contain a very large intron and a small noncoding exon upstream of the first coding exon containing the start codon ATG. The human locus contains a functional p53 DNA-binding element roughly 6.6 kb upstream of the first coding exon. The mouse *BID* locus contains a functional p53 DNA-binding element roughly 5 kb upstream of the first noncoding exon. Two other putative p53-binding elements in the human or the mouse loci are indicated by asterisks. **b**, EMSA^{NI} of the human *BID* (hBID) p53 DNA-binding site. The human *BID* binding site forms a protein–DNA complex with p53 only in the presence of the modifying C-terminal antibody pAb421. L denotes infection with AdLacZ, P denotes infection with Adp53. The p21^{WAF1} p53 DNA-binding element is a positive control for the assay. Mutations in the human *BID* p53-binding sequence eliminate the ability of p53 to bind to the oligonucleotide. **c**, The mouse *BID* (mBID) p53-binding element forms protein–DNA complexes in the presence of p53.

presence of overexpressed or stabilized wild-type p53.

Transcriptional activation by p53 occurs through *BID* genomic loci. To determine whether the potential p53 DNA-binding sites in both the human and the mouse *BID* loci are transcriptionally regulated by p53, we generated luciferase reporter constructs using the genomic regions of *BID* that contain the p53-binding elements. We transfected these constructs into Calu-6 lung carcinoma cells, which do not express endogenous p53, either alone or together with a plasmid expressing wild-type or mutant p53.

Induction of luciferase activity was observed only when the reporter constructs were co-transfected with wild-type p53 (Fig. 4a). Co-transfection of the luciferase reporter with empty vector or mutant p53 did not result in the induction of luciferase activity (Fig. 4a). WWP, a p21 promoter luciferase construct[3], was used as a positive control in these experiments (Fig. 4a, insert). The luciferase activity was almost 3.5-fold higher after transfection with the human *BID* reporter construct than after transfection with empty vector (Fig. 4b). Furthermore, the mouse luciferase reporter construct resulted in an almost sevenfold induction of luciferase activity as compared with empty vector alone (Fig. 4b). The mouse luciferase construct, which was generated through polymerase chain reaction (PCR) amplification of mouse genomic DNA, contained an additional potential p53-binding element, comprising a 90% consensus sequence with four spacers between the two-half sites; however, this site did not form a detectable protein–DNA complex when tested in EMSA^{NI} assays (data not shown).

We used a chromatin immunoprecipitation assay (ChIP) to determine whether the p53-binding element in the human *BID* genomic locus can be bound by p53 *in vivo*. We infected SW480 cells with either AdLacZ or Adp53 and then subjected them to ChIP analysis by immunoprecipitation with an antibody against p53 or a nonspecific antibody raised against human pRb protein. The p53-binding element in the first intron of *BID* was amplified specifically by PCR in cells that had been infected with p53 and then immunoprecipitated for p53 (Fig. 4c). By contrast, the p53-binding element could not be amplified by PCR when the antibody against pRb was used as the precipitating antibody (Fig. 4c). These results, together with the EMSA^{NI} data, indicate that both human and mouse genomic *BID* loci contain functional p53-binding elements.

***BID* contributes to chemosensitivity.** The above results suggested that *BID* is transcriptionally upregulated in response to stabilized p53. To determine whether *BID* has a functional role in response to p53-mediated cellular activities, we treated wild-type and *BID*$^{-/-}$ MEFs with increasing concentrations of the DNA damaging agent adriamycin. Unexpectedly, wild-type MEFs were much more sensitive to treatment with adriamycin than were *BID*$^{-/-}$ MEFs, even at relatively low concentrations of the drug (at 0.4 μg ml^{-1} adriamycin, ~20% of wild-type cells were annexin V positive as compared with about 7% of *BID*$^{-/-}$ cells; Fig. 5a). Indeed, the percentage of annexin-V-positive cells in *BID*$^{-/-}$ MEFs treated with 0.4 μg ml^{-1} adriamycin for 24 h did not differ from that found in untreated control cells (Fig. 5a). At higher concentrations of adriamycin (6 μg ml^{-1}), *BID*$^{-/-}$ MEFs remained resistant to the effects of adriamycin, whereas wild-type MEFs were killed efficiently (Fig. 5a). The expression of BID protein in the MEFs was analysed by western blot (Fig. 5a).

BID$^{-/-}$ MEFs were also resistant to treatment with 5-FU, whereas wild-type MEFs were sensitive to this chemotherapeutic agent (Fig. 5b). Few targets of p53 have been established as determinants of chemosensitivity, but taken together these results indicate that *BID* may be one such target.

Discussion

The search for p53 target-genes involved in cell-cycle arrest and apoptosis has been intense for many years. Although p53-dependent cell-cycle arrest in G1 is known to be mediated primarily by the induction of p21^{WAF1}, p53-mediated apoptosis is less understood[3]. Several p53 target-genes that cause apoptosis have been identified, including *BAX*, *DR5*, *PIDD*, *NOXA*, *PUMA* and *FAS*, but no one target gene seems to be required universally for p53-mediated cell death[5,6,8,10,12]. p53 seems to activate both cell-cycle arrest and apoptosis in most types of cell; however, the mechanism underlying the

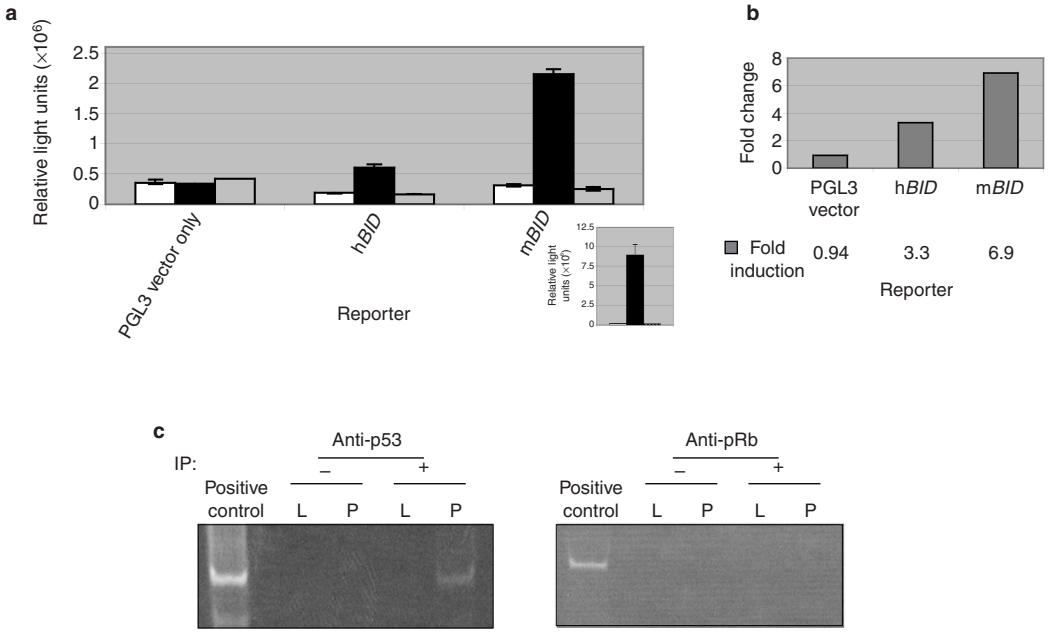

Figure 4 **Luciferase and ChIP assays using *BID* genomic regions. a**, A region of about 360 bp (human, h*BID*) or 770 bp (mouse, m*BID*) of *BID* genomic loci was cloned into the PGL3 promoter vector. White bars represent co-transfection with empty vector, black bars represent co-transfection with wild-type p53, and grey bars represent co-transfection with a tumour-derived mutant p53(273). Activation of reporter activity occurs in the presence of wild-type p53 but not in the presence of the mutant p53. The WWP p21 promoter was used as a positive control (insert). **b**, Induction of luciferase activity by transfection with the human and mouse *BID* reporter constructs relative to that induced by transfection with an empty vector plus p53. **c**, ChIP assay of the p53-binding element in the human *BID* genomic locus. SW480 cells were infected with an adenovirus expressing either β-galactosidase (L) or p53 (P), and chromatin immunoprecipitation was carried out using an antibody against p53. Immunoprecipitation with a nonspecific antibody (anti-pRb) or with no antibody was carried out as a negative control. The genomic region of the human *BID* locus that contains the functional p53 DNA-binding element can be amplified only from cells that express wild-type p53 and are immunoprecipitated by the antibody against p53.

cell fate decision remains unclear[35]. Some studies suggest that phosphorylation of p53 on specific sites, or the binding of p53 to specific loci, may contribute to target gene selectivity[36,37]. The situation has become more complex with the demonstration that DNA-damage-dependent acetylation of the p53 family member p73 contributes to target gene induction and that p63 and p73 function as regulators of p53-dependent apoptosis[35,38].

We have shown here that the pro-apoptotic gene *BID* is transcriptionally regulated by p53. Levels of *BID* mRNA are increased in response to overexpression of p53 both *in vitro* and *in vivo*. Notably, the high induction of *BID* in the colon may offer a clue, in part, to the mechanism of irradiation-induced p53-dependent apoptosis in this tissue. Studies have shown that p53-dependent apoptosis can occur through different mediators in various tissues[32]. *Bcl-2*, but not *BAX*, has a key role in determining the sensitivity of colonic cells to p53-dependent apoptosis after γ-irradiation[29]. Perhaps *BID* may be a regulator for p53-dependent cell death in this tissue.

Both the human and the mouse *BID* genomic loci contain functional p53-binding elements. Both *BID* gene loci contain a large intron and a small noncoding exon upstream of the first coding exon (Fig. 3a). The human intron is about 8.5 kb longer than the mouse intron. We identified a functional p53-binding element in the human intron that is 85% identical to the consensus p53-binding element. The human site is activated in reporter assays and is bound specifically by p53, as shown here by the ChIP assay (Fig. 4). The human and mouse *BID* genomic loci also contain additional putative p53-binding elements either upstream of the noncoding exon (in human) or in the first large intron (in mouse; see Fig. 3a, asterisks). Although a combination of luciferase reporter, ChIP and EMSA[NI] assays did not identify p53 transactivation or specific binding with these other putative sites (data not shown), we cannot rule out the possibility that they may be functional *in vivo*.

Notably, we found a functional p53-binding element upstream of the first noncoding exon in the mouse *BID* genomic locus. This element formed protein–DNA complexes in the EMSA[NI] assays and induced luciferase activity when tested in reporter assays. Both the human and the mouse luciferase reporter constructs could cause transactivation only in the presence of wild-type p53 and not in the presence of a tumour-derived mutant p53. Clearly, there is conservation in the regulation of *BID* by p53, although the precise location of the functional p53-binding sites in the *BID* locus may vary between species or experimental conditions.

p53 can transcriptionally regulate components of an extrinsic pathway mediated by the death receptor and an intrinsic pathway mediated by mitochondria. BID is a Bcl-2 family member that is cleaved by caspase-8 and then translocates to the mitochondria, thereby connecting the death receptor and mitochondrial pathways[17,18]. Although unclear at this time, it is possible that under specific circumstances p53 may transcriptionally regulate *BID* to facilitate the apoptotic process by linking the receptor pathway with the mitochondria. It remains to be determined whether p53-dependent induction of BID protein *in vivo* is sufficient to sensitize cells to killing by DNA damaging agents. Seemingly inconsistent with our model are experiments in which we failed to observe further increases in the quantities of BID protein in cells undergoing p53-dependent apoptosis after the addition of a pan-caspase inhibitor (data not shown). It is conceivable that other proteases may be involved in the processing of BID. We have also not tested the combined effects of p53 overexpression and chemotherapy on the quantities of either full-length or truncated BID protein. We have observed a sensitization effect in cells that are transfected with *BID* and then treated by

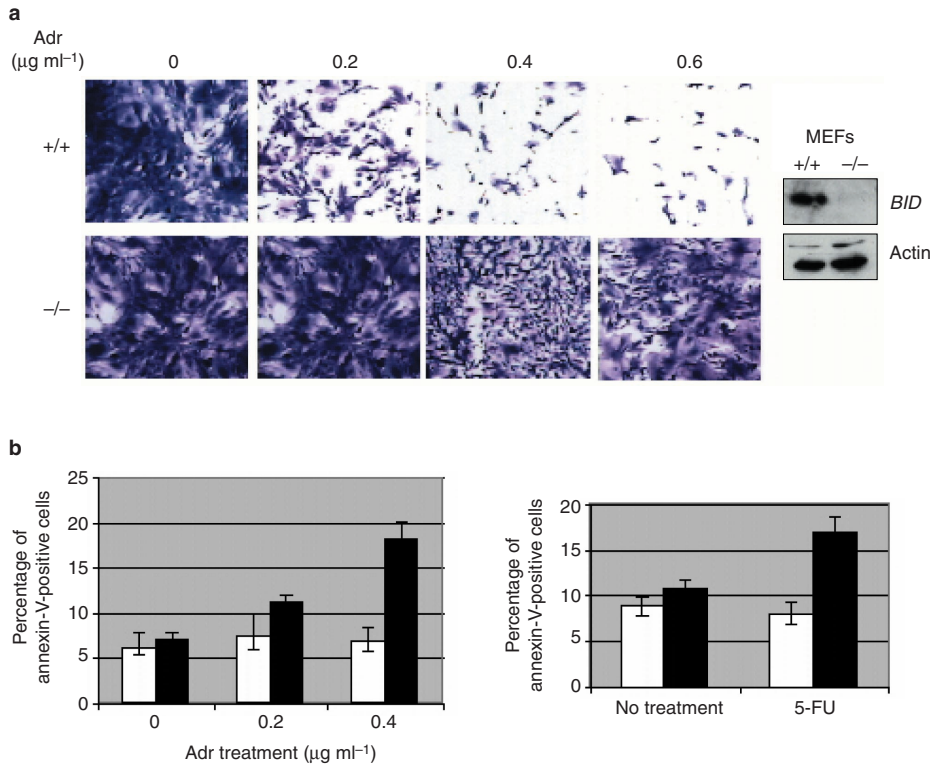

Figure 5 ***BID*$^{-/-}$ MEFs show resistance to adriamycin and 5-FU. a**, Coomassie blue stain of wild-type and *BID*$^{-/-}$ cells and quantification of the effect of adiamycin (Adr) using annexin V staining and analysis by flow cytometry. A western blot of BID protein in the MEFs is shown on the right. **b**, Flow cytometric analysis of annexin-V-positive MEFS after treatment with 5 μM 5-FU. In **a** and **b**, white bars indicate *BID*$^{-/-}$ MEFs and black bars indicate wild-type MEFs.

chemotherapy (data not shown). Future studies that investigate the relationship between *BID* and p53 will help to elucidate the role of both of these molecules in apoptosis and chemosensitivity.

The observations that wild-type p53, but not the tumour-derived mutant p53, can induce luciferase activity and that the p53-binding element can only form a protein–DNA complex in H460-neo cells treated with adriamycin indicate that *BID* may be a p53 target that is inactivated during tumour progression. Treating wild-type and *BID*-null MEFs with increasing concentrations of adriamycin and 5-FU showed that *BID*-null MEFs are much more resistant to these chemotherapeutic agents. Cells with a disrupted *p53* gene are resistant to the effects of 5-FU[39]. Although further investigation is required, we speculate that *BID* may be an important component of the p53-mediated chemotherapeutic sensitization effect. We have not, however, tested the extent to which *BID* is transcriptionally induced by p53 in MEFs or whether the apparent sensitivity of MEFs is due to p53-dependent *BID* induction after exposure to adriamycin or 5-FU.

Notably, the strong induction of *BID* mRNA in wild-type but not p53-deficient colonic epithelia *in vivo* after irradiation (Fig. 2) is consistent with the idea that induction of *BID* may be a candidate determinant of sensitivity in colonic tissue and colon cancer therapy. Both γ-radiation and 5-FU are often used in the therapy of colorectal cancer where p53 mutations constitute a late event that is associated with poor prognosis and poor response to therapy. Future studies should evaluate prospectively the role of *BID* induction in toxicity and clinical response after therapy.

Our current model suggests that p53 directly regulates transcriptional target-genes that can initiate death (such as *KILLER/DR5*, *BAX* or *BID*) or are involved in the execution phase (such as *APAF1* or *CASPASE-6*)[6,10,40,41]. In each group, we think that the induction of certain target-genes by p53 lowers the cell death threshold, leading to 'chemosensitization' effects. The induction of targets such as *BID* mRNA is not sufficient to kill cells in the absence of additional events that promote BID protein processing and activation. The combination of the transcriptional control of *BID* and its subsequent activation provides a molecular mechanism for chemo- and radio-sensitization. In the future it will be important to identify other genes of this class and explore their individual and collective importance to tumour suppression, drug-induced apoptosis and therapeutic response in cancer. It may be useful to examine whether their modulation can reduce toxicity and widen the therapeutic window[2]. At present, there are three recognized p53-regulated target 'chemosensitivity genes', *APAF1*, *CASPASE-6* and *BID*, whose induction can lower the cell death threshold when cells are also exposed to genotoxic agents. □

Methods

Cell lines and culture conditions

We maintained the Vm10 cell line[27] and the murine M3 cell line[11,28] in culture as described. The non-small cell lung cancer line H460, which expresses wild-type p53, was a gift from S. B. Baylin (Johns Hopkins University, Baltimore, MD). The colon cancer cell line SW480, which expresses mutant p53, was maintained in culture as described[42]. We obtained the lung anaplastic carcinoma cell line Calu-6 from ATCC (Manassas, VA) and maintained it in culture in RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Saos2 cells obtained from ATCC were grown in McCoys media (Invitrogen) in 15% FBS and 1% penicillin/streptomycin. Primary wild-type and *Bid*$^{-/-}$ MEFs were grown in Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 20% FBS, L-glutamine, MEM non-essential amino acids, penicillin/streptomycin and β-mercaptoethanol. We collected Vm10 cells 24 h after the temperature shift for all analyses. M3 cells were collected 10 h after the temperature shift for all analyses. SW480 cells were collected 10 h after infection for RNA analysis and 24 h after infection for protein analysis. Saos2 cells were collected 12 h after infection for RNA analysis and 20 h after infection for protein analysis. Adriamycin treatment was done as described[10]. MEFs were treated with 5 μM 5-FU (obtained from the University of Pennsylvania Cancer Center Pharmacy, Philadelphia, PA) for 24 h.

Affymetrix GeneChip

Vm10 cRNA was prepared according to the Affymetrix GeneChip Expression Technical Manual and hybridized to the Murine 11K GeneChip. We carried out hybridization and gene chip expression analysis at the Howard Hughes Medical Institute Biopolymers Laboratory (Cambridge, MA).

Adenovirus preparation and infection

We prepared replication-deficient adenovirus recombinants expressing wild-type p53 (Adp53) and β-galactosidase (AdLacZ) as described[3]. Determination of viral titres and multiplicity of infection (MOI) for each cell line has been described[43]. SW480, Saos2 and Calu-6 cells were infected at a MOI of 50.

Northern analysis

We isolated total RNA and carried out northern blotting as described[3]. A *Not*1 fragment of 2.1 kb from plasmid pCEP4 carrying the *p21* cDNA was used as a probe for northern blots of human *p21*. An *Eco*RI fragment of 2.2 kb from plasmid pZL carrying the mouse *p21* cDNA was used as a probe for northern blots of mouse *p21*. Mouse and human *BID* were cloned by PCR with reverse transcription, inserted into the pcDNA3 vector plasmid and sequenced. We used a *Bam*HI–*Eco*RI fragment of about 600 bp from a pcDNA3 plasmid carrying either the human *BID* or the mouse *BID* cDNA as a probe for northern blots of human and mouse *BID* RNA, respectively.

Mice and treatments

Healthy female wild-type and $p53^{-/-}$ mice aged 5–6 weeks were obtained from Jackson Laboratories (Bay Harbor, ME). Two mice in each experimental group received total body irradiation using a ^{137}Cs γ-ray source at a dose rate of 1.532 Gy min^{-1}. At 0 and 6 h, the mice were killed using an approved Institutional Animal Care and Use Committee Protocol, which followed the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Tissues were collected, fixed in 4% paraformaldehyde at 4 °C overnight and embedded in paraffin.

In situ hybridization

In situ hybridization was done as described[44]. We labelled sense and antisense RNA probes by using the Digoxigenin RNA labelling kit according to the manufacturer's protocol (Roche, Indianapolis, IN).

Western analysis

Western blotting was done as described[45] using rabbit antibodies against human BID and against mouse BID (Cell Signaling Technology, Beverly, MA), mouse antibodies against human actin (Santa Cruz Biotechnology, Santa Cruz, CA) and goat antibodies against mouse BID (Biovision, Moutain View, CA).

Annexin V staining and FACS

Cells were stained with the Clontech ApoAlert Annexin V fluorescein isothiocyanate (FITC) kit according to the manufacturer's protocol (Clontech, Palo Alto, CA). We carried out FACS on a Coulter Epics Elite counter as described[46].

EMSA[NI]

The mouse monoclonal antibody pAb421 against human p53 (Ab-1, Oncogene, San Diego, CA) was used to activate p53 for sequence-specific binding. Assays were carried out using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The double-stranded DNA probes used in the experiments contained the following sequences: *p21* promoter, 5′-ATCAGGAACATGTCCCAACATGTTGAGCTC-3′, site 1; wild-type human *BID* intron 1, 5′-TAGCTGGGCATGATGGTGCATGCCTATAGC-3′; mutant human *BID*, 5′-TAGCTGGGTATTATG-GTGTATTCCTATAGC-3′; and wild-type mouse *BID*, 5′-CTGCCTGGCATGTCCTGGCTAGTTT-TATGT-3′. A 5′-biotin modification was included in all probes except for the competitor oligonucleotide, which did not contain any modifications.

Plasmids

The expression vectors for wild-type p53 (pCEP4-p53)[3] and for mutant p53 (pCEP4-A273H)[47] were provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD). We generated the hBID–luciferase plasmid by a PCR reaction using human genomic DNA as a template to obtain a product of roughly 360 nucleotides containing the consensus p53-binding site (nucleotides 6,930–6,573 upstream of the ATG). The PCR product was then digested with *Kpn* and *Xho*I and ligated into the PGL3 promoter luciferase vector. The mBID–luciferase plasmid was generated by a PCR reaction using mouse genomic DNA as a template to obtain a product of roughly 770 nucleotides containing the consensus p53-binding site (nucleotides 20,313–19,541 upstream of the ATG). The PCR product was digested with *Kpn* and *Xho*I and ligated into the PGL3 promoter luciferase vector. We confirmed the PCR-generated clones by sequencing.

Luciferase assays

Transfections for luciferase assays were carried out as described[48]. Briefly, Calu-6 cells were seeded at 2.5×10^5 cells per well in 12-well plates. The cells were transfected using a Lipofectamine 2000/DNA conjugate suspended in Opti-MEM medium (Invitrogen). A total of 0.8 μg of reporter construct and 0.2 μg of empty vector or p53 plasmid per transfection was used in each assay, with empty vector (pCEP4) added to a total of 2 μg (in all transfections β-galactosidase constituted 10% of the total amount of transfected DNA). We changed the media 4 h after transfection and 24 h later assayed the cell lysates for luciferase and β-galactosidase activity as described[49]. All samples were normalized to β-galactosidase activity.

ChIP assays

We collected SW480 cells for ChIP assay 13 h after infection. ChIP assays were carried out essentially as described[50]. p53 immunoprecipitation was done with 7 μl each of Ab-1 and Ab-2 antibodies against p53 (Oncogene), or with 10 μl of Ab-5 antibodies against pRb (Calbiochem, San Diego, CA) as a negative control. We carried out PCR amplification using primers (forward, 5′-TTAAAGAATCCTTTGCG-GC-3′; reverse, 5′-GTGATTCTCCTGCTTCAG-3′), designed to give a 210-bp product including the p53-binding element. The PCR protocol was 30 cycles of a 45-s denaturation step at 94 °C, a 1-min annealing step at 58 °C and a 1-min extension step at 72 °C.

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.