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Running title: p53 and p73 transactivate Bak

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Abstract

During apoptosis, Bcl-2 proteins control permeabilization of the mitochondrial outer membrane leading to the release of cytochrome c. Essential gate keepers for the cytochrome c release are the pro-apoptotic multidomain proteins, Bax and/or Bak. The expression of *bax* is upregulated upon cellular stress by the tumor suppressor p53. Despite the high functional homology of Bax and Bak little is known about how the *bak* gene is regulated. To investigate its transcriptional regulation in further detail, we have analyzed a region spanning 8200 bp upstream of the *bak* start codon (within exon 2) for transcription factor binding sites and identified three p53 consensus sites (BS1-3). Reporter gene assays in combination with site-directed mutagenesis revealed that only one putative p53-binding site (BS3) is necessary and sufficient for induction of reporter gene expression by p53. Consistently, p53 induces expression of endogenous *bak*. At the mRNA level, induction of *bak* expression is weaker than induction of *puma* and *p21*. Interestingly, *Bak* expression can also be induced by p73 that binds however to each of the three p53 binding sites within the *bak* promoter region. Our data suggest that expression of *bak* can be induced by both, p53 and p73 utilizing different binding sites within the *bak* promoter.

Keywords: Apoptosis / Bak / Bax / transcription / p53 / p73

Abbreviations: BS, binding site; CDK, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; qRT-PCR, quantitative real-time PCR

Introduction

In multi-cellular organisms apoptosis is of utmost importance for organ development, tissue homeostasis and cancer prevention, because superfluous and malfunctional cells are eliminated by this genetically encoded suicide program. The two main apoptosis pathways are triggered either by ligation of death receptors or by the release of cytochrome c from the mitochondrial intermembrane space. The mitochondrial pathway can enhance the response to death receptor ligation or can be activated intrinsically e.g. by genotoxic stress such as γ-irradiation or cytostatic drugs. In the intrinsic pathway members of the Bcl-2 family of proteins constitute the central regulators.[1-3] They share homology in at least one of the four identified Bcl-2 homology (BH) domains and have either anti- or pro-apoptotic capacity. Based on the number of their BH-domains the pro-apoptotic proteins are further subdivided into multi-domain proteins (i.e. Bax and Bak) that contain BH1-3 and so-called BH3-only proteins that only share homology in the BH3-domain with the remainder of the family. Both types of pro-apoptotic proteins have been shown to interact with anti-apoptotic family members such as Bcl-2, Bcl-w, Bcl-xL, and Mcl-1 – either constitutively or after activation.[4-7]

The BH3-only proteins act as sensors for cellular stress and their activity is regulated at the transcriptional level as well as by post-translational modifications. Upon activation the multidomain proteins Bax and/or Bak oligomerize at the mitochondrial outer membrane, leading to its permeabilization and the subsequent release of cytochrome c into the cytosol.[8] Cytosolic cytochrome c associates with Apaf-1, thereby forming the heptameric apoptosome which is the activation platform for the initiator caspase-9.[9] Active caspase-9 then cleaves and activates downstream caspases that dismantle the cell.[10]

Due to the mutual but also selective interaction specificities of pro- and anti-apoptotic proteins the mechanisms underlying Bcl-2 signaling are not fully elucidated yet. Currently, two models of intracellular Bcl-2 family signaling are discussed: the direct activation and the de-repression model. The former proposes that the BH3-only proteins Bim, Bid and Puma are necessary to directly activate Bax and/or Bak via transient interaction. The de-repression model suggests BH3-only proteins to inactivate anti-apoptotic counterparts by binding them and thereby

indirectly releasing Bax and Bak from their inactivating interaction with anti-apoptotic proteins.[7,11] Interestingly, the BH3-only protein Nbk/Bik, which interacts strongly with the Bax antagonizing proteins Bcl-2 and Bcl-x_L but only weakly with Mcl-1 that predominantly interferes with Bak, does not induce apoptosis in Bax-deficient cells, arguing for a de-repression mechanism.[12] In addition to the specific interaction pattern of Bcl-2 proteins, their relative expression level influences the cellular response to apoptosis inducing stimuli. The expression of the upstream acting BH3-only proteins Puma and Noxa is induced by p53 in response to DNA damage.[13,14] p53 also upregulates the expression of the downstream acting protein Bax[15] resulting in a cooperative sensitization of cells to apoptosis. Furthermore, p53 negatively influences the expression of the anti-apoptotic protein Bcl-2[16] which can lead to an enhanced expression of Bcl-2 in p53 deficient cells.[17,18]

Since *bax* is a transcriptional target of p53, we wondered whether also expression of Bak, the other main pro-apoptotic multidomain protein, is regulated by p53. We therefore analyzed the human *bak1* gene locus for transcription factor binding sites. Indeed, we identified a functional p53 consensus binding site and verified p53-mediated regulation of the *bak1* promoter region by luciferase reporter gene assays, qRT-PCR, and Western blot analysis. Upregulation of Bak in response to the DNA-damaging agent etoposide occurred later than p53-mediated induction of p21 expression and cell cycle arrest. Our data suggest a model in which cells in sustained cell cycle arrest are sensitized for apoptosis by enhanced Bak expression that shifts the Bcl-2 rheostat of anti-apoptotic proteins and pro-apoptotic multidomain proteins.

Results

Identification of putative p53-binding sites in the bak1 gene locus. The tumor suppressor protein p53 suppresses the expression of Bcl-2[17], whereas it induces expression of several BH3-only proteins, such as Puma and Noxa, and the pro-apoptotic multidomain protein Bax.[15] This prompted us to analyze the possibility that p53 in addition to Bax also regulates the expression of the homologous pro-apoptotic multidomain protein Bak. We initially analyzed 8200 bp of the

bak1 gene locus on chromosome 6p21.31 for potential promoter regions using the "Gene2Promotor" and "ElDorado" software (Genomatix Software GmbH, www.genomatix.de). These 8200 bp span a region starting 5550 bp upstream of exon 1, include exon 1, intron 1, and the first 16 codons of the coding sequence within exon 2. The software indicated two potential promoter regions, the first starting upstream of and extending into exon 1 (bp -3188 to -2115) and the second starting within intron 1 and extending into the coding region of exon 2 (-508 to 50; Fig. 1a). Based on these findings we analyzed a total of 3350 bp (-3300 to 50) including both promoter regions for transcription factor-binding sites using the "MatInspector" (Genomatix) software. The MatInspector software identified binding sites for different transcription factors, e.g. SP1/2, STAT, NF-κB and also p53 (Fig. 1a). Because p53 transactivates expression of several pro-apoptotic Bcl-2 proteins we analyzed the three potential p53-binding sites in this region - the first one directly upstream of exon 1 and the other two following exon 1. To test whether these putative p53-binding sites were functional, we cloned the region containing all 3 potential p53 binding sites and exon 1 (-2790 to -2020; a total of 770 bp) into the pGL3-basic vector generating pGL3-p53BS and performed luciferase reporter gene assays.

p53 induces target gene expression from bak promoter constructs. In a next step we co-transfected HeLa cells with different amounts of a p53 expression vector together with the reporter plasmid pGL3-Basic-p53BS that contains all three putative p53-binding sites. Cell extracts were prepared 48 h post transfection and luciferase activity measured luminometrically. Clearly, co-transfection of p53 induced luciferase expression in HeLa cells (Fig. 1b, left panel). Induction of luciferase expression from pGL3-Basic-p53BS was higher when cells were co-transfected with 1.0 µg of the p53 expression plasmid as compared to transfection with 0.5 µg, indicating specific target gene expression from the *bak*-promoter construct in a concentration-dependent manner. To further confirm these data we also performed similar experiments in HCT116/p53$^{-/-}$ cells. Again, cells were co-transfected with either 0.5 µg or 1.0 µg of the p53 expression construct together with pGL3-Basic-p53BS. Also in the p53-negative cellular background exogenous p53 specifically induced luciferase activity (Fig. 1b, right panel). These results show that p53 can transactivate gene expression from the cloned region of the *bak1* gene locus.

We next set out to investigate the specific role of each of the putative p53-binding sites within the 770 bp region and therefore generated 4 more luciferase expression vectors containing either mutations in one of each putative p53 consensus sequence or mutations in all three putative p53-binding sites as described in materials and methods. The p53 consensus binding site consists of two half sites of the sequence 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' separated by 0 – 13 bp.[19] We introduced mutations in each of the p53 binding sites separately by exchanging the essential cytosine and guanine residues for adenine and thymine (Suppl. Table 1). Additionally, we created a luciferase reporter gene vector in which all three p53-binding sites were mutated simultaneously. These four additional reporter gene plasmids, pGL3-Basic-mutBS1, -mutBS2, -mutBS3, and -mutBS1-3 were then co-transfected together with the p53 expression construct into HeLa cells and luciferase activity in cellular extracts was assessed 48 h post transfection (Fig. 2a). Unexpectedly, mutation of BS1 or BS2 resulted in negligible changes of luciferase activity compared to the non-mutated construct, whereas mutation of BS3 caused a significant reduction of luciferase activity by about 60% (23-fold compared to 61.7-fold; Fig. 2a). The mutation of BS3 had a similar effect when assays were conducted in HCT116/p53$^{-/-}$ cells. Again, mutation of BS1 or BS2 did not significantly influence reporter gene expression, whereas luciferase activity induced from pGL3-Basic-mutBS3 was reduced by about 60% (6.2-fold compared to 15.4-fold) (Fig. 2b). The simultaneous mutation of all three putative p53-binding sites did not result in a further reduction of luciferase activity compared to the single mutation of BS3 in either cell line. In agreement with the previous results, overall induction of luciferase expression was higher in HeLa than HCT116/p53$^{-/-}$ cells. These data do not only demonstrate that p53 induces target gene expression from the *bak1* promoter region but, moreover, show that exclusively BS3 is necessary and sufficient for p53-driven gene expression, whereas BS1 and BS2 are dispensable.

p53 directly binds to BS3. Because mutation of BS3 largely reduced reporter gene activity, we wanted to confirm p53 binding to the newly identified BS3. To this end, we performed electrophoretic mobility shift assays (EMSAs) using radiolabeled ds-oligonucleotides containing the sequence of each putative binding site. We also included the mutated BS3 and, as a positive control, the p53 consensus binding site in these assays. The radiolabeled probes were incubated with nuclear extracts from HCT116/wt cells that had been treated for 48 h with the DNA-

damaging agent etoposide. To further analyze specificity of the DNA/protein complex, an anti-p53 antibody was added to the samples. In samples containing the p53 consensus binding site a protein/DNA complex was readily detected and addition of the p53 antibody produced a supershift proving the identity of the p53/DNA complex (Fig. 2c, left lane). Although each of the binding site oligonucleotides was unexpectedly shifted in EMSAs, exclusively the BS3 oligonucleotide was supershifted by the p53 antibody. Mutation of BS3 abolished protein binding in the presence or absence of the p53 antibody. Hence, in line with our results from luciferase reporter gene assays, p53 directly binds to BS3 and mutation of the essential cytosine and guanine residues in the BS3 sequence disrupts p53 binding. In further mobility shift assays that were performed with extracts from etoposide treated HCT116/p53$^{-/-}$ cells we also detected protein/DNA complexes of each of the binding sites but, as expected, no supershift was produced by addition of the p53 antibody. These results confirm direct binding of p53 to BS3, but also show that BS1-3 are bound by additional proteins. Lastly, we performed chromatin immunoprecipitation (ChIP) assays to analyze binding of p53 to BS1, BS2 and BS3 within the *bak* promoter in a cellular context. In anti-p53 ChIPs from samples of etoposide-treated HCT116/wt cells we found enrichment of BS3, but not BS1 or BS2 (Fig. 2d), thus confirming binding of endogenous p53 specifically to BS3 within the *bak* promoter region.

p53 upregulates expression of endogenous bak. The results obtained so far clearly demonstrate that p53 can induce target gene expression from constructs containing the identified promoter region by binding to BS3. Consequently, we next investigated endogenous *bak* gene expression. In initial experiments we transduced HCT116/p53$^{-/-}$ cells with different MOIs of an adenovirus encoding for p53 and analyzed Bak mRNA expression three days later. Bak mRNA levels were elevated (about 6-fold) in transduced cells (Fig. 2e, left bars). Because adenoviral transduction induces high levels of p53, we also aimed to achieve p53 upregulation in a more physiological manner. To this end, we transduced HCT116/wt and HCT116/p53$^{-/-}$ cells with an adenovirus for the expression of p14^{ARF}. p14^{ARF} inhibits MDM2-mediated p53 degradation and induces elevated levels of p53. Enhancing the level of endogenous p53 clearly resulted in elevated levels (more than 10-fold) of Bak mRNA (Fig. 2e, middle bars) in HCT116/wt cells.

The specificity of these results was underlined by transduction of HCT116/p53$^{-/-}$ cells with Ad-p14^{ARF}. In the absence of p53 p14^{ARF} did not induce *bak* mRNA expression (Fig. 2e, right bars).

We then investigated whether activation of the endogenous p53 signaling pathway in response to DNA damage also drives Bak mRNA expression. To this end, HCT116/wt cells were treated with the topoisomerase II inhibitor etoposide and Bak mRNA expression was analyzed by quantitative real-time PCR (qRT-PCR). As a control we also investigated Bak mRNA expression in response to etoposide treatment in the isogenic HCT116/p53$^{-/-}$ cell line. The results of qRT-PCR were normalized to internal GAPDH mRNA expression. Bak mRNA was upregulated over time in HCT116/wt cells following DNA damage due to etoposide treatment (Fig. 3a, left panel). Bak mRNA expression was 1.75-fold after 24 h, 2-fold after 48 h and increased up to 4.5-fold after 72 h. In HCT116/p53$^{-/-}$ cells treated with etoposide induction of Bak mRNA expression was less pronounced (1.5-fold after 24 h and 48 h and 2.7-fold after 72 h; Fig. 3a, right panel).

The results from EMSA experiments indicated binding of proteins different from p53 to BS1, 2 and 3, which is reflected by Bak mRNA induction in p53-negative cells. To test whether upregulation in the absence of p53 is specific for Bak or does also occur for other p53 target genes, we investigated the induction of several other p53 target genes in HCT116/wt and HCT116/p53$^{-/-}$ cells. We chose to analyze mRNA expression of the cyclin-dependent kinase (CDK) inhibitor p21[20,21], the pro-apoptotic multidomain protein Bax[15] and the BH3-only protein Puma[13,22], which all are well established target genes of p53. In line with data from the literature, qRT-PCR analysis showed induction of all these target genes in response to etoposide treatment of HCT116/wt cells. Target gene induction was time-dependent and most pronounced after 72 h. Induction was strongest for p21 reaching a 55-fold expression level after 72 h (Fig. 3b). Induction of Puma was below that of p21 (25-fold after 72 h, Fig. 3c) and that for Bax was weakest (7-fold after 72 h, Fig. 3d). Comparable to the results obtained for Bak, all these established p53 target genes were also induced in p53-deficient HCT116/p53$^{-/-}$ cells, although to a lesser extent compared to wild type HCT116. We therefore conclude that *bak* can be considered a bona fide p53 target gene, although a weaker expression is also detectable in the absence of p53, similarly to the established p53 targets *p21*, *puma* and *bax*.

We also tested whether the p53-mediated induction of target genes correlates with the strength of DNA damage. To this end we treated HCT116/wt and HCT116/p53$^{-/-}$ cells with 10 µM, 50 µM, and 100 µM etoposide for 48 h and performed qRT-PCR analysis. These experiments showed that Bak mRNA induction is concentration-dependent. Whereas the Bak mRNA level was about 2.5-fold in cells treated with 10 µM and 50 µM etoposide, it was 3-fold in response to treatment with 100 µM etoposide in HCT116/wt cells (Fig. 4a). Again, etoposide treatment affected Bak mRNA expression in HCT116/p53$^{-/-}$ only marginally (Fig. 4a). For comparison we also analyzed induction of Bax, p21, and Puma mRNAs in similarly treated HCT116/wt and HCT116/p53$^{-/-}$ cells. In HCT116/wt cells induction of Bax mRNA was comparable to that of Bak (Fig. 4b) and induction of Puma mRNA expression was higher than that of Bax and Bak (up to 4.5-fold) (Fig. 4c). Notably, p21 was induced about 16-fold regardless of the etoposide concentration (Fig. 4d). The mRNA expression of Bax and Bak was significantly reduced in HCT116/p53$^{-/-}$ cells compared to that in HCT116/wt cells (Fig. 4a-b). Expression of p21 and Puma mRNA after etoposide treatment was slightly elevated in HCT116/p53$^{-/-}$ cells (Fig. 4c-d).

P53-dependent upregulation of Bak protein level in response to DNA damage. We clearly demonstrated p53 specific regulation of *bak* gene expression and finally wanted to confirm Bak upregulation at the protein level. Therefore, we analyzed Bak protein expression by Western blot analysis of HCT116/wt and HCT116/p53$^{-/-}$ cells that had been treated with 50 µM etoposide for 24 h, 48 h, and 72 h or had been left untreated. Confirming our qRT-PCR results Bak was significantly upregulated at the protein level in response to etoposide treatment in HCT116/wt cells, whereas induction of Bak expression was less pronounced in HCT116/p53$^{-/-}$ (Fig. 4e). We also analyzed the expression of the CDK inhibitor p21 and Bax by Western blotting. Again, confirming qRT-PCR data expression of p21 was highly induced already 24 h after etoposide treatment. However, the protein level of p21 appeared to slightly decline over time. Western blot analysis also confirmed p53-dependent induction of Bax (Fig. 4e). Interestingly, the basal level of Bak was reduced in HCT116/p53$^{-/-}$ cells compared to the wild type cell line. This finding might be indicative of the basal p53 protein expression in HCT116/wt cells upregulating Bak expression on the one hand, and of Bak regulation by other transcription factors than p53 in HCT116/p53$^{-/-}$

cells on the other. The results obtained from Western blots of dose-response experiments showed that expression of p21 and Bax was already induced by low doses of etoposide, whereas Bak expression increased in a dose-dependent manner (Fig. 4f).

p73 transactivates bak. Since we had conclusively established p53-dependent upregulation of the pro-apoptotic protein Bak in response to DNA damage, we were left with the interesting finding that Bak expression, albeit to a weaker extent, is also upregulated in the absence of p53. Bak induction in the absence of p53 could be due to redundancy of the p53 family proteins regarding their consensus binding sites. Because p73 has been shown to induce expression of Puma in response to DNA-damage by γ-irradiation[23], we hypothesized that regulation of Bak expression might also be affected by p73. To test this we co-transfected HCT116/p53$^{-/-}$ cells with expression plasmids encoding for different p73-isoforms together with the *bak* promoter construct. All p73 isoforms (α, β, γ, δ) induced luciferase expression to various degrees (data not shown). p73δ showed the highest induction and was used as p73 in subsequent experiments. We next co-transfected the different luciferase reporter gene plasmids containing the *bak* promoter region and mutations in the p53 consensus sequences together with the p73 expression vector. Then, 48 h post transfection, lysates were analyzed for luciferase activity. Indeed, p73 induced luciferase expression from the *bak* promoter region (Fig. 5a). Interestingly, the mutation of each of the putative p53-binding sites reduced p73-mediated reporter gene induction by 20–30%. Simultaneous mutation of all three binding sites had an additive effect and reduced reporter gene expression by about 60%. These results show that *(i) bak* gene expression is induced by p73 and *(ii)* this induction is regulated by all three binding sites and not exclusively by BS3 as is the case for p53. In EMSAs we analyzed whether p73 can bind to all three putative p53 consensus sequences supporting the luciferase data. Indeed, addition of a p73 antibody to the samples produced a supershift of all unmutated ds-oligonucleotides used, showing that p73 binds to BS1, BS2 and BS3 (Fig. 5b).

Both, p53 and p73, can also be expressed as N-terminal truncated isoforms that lack the transactivation domain and these ΔN-isoforms act dominant negative on their respective transactivation competent TA-isoforms.[24] We investigated the impact of ΔN-isoforms on p73-

mediated reporter gene induction by co-transfection of expression plasmids for Δ133p53 and DNp73, respectively. As expected, p73-induced reporter gene expression from the *bak* promoter was reduced by co-expression of DNp73 in both, HeLa (Fig. 5c, left panel) and HCT116/p53$^{-/-}$ cells (Fig. 5c, right panel) to basal level. Interestingly, p73-mediated reporter gene induction was not reduced by co-expression of Δ133p53. In complementary experiments we investigated the influence of DNp73 and Δ133p53 on p53 induced reporter gene expression. In HeLa cells reporter gene induction by p53 was reduced by 70% when DNp73 was co-expressed, and by 30% in case of Δ133p53 co-expression (Fig. 5d, left panel). Likewise, in HCT116/p53$^{-/-}$ cells co-expression of DNp73 diminished p53-mediated luciferase expression from 12.5-fold to 2.2-fold and Δ133p53 again showed less reduction of luciferase activity (12.5-fold vs. 7.6-fold) (Fig. 5d, right panel).

We finally tested whether p73 induces expression of endogenous Bak. To this end HCT116/p53$^{-/-}$ cells were transduced with 50 MOI of an adenovirus encoding for p73α and 72 h post transduction cells were harvested and analyzed for Bak mRNA. Quantitative real time PCR showed a 2.5-fold increase of Bak mRNA expression in p73-expressing HCT116/p53$^{-/-}$ cells as compared to control cells (Fig. 5e). p73-mediated upregulation of Bak mRNA expression in response to etoposide treatment for 72 h was furthermore analyzed in HCT116/wt cells tranfected with an p73-shRNA expression vector and control cells. Expression of p73 was upregulated 4-fold in etoposide treated control cells and only 1.5-fold in shp73 transfected cells (data not shown). In line, the 8-fold induction of Bak mRNA in etoposide treated cells was reduced to about 4-fold in p73-shRNA-expressing cells (Fig. 5f, left panel), once more verifying p73 mediated-upregulation of endogenous Bak. To further investigate whether p73 regulates Bak mRNA expression we also incubated HCT116/p53$^{-/-}$ cells that had been transfected with the p73-shRNA construct in the absence or presence of 50 µM etoposide for three days. Subsequently, Bak mRNA expression was analyzed by qRT-PCR. Upregulation of Bak mRNA expression in response to etoposide treatment was reduced from 2.3-fold in etoposide-treated control cells to 1.7-fold in p73-shRNA expressing cells (Fig. 5f, right panel). These results not only confirm that p73 modulates Bak mRNA expression but, moreover, indicate that p73 modulates expression of Bak mRNA independently of p53.

To investigate the relevance of Bak expression for DNA damage-induced apoptosis, we compared sub-G1 populations in different etoposide-treated HCT116 cell lines that were either wild type, devoid of Bax or Bak, or both Bax and Bak expression, as verified by Western Blot analysis (Fig. 6a). The various cell lines were incubated in the presence of 50 µM etoposide for 24 h, 48 h and 72 h and the percentage of sub-G1 (referred to as apoptotic) cells was assessed by flow cytometry. As expected, Bax knock-out as well as Bak knock-down reduced the number of apoptotic cells at each time point (Fig. 6b). Interestingly, after 72 h of treatment, combined knock-out of Bax and knock-down of Bak had an additive effect that was not evident after 24 h and 48 h. At this late time point the number of sub-G1 cells was reduced by about 50% in HCT116/Bax$^{-/-}$/Bak^{kd} and only about 25% in either HCT116/Bax$^{-/-}$ or HCT116/Bak^{kd} compared to HCT116/wt (Fig. 6b).

Discussion

In this study we have identified the pro-apoptotic multidomain protein Bak as a transcriptional target of the tumor suppressor p53 and we have furthermore for the first time characterized the *bak1* promoter region with respect to p53 binding sites. We found that p53-mediated reporter gene upregulation exclusively depends on the p53-BS3 spanning bp -2085 to -2066. Binding of p53 to this region was verified by a supershift in EMSAs. Induction of endogenous Bak mRNA and protein expression by p53 was confirmed by qRT-PCR and Western blot analyses in HCT116/wt cells and the isogenic p53 knock-out cell line HCT116/p53$^{-/-}$. Hence, like other Bcl-2 family proteins, e.g. Puma, Noxa, Bax and Bcl-2, also Bak expression is transcriptionally regulated by p53. The location of the p53 response element downstream of exon 1 within the first intron is reportedly a common feature of some p53 target genes, such as GADD45α, KILLER/DR5 and the p53 negative regulator MDM2.[19,25-27]

Recent analyses of apoptosis regulation by Bcl-2 proteins have revealed specific interactions of BH3-only proteins with anti-apoptotic Bcl-2 proteins. Likewise, anti-apoptotic Bcl-2 proteins specifically interact with the pro-apoptotic multidomain proteins Bax and Bak. In

the de-repression model interaction of BH3-only proteins with anti-apoptotic Bcl-2 proteins results in their inactivation. Therefore, the anti-apoptotic proteins no longer counteract Bax and Bak, which then can oligomerize at the mitochondrial outer membrane and induce its permeabilization. Although further complexity is added to the Bcl-2 network by post-translational modifications of Bcl-2 proteins that modulates their activity[28], transcriptional upregulation of Puma and Noxa by p53 is paramount for apoptosis induction. Also the downstream acting pro-apoptotic multidomain protein Bax is a transcriptional target of p53[15], whereas the expression of the Bax-antagonizing protein Bcl-2 is reduced in the presence of p53.[17,18]

Our qRT-PCR data show that Puma induction by p53 is stronger than that of either Bak or Bax but still does not reach the level of p53-mediated p21 induction. This reflects that cell cycle-regulatory genes contain more robust p53REs than do pro-apoptotic genes.[29] The strength of p53 mediated induction of the pro-apoptotic genes *puma*, *bax* and *bak1* correlates with the distance of their respective p53REs from the transcriptional start site, as is the case for many p53 target genes.[30] The *puma* p53RE is located in the promoter region (-145/-126), those for *bax* (354/373) and *bak1* (596/617, corresponding to BS3) are located in the first intron. Although several other mechanisms might regulate p53 target gene expression, the relative induction of these proteins (p21, Puma, Bax and Bak) reflects the cellular response that is established by p53. The first event to take place is cell cycle arrest via strong induction of p21. Under sustained cell cycle arrest and as long as Puma expression is not sufficient to induce apoptosis, expression of the pro-apoptotic multidomain proteins Bax and Bak rises and both accumulate. Finally, cells cross the borderline to apoptosis due to the overall shift in the Bcl-2 rheostat that drives them into cell death rather than cell cycle arrest.

Our data furthermore show that, in addition to p53, another member of the p53 family of transcription factors, namely p73, can drive gene expression from the *bak* promoter. We found that p73 induces reporter gene expression from the *bak* promoter construct and that transgenic expression of p73 and also etoposide treatment induce Bak expression in p53-negative HCT116/p53$^{-/-}$ cells. With regard to the reporter gene assays, it is most interesting to note that all

three investigated binding sites equally contribute to p73-mediated reporter gene induction, whereas p53-mediated reporter gene induction exclusively depends on the presence of BS3. These findings were verified by supershift assays that showed that p53 exclusively binds to BS3, whereas p73 binds each of the binding sites. An overlapping response element affinity by p53 and p73 has already been shown by Osada et al.[31] However, in addition to binding to p53 consensus sites, p73 has also its own specific consensus sequence.[31] The presence of additional specific p73 consensus sites in the *bak* promoter region is presumable, as mutation of all three putative p53 binding sites does not completely abrogate p73-driven reporter gene activity. Transactivation by p73 in addition to p53 has also been shown for the BH3-only protein Puma.[23] We have also detected upregulation of Puma mRNA in etoposide-treated p53-negative HCT116/p53$^{-/-}$ cells, which might be due to a regulation by p73.[23]

We furthermore found that ΔN-isoforms differentially influence p53- and p73-induced reporter gene expression. Whereas DNp73 suppresses reporter gene induction by both, p53 and p73, the ΔN133p53-isoform specifically reduces reporter gene induction by p53 but not p73. However, the impact of ΔN133p53 and DNp73 on transactivation by p53 and p73 not only depends on binding specificity and binding affinity, but is highly dependent on the relative expression level of transcription factors. Therefore, transactivation of the *bak* promoter by endogenous p53- and p73-isoforms might differ to some degree from results of reporter gene assays.

The expression of several Bcl-2 family proteins has been shown to be induced by transcription factors that do not belong to the p53 family. Expression of Puma for instance can also be induced by FoxO3[32] and SP1.[33] Interestingly, Chirakkal et al. identified a SP1-binding site within the promoter region of *bak1*[34] that was also indicated in our MathInspector analysis (Fig.1). This SP1 binding site mediates upregulation of Bak in response to butyrate as well as basal Bak expression. The presence of this SP1 binding site in our reporter gene constructs might therefore explain that mutation of p53 binding sites does not completely abrogate reporter gene induction. Most recently NF-κB was shown to induce expression of Puma, Noxa, Bad, and Bim, thereby contributing to apoptosis induction by TNF-α[35]. As our software analysis also indicated

the presence of several NF-κB binding sites in the Bak promoter region, it is likely that apart from p53 other transcription factors influence expression of Bak under certain conditions. For example, in embryogenesis and tissue development the transcription factor WT1 (Wilm's tumor suppressor protein 1) has been implicated in regulation of Bak expression.[36] Mutations in WT1 cause Wilm's tumors and knock-out of WT1 results in agenesis of kidneys associated with embryonic lethality. Transactivation of the *bak* promoter during differentiation appears to be negatively regulated by brain acid soluble protein 1 (BASP1).[37] In summary, all these findings reflect that, despite the ever increasing advances in our understanding of signaling by Bcl-2 proteins, the regulation of their expression by numerous transcription factors is still poorly investigated. A comprehensive model for the complex mechanisms that underlie control of apoptosis by Bcl-2 proteins would need to incorporate both, transcriptional and post-transcriptional regulation.

Materials and methods

Cell lines, reagents and antibodies. HCT116/wt, HCT116/*p53*$^{-/-}$, HCT116/*bax*$^{-/-}$ (kindly provided by B. Vogelstein)[38] and HCT116 cells stably expressing Bak siRNA (HCT116/*bak*^{kd} and HCT116/*bax*$^{-/-}$/*bak*^{kd})[39] were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine and antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin) (PAA Laboratories, Linz, Austria). HeLa cells were cultured in RPMI-1640 (PAA) with identical supplements. All chemicals were purchased from Sigma (Munich, Germany). The following antibodies were used: monoclonal mouse anti-Bax (6A7, Trevigen, Gaithersburg, MD), polyclonal rabbit anti-Bak (Upstate, Temecula, CA), monoclonal mouse anti-p53 (Ab-6, Calbiochem/EMD Biosciences, Darmstadt, Germany), monococlonal mouse anti-p73 (ER-13, Sigma), monoclonal mouse anti-p21 (Becton Dickinson, Heidelberg, Germany) and monoclonal mouse anti-β-actin (Sigma). Secondary antibodies to mouse and rabbit IgG were from Promega (Mannheim, Germany).

Cloning and mutagenesis. The *bak1* promoter region was amplified by PCR from clone RPCIP704J10291Q (RZPD, Berlin, Germany) using the primer pair BS-for/BS-rev (Suppl. Table 1) that contained the XhoI and HindIII restriction sites. After restriction with XhoI and HindIII the PCR product was ligated into the pGL3-Basic vector (Promega) generating pGL3-Basic-p53BS. Vectors containing mutations in the putative p53 consensus binding sites 1 and 3 were generated by PCR amplification using the primer pair mutBS1-for/BS-rev and BS-for/mutBS3-rev (Suppl. Table 1), respectively. Subsequent ligation of the PCR products into pGL3-Basic resulted in pGL3-Basic-mutBS1 and pGL3-Basic-mutBS3. Binding site 2 was mutated using the Quick Change Mutagenesis kit (Stratagene, La Jolla, CA). The vector pGL3-Basic-p53BS served as template and the two complementary primers mutBS2-for and mutBS2-rev (Suppl. Table 1) introduced the desired mutations resulting in pGL3-Basic-mutBS2. To generate the vector pGL3-Basic-mutp53BS containing mutations in all three binding sites simultaneously the template pGL3-Basic-mutBS2 was PCR amplified using the primers mutBS1-for and mutBS3-rev. All vector sequences were confirmed by DNA sequencing.

Western blot analysis. Cells were harvested by scraping, washed in ice-cold PBS and lysed in buffer containing 1% Nonidet P-40, 20 mM HEPES (pH 7.9), 2 mM PMSF, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined using the BCA assay kit (Thermo Fischer Scientific, Bonn, Germany) and 15 µg of protein per lane were loaded onto standard SDS-PAGE gels and separated at 200 V. The proteins were transferred onto a polyvinylidene difluoride membrane (Amersham Bioscience GmbH, Freiburg, Germany) by tank blotting. The membranes were blocked in PBS containing 4% BSA and 0.05% Tween-20 for 1 h followed by an overnight incubation with the primary antibody diluted in blocking buffer at 4 °C. After washing the membranes thrice in blocking buffer the appropriate secondary antibodies (1:5000) were applied for 1 h. Finally, membranes were washed for an additional 2 h in PBS, 0.05% Tween-20, before proteins were visualized using ECL reagents (Amersham Biosciences).

Transfection and viral transduction. Cells were seeded 24 h prior to transfection in 6-well plates or culture dishes at a density of $7.5x10^4$ cells per cm^2. Transfection was performed using the Fugene 6 (Roche) or jetPEI (PEQLAB, Erlangen, Germany) reagent according to the manufacturer's protocol. Transfected cells were grown for various time periods before harvesting. Preparation of $p14^{ARF}$, p53 and p73 adenoviruses[40,41] and transduction of cells were performed as described.[42]

Luciferase reporter gene assays. Cells were transfected with the respective pGL3-Basic reporter plasmid together with expression vectors for p53, Δ133p53, DNp73, p73δ or empty vector.[43,44] 48 h post transfection cells were harvested by scraping and lysed in LLB [25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA (pH 8.0), 1 mM DTT, 1% Triton X-100] for 5 min on ice. After centrifugation (5 min, 10000 x g) 10 µl of the supernatants were assayed in 100 µl luciferase assay buffer [15 mM potassium phosphate (pH 7.8), 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP] using a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). Light emission was measured after injection of 100 µl of luciferin (0.3 mg/ml).

Electrophoretic mobility shift assays. The p53-consensus oligonucleotide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the oligonucleotides representing BS1, BS2, BS3 and mutBS3 (Suppl. Table 2) were from Sigma. Hybridized oligonucleotides were radiolabelled by Klenow-reaction in the presence of [γ]–^{32}P-ATP. 10 µg of nuclear extracts from etoposide (50 µM, 48 h) treated HCT116/wt or HCT116/$p53^{-/-}$ cells were incubated with radiolabeled oligonucleotides for 30 min at room temperature. For supershift assays 1 µg of the respective antibody was added to the samples followed by an additional incubation for 30 min on ice. Samples were separated on an 8% 0.5 x TBE polyacrylamide gel at 160 V, dryed and autoradiagraphs were taken by exposure to an x-ray film for 3-5 h.

Quantitative real-time PCR. Cells were seeded at a density of $1x10^5$ cells per cm^2. 24 h later the culture medium was changed for fresh medium with or without etoposide (50 µM). After the indicated periods of time cells were harvested by scraping and total RNA was isolated using the RNeasy mini kit from Qiagen (Hilden, Germany) according to the manufacturer's protocol.

cDNA was reverse transcribed from 1 µg total RNA using a 2-step protocol: first RNA was incubated with 50 µM random hexamer primers (Fermentas GmbH, Leon-Rot, Germany) and 40 units RNase-Inhibitor (RiboLock RNase Inhibitor, Fermentas) at 65°C for 10 min. Then, 200 units of reverse transcriptase (Revert Aid H Minus M-MuLV Reverse, Fermentas), 10 mM DTT, 400 µM dNTPs and water were added to a final volume of 25 µl and samples were incubated at 42°C for 50 min followed by final heat inactivation at 72°C for 10 min. qPCR was performed in an ABI7300 instrument (Applied Biosystems, Darmstadt, Germany) using 1x Maxima SYBR Green qPCR Mastermix (Fermentas), 300 nM sense-/antisense primers and 80 ng cDNA. The qPCR programme started with an initial denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed by heating the samples twice to 95°C for 15 s and 60°C for 1 min. Negative control reactions contained water instead of cDNA and were included in each run to ensure absence of contamination. Primers used for analysis of *p21* (Hs_CDKN1A_1_SG), *puma* (Hs_BBC3_1_SG), and *bak* (Hs_BAK1_1_SG) expression were purchased from Qiagen and primers for *bax* expression (forw: 5'-CACGGCAGAGAATGCCTATGA-3', reverse: 5'-CCCAATTGATGCCACTCTCAA-3') were kindly provided by Dr. K. Lauber (University Hospital Tübingen). Results were normalized to GAPDH and analyzed by the ΔΔCt method to give fold-induction as compared to untreated control samples.

Chromatin immunoprecipitation (ChIP) assay. Cells were seeded at a density of $1x10^5/cm^2$. 24 h later the culture medium was changed for fresh medium with or without 50 µM etoposide. After three days, cells were harvested by scraping, crosslinked and ChIPs were performed from $5x10^7$ cells using an anti-p53 antibody (Ab-6) as described previously.[45] Quantitative real-time PCR was performed on a LightCycler480 instrument using the SYBR Green I Master kit (Roche) according to the manufacturer's protocol. Primers specific for amyloid precursor protein (APP), BS1, BS2 and BS3 are indicated in Suppl. Table 3. Values were normalized to APP and fold enrichment over input chromatin was calculated using the 2exp{-ΔΔCt} method.

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Legends to figures

Figure 1: Representation of the *bak1* gene locus and reporter gene assays. (**a**) Schematic representation of the analyzed region of the *bak1* gene locus on 6p21.31. The promoter regions predicted by the Matinspector software, positions of exon 1, intron 1 and exon 2 and positions of the three putative p53-binding sites are depicted. Also shown is the sequence of the cloned *bak* promoter region. The putative p53-binding sites are printed in bold and highlighted by grey boxes, the position of exon 1 is highlighted in grey, potential binding sites for SP1 and STAT are underlined. (**b**) Luciferase reporter gene assay using the reporter vector containing the illustrated region (bp -2790 to -2020) of the *bak1* gene locus. HeLa and HCT116/p53$^{-/-}$ cells were co-transfected with pGL3-Basic-p53BS together with different amounts of a p53 expression vector. p53 induces luciferase expression in a concentration-dependent manner in HeLa and HCT116/p53$^{-/-}$ cells. Values are means ± S.D. from 6 independent experiments.

Figure 2: Identification of BS3 as the functional p53-binding site. HeLa (**a**) and HCT116/p53$^{-/-}$ (**b**) cells were transfected with the indicated reporter gene constructs together with a vector for the expression of p53. Luciferase reporter gene induction by p53 is not affected by mutation of BS1 or BS2, whereas mutation of BS3 reduces luciferase activity significantly. (**c**) EMSA using the p53 consensus sequence (p53con), BS1, BS2, BS3 or mutated BS3 (mutBS3) and extracts from etoposide-treated HCT116/wt and HCT116/p53$^{-/-}$ cells. Addition of a p53 antibody produces a supershift (indicated by *) of p53con and BS3 in samples from wild type but not from p53-deficient cells. No shift of BS1, BS2, or mutBS3 is detected. (**d**) Anti-p53 ChIP assay from HCT116/wt cells incubated in the absence or presence of 50 µM etoposide for 72 h. The graph shows enrichment of BS3 but not BS1 and BS2 over input DNA. Values are averages of two independent experiments. (**e**) HCT116 cells were transduced with 10 or 50 MOI of adenoviruses for the expression of p53 or p14^{ARF}.

After 3 days cells were harvested and total RNA analyzed by qRT-PCR. Expression of p53 in HCT116/p53$^{-/-}$ and p14^{ARF} in HCT116/wt cells induces Bak mRNA expression, whereas p14^{ARF} does not trigger upregulation of Bak mRNA in HCT116/p53$^{-/-}$ cells. Values represent means ± S.D. from at least three independent experiments.

Figure 3: Time course of p53-dependent gene induction in response to DNA damage. HCT116/wt and HCT116/p53$^{-/-}$ cells were treated with 50 µM etoposide for 24 h, 48 h and 72 h and expression of Bak (**a**), p21 (**b**), Puma (**c**) and Bax (**d**) mRNA was analyzed by qRT-PCR. Bak, Bax, Puma and p21 mRNA expression rises over time in response to treatment with etoposide in HCT116/wt and to a lesser extent in HCT116/p53$^{-/-}$ cells. Values are means ± S.D. from three independent experiments.

Figure 4: Analysis of mRNA and protein expression of p53 target genes in response to etoposide. HCT116/wt and HCT116/p53$^{-/-}$ cells were treated with 10 µM, 50 µM and 100 µM etoposide for 48 h and expression of Bak (**a**), Bax (**b**), Puma (**c**) and p21 (**d**) mRNA was analyzed by qRT-PCR. Expression of Bak and Puma mRNA increases with concentration of etoposide, whereas induction of Bax and p21 mRNA expression is independent of the concentration of etoposide. (**e**) HCT116/wt and HCT116/p53$^{-/-}$ cells were incubated with 50 µM etoposide for 24 h, 48 h and 72 h and expression of p53, Bak, Bax and p21 was analyzed by Western blotting. (**f**) HCT116/wt and HCT116/p53$^{-/-}$ cells were incubated with the indicated concentrations etoposide for 48 h and expression of p53, Bak, Bax and p21 protein was analyzed likewise. In HCT116/wt and to a lesser extent also in HCT116/p53$^{-/-}$ cells the level of Bak expression rises in a time- (**e**) and concentration- (**f**) dependent manner. Values are means ± S.D. from three independent experiments.

Figure 5: p73-mediated induction of Bak reporter gene expression, DNA binding, and repression by Δ133p53, DNp73, and p73 knock-down. (**a**)

HCT116/p53$^{-/-}$ cells were co-transfected with the indicated reporter gene plasmids and a p73α expression vector. After 48 h cells were harvested and luciferase activity was assessed. Values are expressed as fold induction normalized to the activity induced from pGL3-Basic alone. Reporter gene induction was reduced by the single mutation of each of the putative p53-binding sites, while mutation of all three binding sites had an additive effect. (**b**) HCT116/p53$^{-/-}$ cells were treated with 50 µM etoposide for 48 h and EMSAs were performed on nuclear extracts using ^{32}P labeled ds-oligonucleotides for the p53 consensus sequence (p53con), BS1, BS2, BS3, and mutated BS3 (mutBS3). Addition of a p73 antibody produces a supershift (*) of p53con, BS1, BS2 and BS3 indicating binding of p73α to these oligonucleotides. (**c**) HeLa and HCT116/p53$^{-/-}$ cells were co-transfected with p73 and Δ133p53 or DNp73 together with the *bak* promoter reporter construct. After 48 h cells were harvested and luciferase activity detected luminometrically. Induction of luciferase activity by p73 was reduced to basal level in cells co-transfected with the DNp73 expression plasmid, whereas no reduction was seen in Δ133p53 co-expressing cells. (**d**) HeLa and HCT116/p53$^{-/-}$ cells were co-transfected with p53 and Δ133p53 or DNp73 together with the *bak* promoter reporter construct. Induction of luciferase activity by p53 was measured 48 h post transfection and reduced in both, Δ133p53 and DNp73 co-expressing cells. (**e**) HCT116/p53$^{-/-}$ cells were transduced with 50 MOI of Ad-p73α and after 72 h samples were analyzed for Bak mRNA expression by qRT-PCR. The Bak mRNA expression was upregulated 2.5-fold in Ad-p73 transduced cells as compared to control cells. (**f**) HCT116/wt and HCT116/p53$^{-/-}$ cells were transfected with a vector for the expression of p73-shRNA and incubated in the absence or presence of 50 µM etoposide for 72 h. qRT-PCR revealed inhibition of etoposide-induced Bak mRNA expression by the p73-specific shRNA as compared to the control in both wildtype and p53-deficient HCT116 cells.

Figure 6: Apoptosis induction in Bak- or Bax-deficient and proficient HCT116 cells. (**a**) Western blot analyses show the status of Bax and Bak expression in the

individual cell lines; actin served as loading control. (**b**) The indicated Bax- or Bak-proficient and deficient HCT116 cell lines were incubated with 50 µM etoposide for up to 72 h and DNA content of cell nuclei was analyzed by flow cytometry. Nuclei with less than 2N of DNA content (sub-G1) were quantified as apoptotic. Knock-out of Bax protects HCT116 cells more efficiently from apoptosis than knock-down of Bak at 24 h and 48 h. At later time points combined Bak knock-down and Bax knock-out have an additive protective effect.

Figure 1
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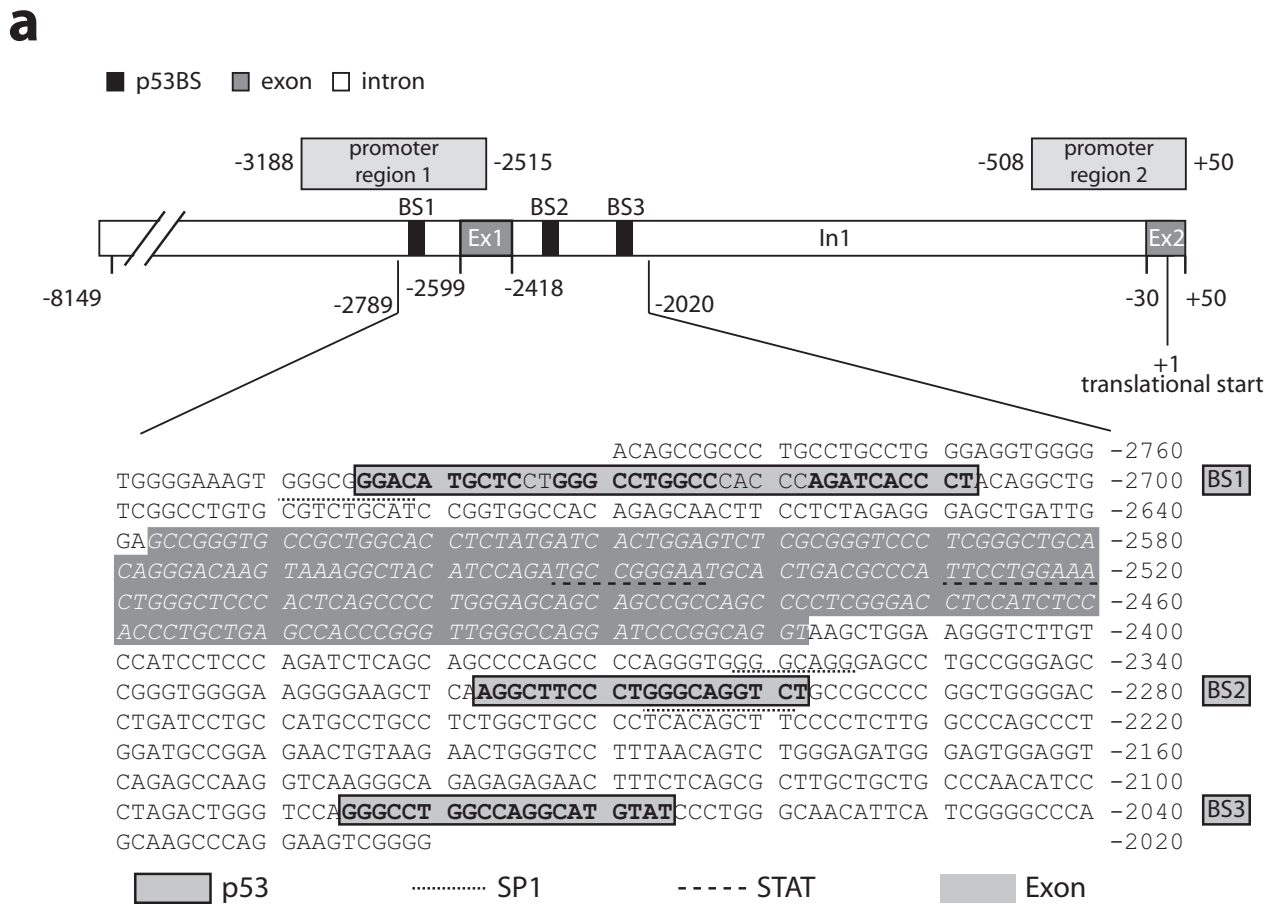

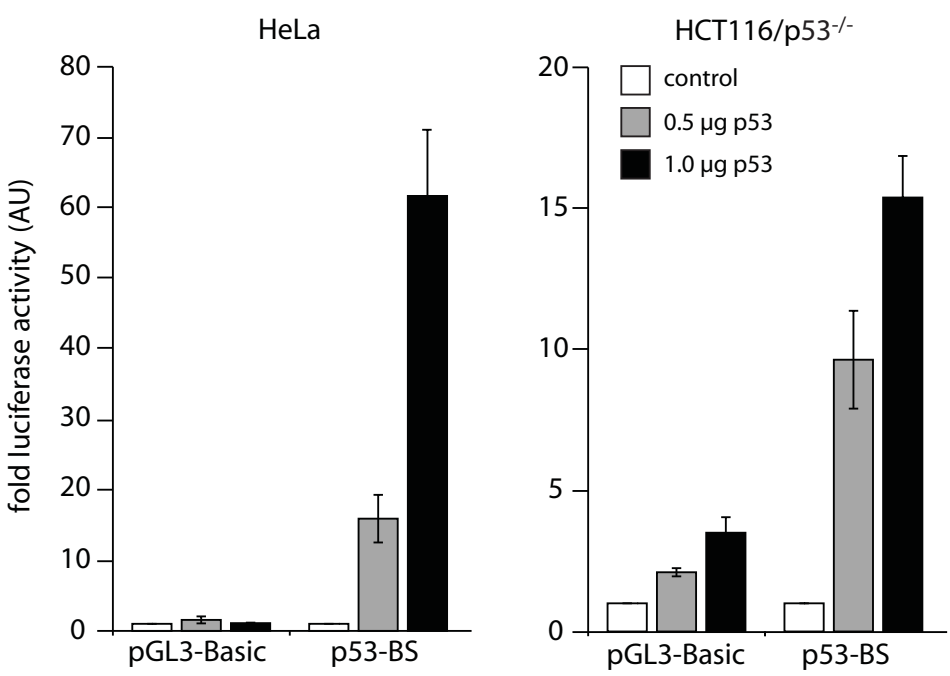

Figure 2
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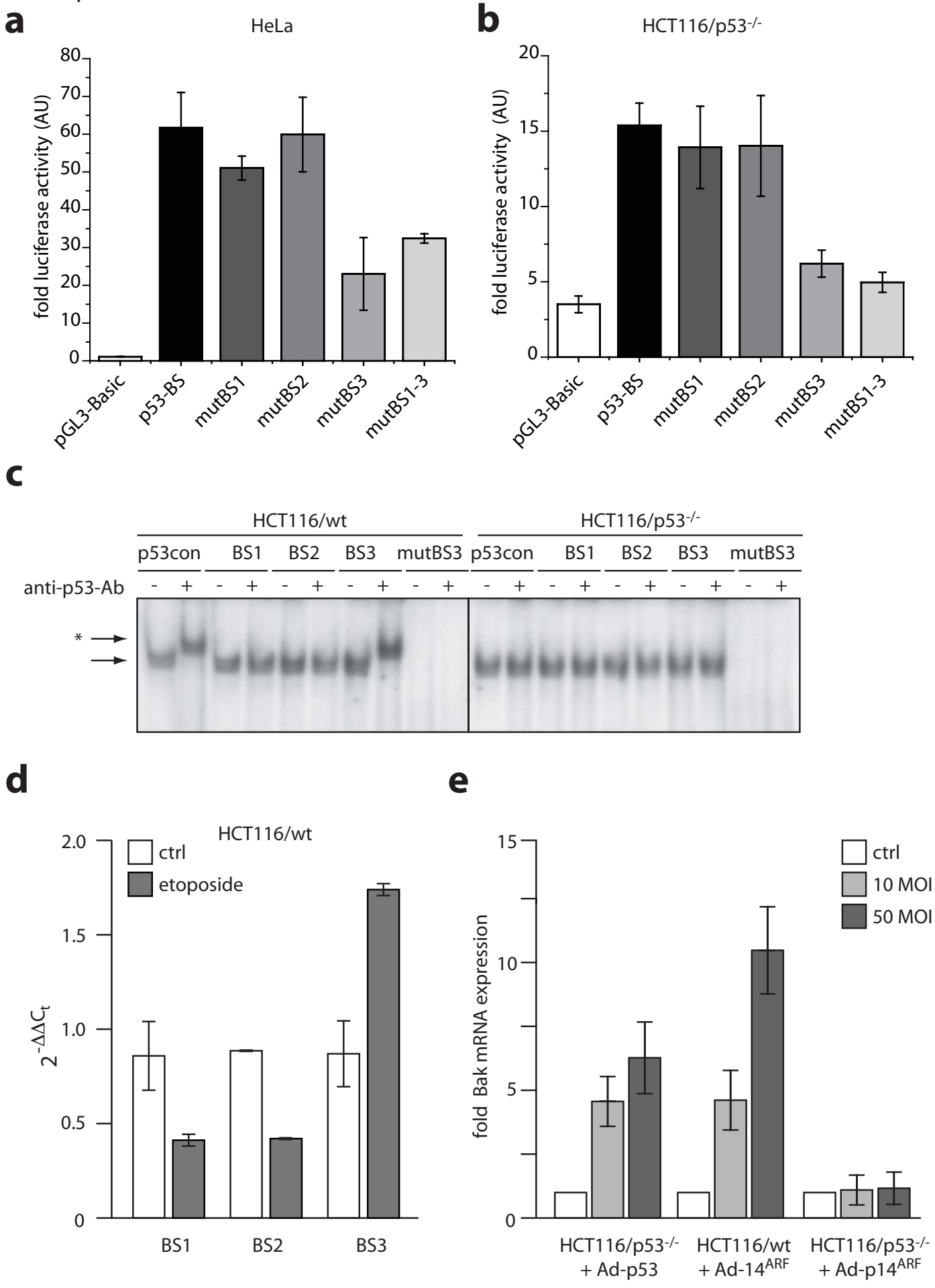

Figure 3
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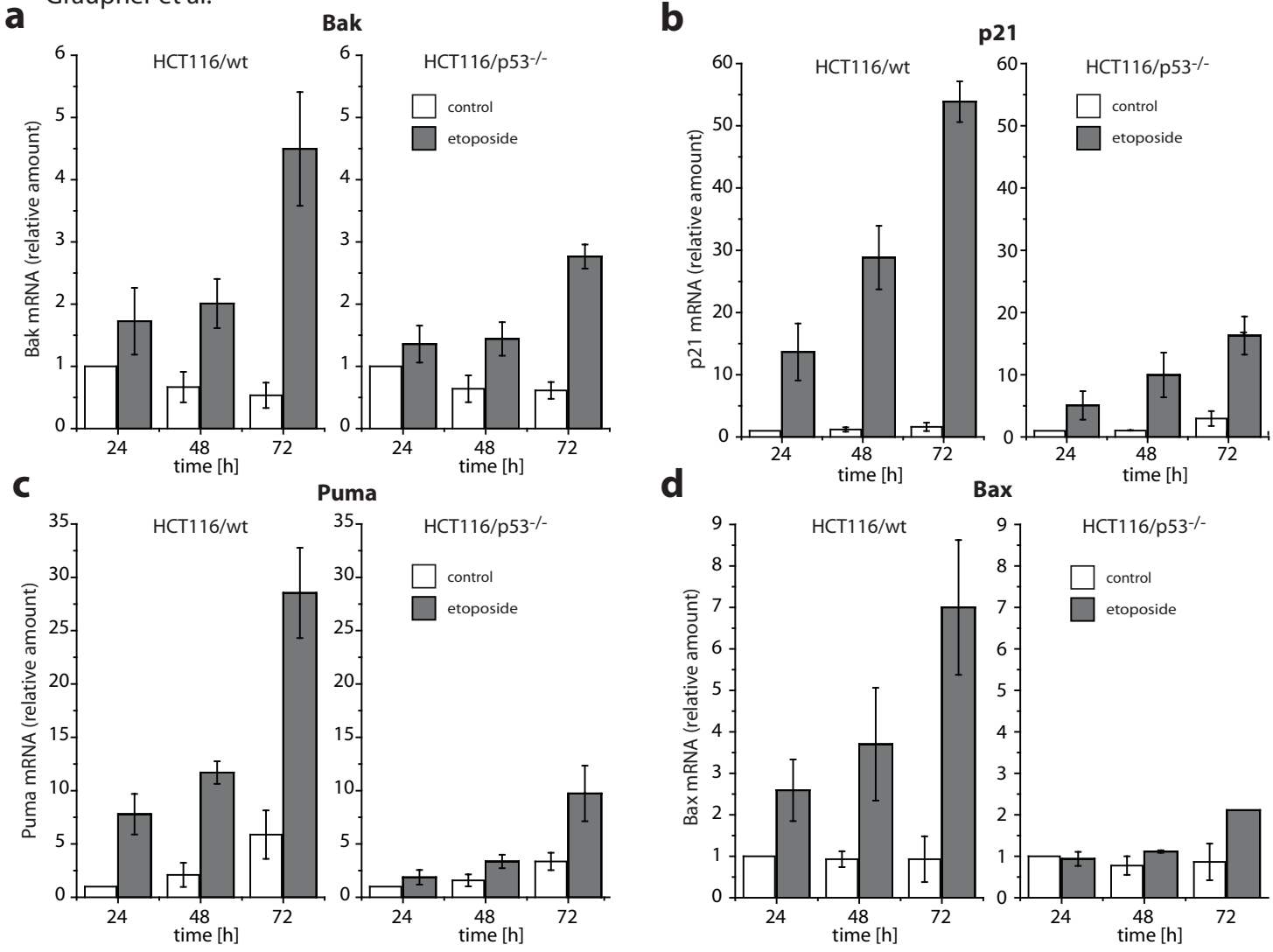

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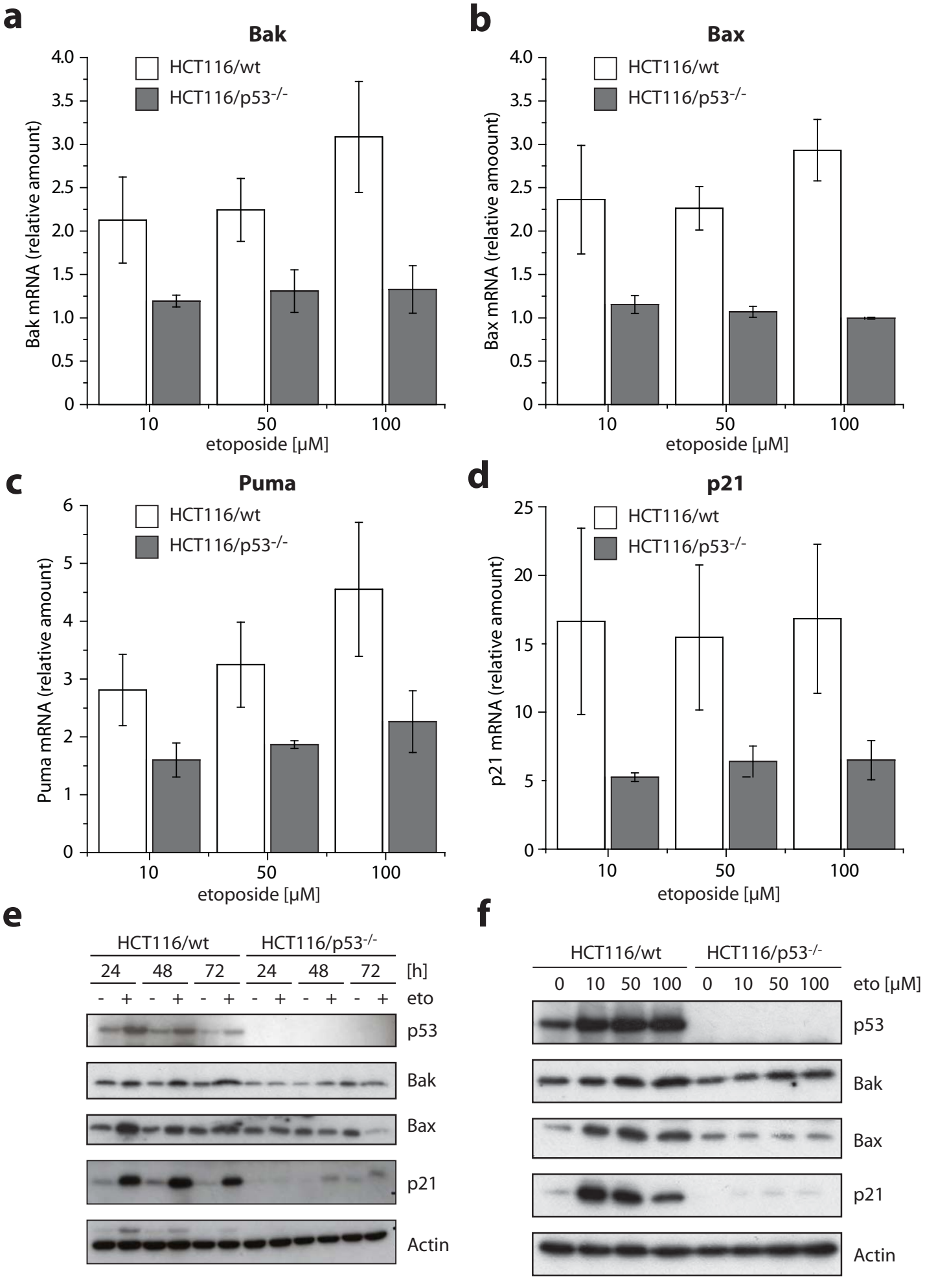

Figure 5
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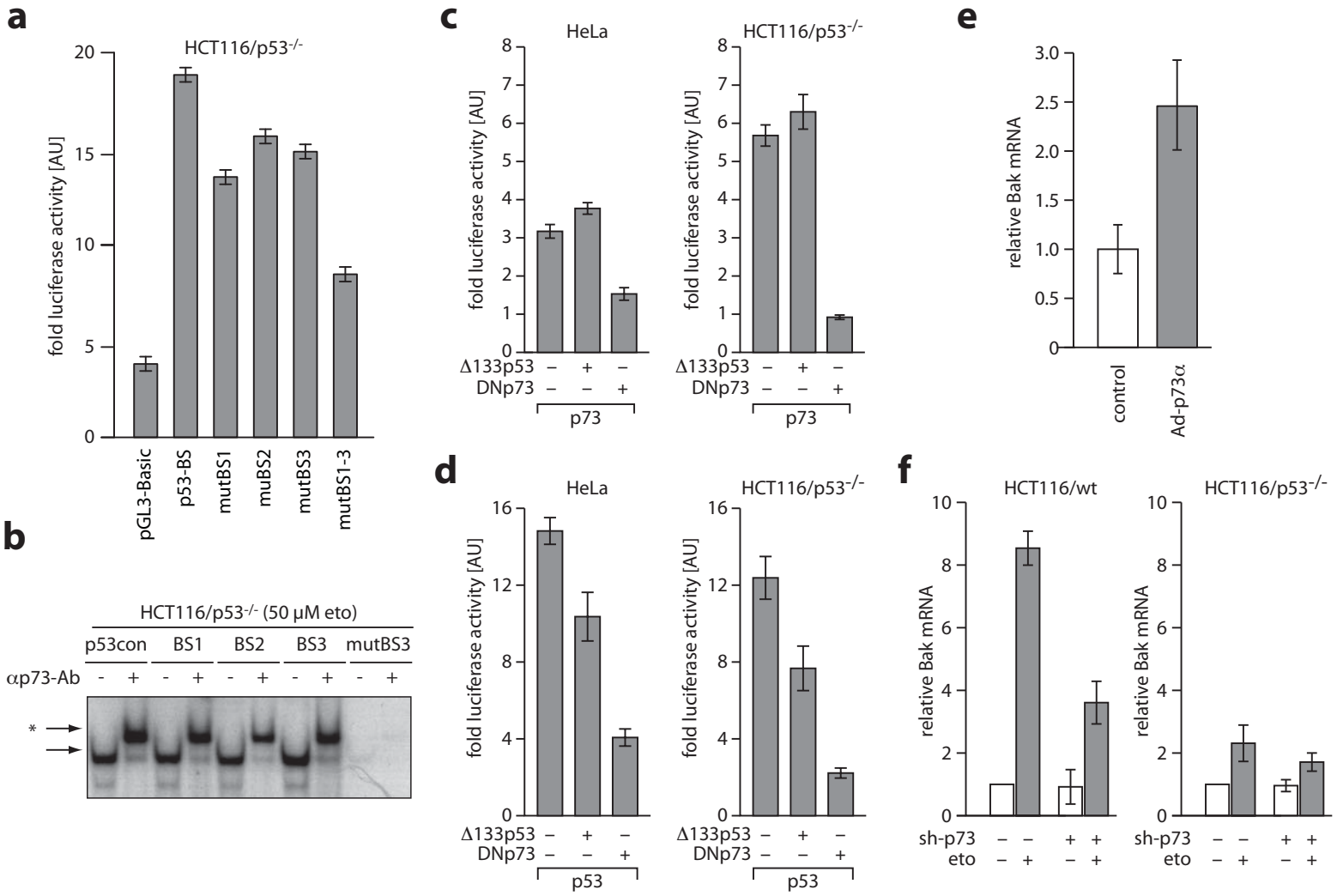

Figure 6
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