# p53 triggers apoptosis in oncogene-expressing fibroblasts by the induction of Noxa and mitochondrial Bax translocation

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## Abstract

**The mechanism of p53-dependent apoptosis is still only partly defined. Using early-passage embryonic fibroblasts (MEF) from wild-type (wt), p53$^{-/-}$ and bax$^{-/-}$ mice, we observe a p53-dependent translocation of Bax to the mitochondria and a release of mitochondrial Cytochrome $c$ during stress-induced apoptosis. These events proceed independent of zVAD-inhibitable caspase activation, are not prevented by dominant negative FADD (DN-FADD), but are negatively regulated by Mdm-2. Bcl-x$_L$ expression prevents the release of mitochondrial Cytochrome $c$ and apoptosis, but not Bax translocation. At a single-cell level, enforced expression of p53 is sufficient to induce Bax translocation and Cytochrome $c$ release. Real-time RT-PCR analysis reveals a significant induction of RNA expression of Noxa and Bax in p53$^{+/+}$, but not in p53$^{-/-}$ MEF. Noxa protein expression becomes detectable prior to Bax translocation, and downregulation of endogenous Noxa by RNA interference protects wt MEF against p53-dependent apoptosis. Hence, in oncogene-expressing MEF p53 induces apoptosis by BH3 protein-dependent caspase activation.**
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**Keywords:** apoptosis; p53; Bax; Cytochrome $c$; Noxa

**Abbreviations:** DMEM, Dulbecco's modified essential medium; FADD, Fas-associated death domain; MEF, murine embryonic fibroblasts; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; RNAi, RNA interference; TMRE, tetramethyl-rhodamine-ethylester; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

## Introduction

The activation of oncogenes sensitizes primary cells towards a p53-dependent stress response, which includes cell cycle arrest, cellular senescence and apoptosis. In order to overcome this endogenous defense mechanism, immortalized cells are strongly selected for mutations of the p53 pathway.[1–3] This observation is supported *in vitro* and *in vivo* by genetic mouse models, which demonstrate enhanced tumorigenesis as well as increased resistance towards various stresses or anticancer treatment on the background of a deficiency for p53 and the gene products of the *INK4A/ARF* locus.[4–10] Additionally, in some experimental systems, endogenously arising murine tumors are selected for mutations of p53 and ARF, or for amplification of the p53 repressor Mdm-2.[8] This confirms the functional importance of the p53 tumor suppressor *in vivo*, which is implicated by its frequent mutation in human cancers.[11] Further understanding of the molecular events of these genetically defined pathways will help to devise specific strategies for cancer treatment and prevention.

While the p53-dependent cell cycle checkpoints are well characterized, the actual mechanism whereby p53 activates programmed cell death is still not fully understood.[3] Through its activity as a sequence-specific transcription factor, p53 can induce expression of several proapoptotic genes including the death receptors CD95/Fas/APO-1,[12,13] KILLER/DR5,[14] and the proapoptotic Bcl-2 family members Bax,[15] Noxa[16] and PUMA.[17,18] Additional genes, such as the PIGs,[19] PIDD,[20] IGF-Binding Protein 3,[21] PERP[22] or p53AIP1,[23] were identified to be transcriptionally upregulated during p53-dependent cell death. However, their roles in the apoptotic pathways, as they are currently understood, remain to be defined.

Changes in the intracellular localization of molecules are crucial to the regulation of apoptosis. The 'mitochondrial' apoptotic pathway is activated upon the formation of the Apaf-1-Caspase 9 'apoptosome', which requires release of Cytochrome $c$ from the mitochondrial intermembrane space into the cytoplasm.[24] In addition, the proapoptotic molecules Smac/DIABLO and HtrA2/Omi are released from the mitochondria most likely to derepress caspase activation through interaction with IAP proteins.[25–30] Mitochondrial Cytochrome $c$ release is regulated by the various pro- and antiapoptotic Bcl-2 family members.[31] It can either be triggered via the death receptor pathway, involving the cleavage and activation of proapoptotic Bid by Caspase 8,[32–35] or by the stress pathway, which activates proapoptotic Bcl-2 family members through an unknown mechanism. Recently, it has been reported that during death receptor-dependent and -independent apoptosis the proapoptotic Bcl-2 family member Bax undergoes a conformational change, followed by its redistribution from the cytoplasma to the outer mitochondrial

membrane, into which it inserts to induce the release of Cytochrome $c$.[36–40] Bim,[41] another proapoptotic Bcl-2 family member, is also regulated via its subcellular localization. Upon certain stresses, Bim translocates from the microtubule-associated dynein motor complex to the mitochondria and cellular membranes to promote caspase activation and apoptosis.[42]

We have previously shown in cancer cell lines that p53 induces apoptosis by a death receptor-independent induction of Cytochrome $c$ release.[43] Moreover, primary murine cells lacking Cytochrome $c$,[44] Apaf-1[45–47] or Caspase 9,[47–49] all essential components of the 'mitochondrial' pathway, were protected against stress- or oncogene-induced apoptosis. In the present work, we attempt to further delineate the p53-dependent apoptotic pathway in a genetically defined tumor model. We address the hypothesis that p53 triggers cell death by caspase-independent regulation of the activation and intracellular localization of proapoptotic Bcl-2 family proteins.

## Results

### Bax is involved in p53-dependent apoptosis and caspase activation of oncogene-expressing murine embryonic fibroblasts

In a cell-free system using extracts from Saos-2 osteosarcoma cells, we showed that the p53-induced release of mitochondrial Cytochrome $c$ requires cytosolic Bax.[43] In order to extend this observation *in vivo*, we obtained MEF deficient in p53 or Bax,[50] and their respective wild-type controls, and retrovirally transduced those cells to express the oncogenes *E1A*, *Myc* and H-*ras*G12 V. Following short-term selection, the MEF were treated under various conditions to induce apoptosis. In agreement with previous observations,[51] p53-deficient MEF as well as Bax-deficient MEF were protected against apoptosis induced by DNA-damaging agents and UV radiation (Figure 1a). To study the requirement of caspase activation in this system, wt MEF were treated with proapoptotic stimuli in the presence or absence of the broad-spectrum caspase inhibitor zVAD-fmk. Blocking caspase activity effectively prevented the onset of cell death (Figure 1a), confirming its requirement for p53-dependent apoptosis in fibroblasts.[52] The extent of death observed in p53$^{-/-}$ or bax$^{-/-}$ MEF at low and intermediate stress intensity roughly equaled the amount of cell death found in wt MEF in the absence of caspase activity (Figure 1a). Thus, caspase activation in oncogene-expressing MEF by low or intermediate doses of DNA damage depended on p53 and Bax. At high doses, however, p53$^{-/-}$ and bax$^{-/-}$ MEF were only partly protected as compared to caspase inhibition by zVAD-fmk (Figure 1a). This argues for p53- and Bax-independent pathways of caspase activation in MEF, which can be triggered by very high doses of DNA-damaging agents. Similar observations have been reported in cancer cell lines.[53]

The Myc-induced apoptotic response of fibroblasts was found to involve the activation of death receptor pathways as well as mitochondrial Cytochrome $c$ release.[54] Furthermore, it was shown to require the formation of the Apaf-1-Caspase 9 'apoptosome', as Myc-transformed fibroblasts from mice

deficient in Apaf-1 or Caspase 9 were largely protected against stress-induced, p53-dependent apoptosis.[47] Accordingly, the clonogenic growth of early-passage MEF expressing Myc and H-rasG12 V was rescued by p53-deficiency, and was partially rescued by Bax-deficiency. Enumeration revealed that p53$^{-/-}$ MEF formed 25.9 ($\pm$4) times more foci, and bax$^{-/-}$ MEF formed 12.1 ($\pm$2) times more foci than wt MEF following expression of c-Myc and H-rasG12 V (Figure 1b).
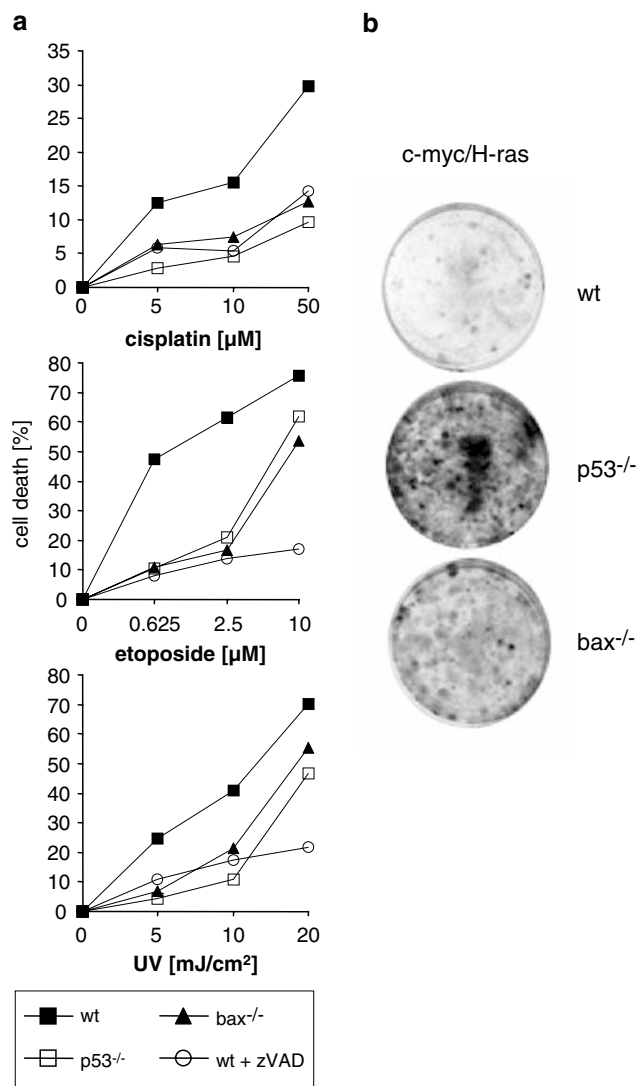


**Figure 1** Stress-induced apoptosis of oncogene-expressing MEF involves p53 and Bax signaling. (**a**) Early-passage MEF of the respective genotype expressing E1A and H-rasG12 V were treated with cisplatin (upper), etoposide (middle) or UV radiation (lower panel). In addition, wt MEF were also treated in the presence of the broad-spectrum caspase inhibitor zVAD-fmk, and cell death was assessed by flow cytometry (sub-$G_1$ fraction) 24 h following treatment. Similar results were obtained by annexin-V-PI staining (not shown). Mean values were normalized[47] to the rate of spontaneous cell death of untreated early-passage MEF ($<$10% in transformed p53$^{-/-}$ and bax$^{-/-}$ MEF, and $<$25% in transformed wt MEF). (**b**) Early-passage MEF were retrovirally transduced to express Myc and H-rasG12 V, and were plated at low densities in 35 mm dishes. After selection for 1 week, cells were fixed and stained for analysis. A representative result of three independent experiments is shown

In cancer cell lines or in cell-free reconstituted systems, Bax induces the release of Cytochrome *c* from the mitochondrial intermembrane space into the cytosol.[55,56] This enables the formation of the Apaf-1–Caspase 9 'apoptosome', which activates the effector caspases.[31,57] To study these events in oncogene-expression MEF, subcellular fractionation was performed before and after 24 h of treatment with etoposide, and the mitochondria-containing fractions were analyzed by immunoblotting. Following etoposide treatment, a loss of mitochondrial Cytochrome *c* was observed only in wt MEF, but not in p53$^{-/-}$ or bax$^{-/-}$ MEF (Figure 2a). The release of Cytochrome *c* corresponded with caspase activation, as indicated by the detection of processed Caspase 3 and the cleavage of the caspase substrate PARP in treated wt MEF (Figure 2a). Time course experiments demonstrated that the onset of p53-dependent apoptosis paralleled the release of mitochondrial Cytochrome *c* (Figure 2b). Despite low-level apoptosis, no Cytochrome *c* release was observed following etoposide treatment of bax$^{-/-}$ MEF, whereas at a late time point, some Cytochrome *c* was lost from the mitochondria of p53$^{-/-}$ MEF (Figure 2b).

Taken together, the p53-dependent apoptotic response of oncogene-expressing early-passage MEF at least in part involves caspase activation through the proapoptotic Bcl-2 family member Bax and mitochondrial Cytochrome *c* release.
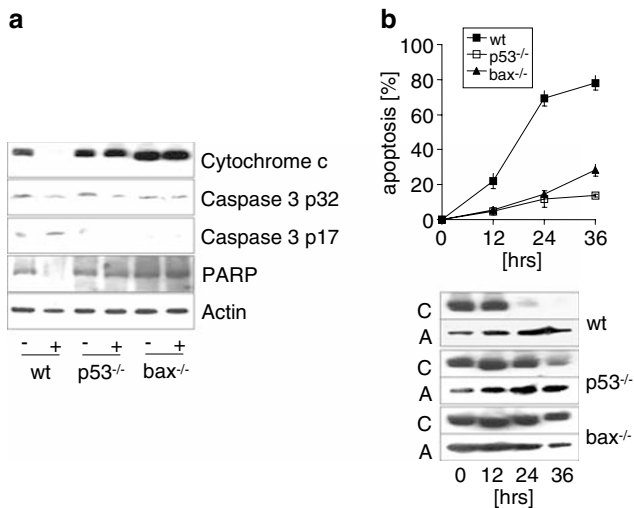
## Cytosolic Bax redistributes to mitochondria during p53-dependent apoptosis of oncogene-expressing MEF, independent of Cytochrome *c* release or caspase activation

The release of mitochondrial Cytochrome *c* can be directly induced by the proapoptotic Bcl-2 family member Bax.[55,56] *In vivo*, this activity appears to require a conformational change of Bax, which then enables its homo-oligomerization and insertion into the outer mitochondrial membrane.[58–60] Accordingly, we found an accumulation of Bax in the mitochondria-containing fraction following etoposide treatment of wt MEF, but not of p53$^{-/-}$ or bax$^{-/-}$ MEF (Figure 3). Both, mitochondrial accumulation of Bax and Cytochrome *c* release, were not prevented by the caspase inhibitor zVAD-fmk or by expression of DN-FADD, which is consistent with a death receptor-independent and caspase-independent engagement of the mitochondrial pathway by p53. This contrasts reports implying the activation of Caspase 8 as an upstream event in p53-dependent apoptosis in different experimental systems.[61–63]

p53 deficiency or expression of Mdm-2, which destabilizes p53 by targeting for proteasomic degradation,[64] prevented mitochondrial accumulation of Bax and Cytochrome *c* release. Again, no Cytochrome *c* release was observed in bax$^{-/-}$ MEF under the present experimental conditions (Figure 3). In contrast, expression of Bcl-x$_L$, which effectively prevents the release of mitochondrial Cytochrome *c* (Figure 3) and the induction of apoptosis (not shown), did not influence the mitochondrial translocation of Bax. Similar to a recent study using human fibroblasts,[65] neither mitochondrial Cytochome *c* release, nor Bax translocation were observed upon etoposide treatment of nontransformed MEF (data not shown).

These results are consistent with a model of stress-induced apoptosis, in which activated p53 signals to Bax (or other proapoptotic Bcl-2 proteins) to engage the 'mitochondrial'
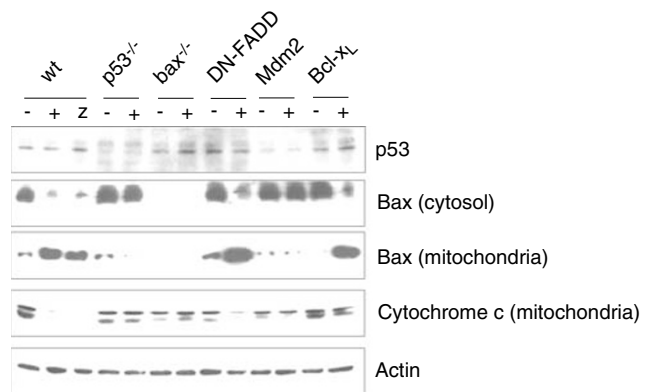


**Figure 2** Stress-induced apoptosis of oncogene-expressing MEF involves Bax-dependent mitochondrial Cytochrome *c* release and caspase activation. (**a**) Oncogene-expressing MEF were treated with 2.5 $\mu$M etoposide. Floating and adherent cells were harvested before (−) and 24 h after (+) treatment, the mitochondria-containing fraction was obtained and analyzed by immunoblotting with the indicated primary antibodies. Note the loss of mitochondrial Cytochrome *c*, the appearance of processed Caspase 3 and the degradation of PARP in treated wt MEF. (**b**) Oncogene-expressing MEF were treated with 2.5 $\mu$M etoposide, and the fraction of apoptotic cells with sub-G1 DNA content (mean ± S.D.) was determined by cell cycle analysis and flow cytometry (upper panel). In parallel, the mitochondria-containing fraction was obtained from identically treated MEF, and was analyzed by immunoblotting for the loss of Cytochrome *c* (C). The blots were reprobed for actin (A) as loading control (lower panel)



**Figure 3** p53-dependent Bax translocation and Cytochrome *c* release in oncogene-expressing MEF. Early-passage oncogene-expressing MEF of the indicated genotypes and wt MEF expressing DN-FADD, Mdm-2 or Bcl-x$_L$ were left untreated (−), or were treated with 2.5 $\mu$M etoposide (+) or 2.5 $\mu$M etoposide and zVAD-fmk (z). Floating and adherent cells were harvested after 18 h of treatment, and fractionated as described. The cytosolic and mitochondria-containing fractions were analyzed by immunoblotting using the respective primary antibodies. Note the redistribution of Bax from the cytosolic to the mitochondrial fraction in wt, DN-FADD and Bcl-x$_L$-expressing MEF, and the loss of mitochondrial Cytochrome *c* in wt and DN-FADD-expressing MEF

death pathway independent of caspase activation or death receptor signals. Activation of the caspase cascade seems to occur downstream of mitochondria, most likely via the formation of the Apaf-1–Caspase 9 'apoptosome'.[47]

## p53 expression is sufficient to induce caspase-independent translocation of Bax from the cytosol to mitochondria, and release of Cytochrome *c* from the mitochondria into the cytosol

To study the changes of intracellular localization of Cytochrome *c* and Bax during p53-dependent apoptosis at a single-cell level, we devised HeLa cells, and adenovirus-mediated p53 gene transfer.[43] Previously, it has been reported that adenovirus-mediated expression of p53 can induce apoptosis in HeLa cells independent of Bax transloca-tion and Cytochrome *c* release.[66] Using a similar adenoviral p53 vector, we confirmed that p53 expression can lead to apoptosis of HeLa cells (Figure 4). However, by means of time-lapse confocal microscopy,[67] we found that the release of Green-fluorescent protein (GFP)-tagged Cytochrome *c* from the mitochondria into the cytosol always preceded apoptotic cell death of HeLa cells induced by the expression of p53 (Figure 5a). The p53-induced release of Cytochrome *c* in HeLa cells occurred independent of caspase activity, as treatment with the broad-spectrum caspase inhibitor zVAD-fmk directly after adenoviral transduction could not prevent the release of GFP-Cytochrome *c* from mitochondria to the cytosol (Figure 5a).

To study whether p53 expression is sufficient to induce the mitochondrial translocation of Bax, we stably expressed GFP-tagged Bax[68] in HeLa cells. When apoptosis was induced by adenoviral expression of p53, GFP-Bax was redistributed

from a diffuse pattern to a predominantly perinuclear, punctate pattern (Figure 5b). Following redistribution, the GFP-Bax signal colocalized with the signal obtained by the mitochondrial dye TMRE (Figure 5c), suggesting a trans-location of GFP-Bax from the cytosol to mitochondria in response to p53 expression. Like p53-induced Cytochrome *c* release, Bax translocation also occurred in the presence of the caspase inhibitor zVAD-fmk, ordering this event upstream of zVAD-inhibitable caspase activation in p53-induced apoptosis.

Similar results were obtained by immunofluorescence detection of endogenous Bax in oncogene-expressing MEF treated with various apoptotic stimuli. A conformational change of Bax, which precedes its mitochondrial transloca-tion, and which can be detected by an antiserum specific against the N-terminus of the molecule,[37,40] was induced by DNA-damaging agents in the absence of caspase activation (data not shown).

## Induction of the BH3-protein Noxa is essential for p53-dependent apoptosis of oncogene-expressing fibroblasts

p53 is thought to exert most of its functions through its activity as a sequence-specific transcriptional activator.[1] In order to assess the impact of p53 on the RNA expression of proapoptotic molecules, we isolated and reverse-transcribed RNA from oncogene-expressing wt and p53$^{-/-}$ MEF following treatment with etoposide. By quantitative real-time PCR analysis, we found a dramatic induction of Bax and the BH3-domain containing Noxa[16] in wt MEF. RNA expression peaked at 7 h post-treatment and decreased later on (Figure 6a, and not shown). In addition, the BH3-protein PUMA,[17,18] the zinc finger protein Peg3,[69] and the p53 target gene p21/WAF1[70] were induced in a p53-dependent manner (Figure 6a). In contrast, the expression of proapopto-tic Bak, which acts at the same level as Bax during stress-induced apoptosis in MEF,[71,72] remained unchanged following etoposide treatment (Figure 6a). Time-course experiments revealed that the RNA induction of *Noxa* and *Bax* occur as early as 3 h after etoposide treatment, which is prior to Bax translocation and mitochondrial Cytochrome *c* release, that become detectable after 8 h of treatment (Figure 6b). At the protein level, Noxa expression became detectable after 4 h treatment with etoposide, and thus earlier than the redistribution of Bax and Cytochrome *c* can be observed (Figure 6b).

To assess whether the induction of Noxa is essential for p53-dependent apoptosis of oncogene-expressing MEF, we devised RNA interference[73] to knock down endogenous *Noxa* RNA expression in our experimental system. Stable expression of Noxa-specific interfering RNA oligonucleotides[74,75] protected oncogene-expressing wt MEF against p53-dependent apoptosis induced by etoposide, UV radiation, staurosporine and actinomycin D (Figure 6c). Hence, the induction of the BH3-only protein Noxa, which indirectly acts on mitochondria via Bax or Bak,[76] is essential for p53-dependent apoptosis in the present experimental system.
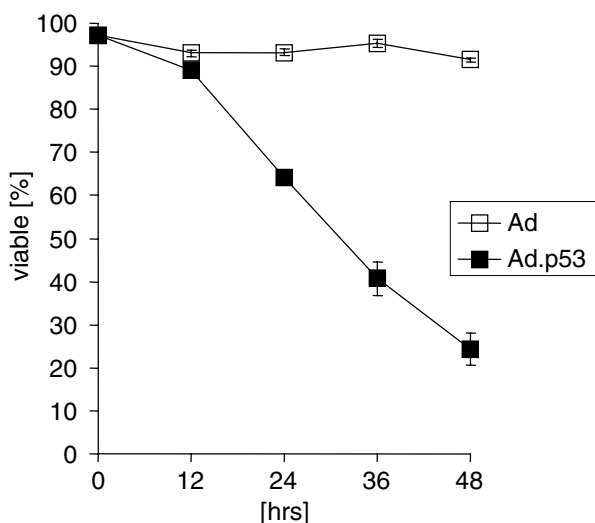


**Figure 4** Adenovirus-mediated expression of p53 induces apoptosis in HeLa cells. HeLa cells were adenovirally transduced ($5 \times 10^9$ viral particles/ml for 1 h, as described[43]) to express high levels of p53 (Ad.p53) or control vector (Ad). Apoptosis was measured at the indicated time points following transduction by staining with annexin-V-FITC and PI, and dual-color flow cytometry. The mean percentage ($\pm$S.D.) of annexin-V-negative/PI-negative viable cells is given
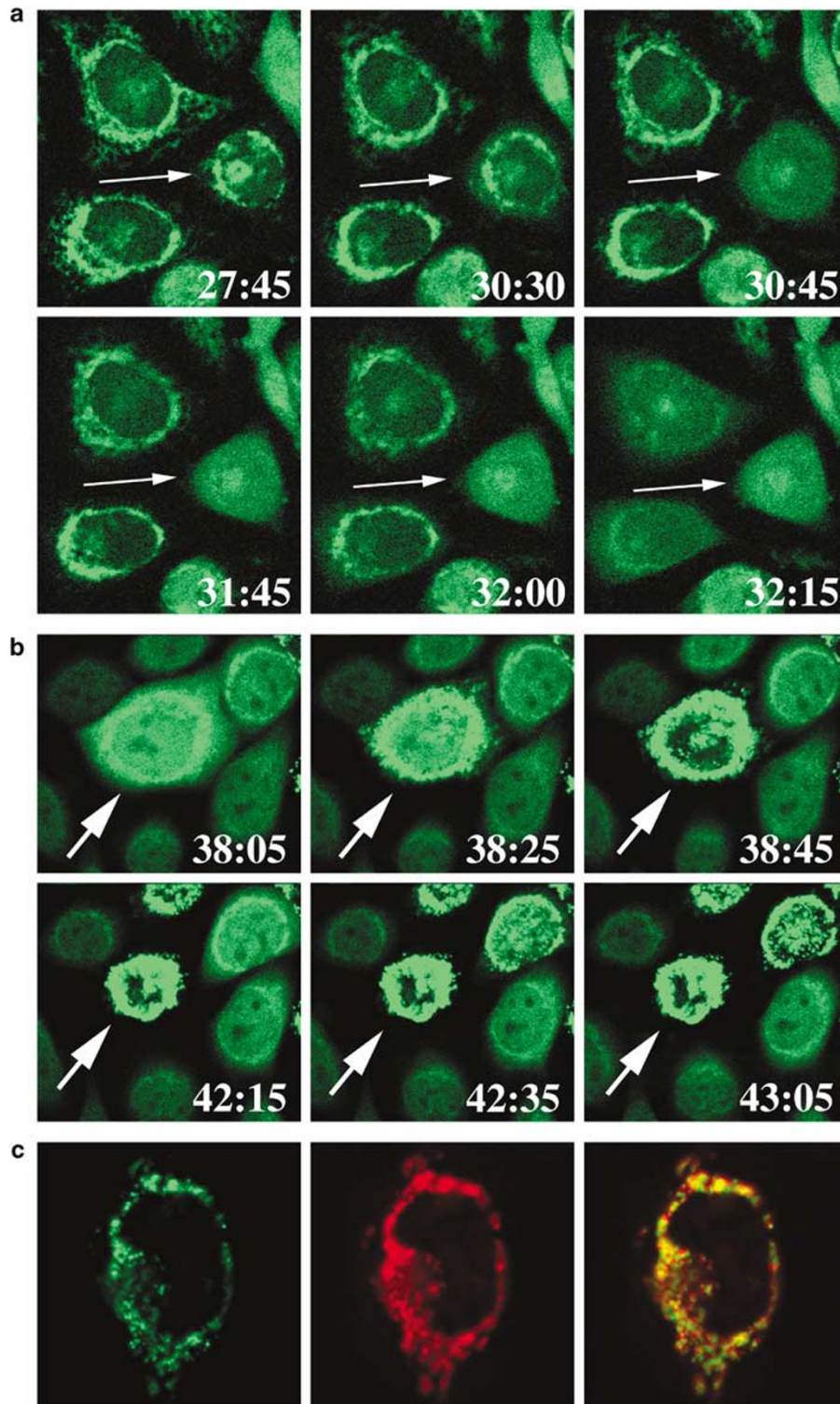
**Figure 5** p53 expression, induces caspase-independent redistribution of Bax and release of mitochondrial Cytochrome *c*. (**a**) A time lapse of HeLa cells undergoing apoptosis following adenovirus-mediated p53 expression. GFP-Cytochrome *c* moves from a mitochondrial staining pattern to a diffuse cytoplasmic distribution in the presence of the broad-spectrum caspase inhibitor zVAD-fmk. The arrow indicates a Cytochrome *c*-releasing cell; the number at the bottom right of each frame represents the time after transduction. Images were taken with a 40 × objective and a zoom of 2. (**b**) A time lapse of GFP-Bax localization following p53 expression. HeLa cells with a mostly diffuse cytoplasmic, but some perinuclear GFP-Bax localization change to a perinuclear pattern in the presence of zVAD-fmk. The arrow indicates a cell with Bax translocation to the mitochondria; the number at the bottom of each frame represents the time after transduction. Images were taken with a 40 × objective and zoomed 1.75 times. (**c**) Perinuclear GFP-Bax colocalizes with a mitochondria stain. Left image (green) is GFP-Bax following translocation. Middle image (red) is the mitochondrial marker TMRE (40 $\mu$M). The result of a merge of the left and middle images is on the right (40 × objective, zoom 3). The colocalization of GFP-Cytochrome *c* with mitochondria in nonapoptotic cells was established previously.[67]
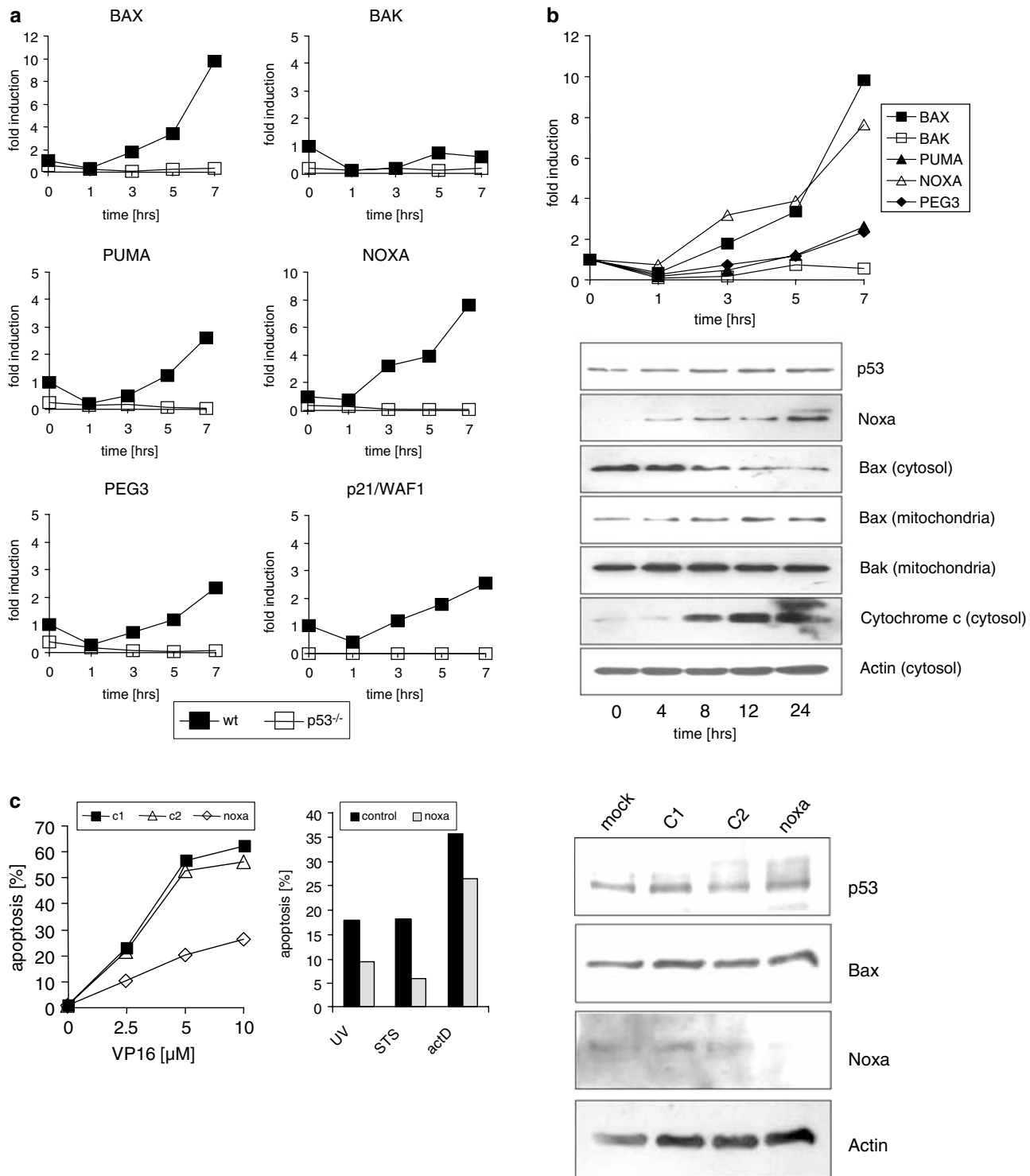
**Figure 6** Induction of Noxa is necessary for p53-dependent apoptosis of oncogene-expressing MEF following DNA damage. (**a**) Oncogene-expressing wt (closed squares) and p53$^{-/-}$ (open squares) MEF were treated with 2.5 $\mu$M etoposide, and total RNA was isolated at the respective time points. Following reverse transcription, the expression of BAX, BAK, PUMA, NOXA, PEG3, p21/WAF1 (**a**), BCL-2, ACTIN and 18S RNA (not shown) was quantitated by real-time PCR as described. Mean values of triplicates of a representative experiment are shown. (**b**) (Upper panel) Timing and relative induction of genes implicated in mitochondrial Cytochrome $c$ release in oncogene-expressing wt MEF treated with 2.5 $\mu$M etoposide. (Lower panel) Timing of protein expression and redistribution of Cytochrome $c$ and Bax in oncogene-expressing wt MEF following treatment with 2.5 $\mu$M etoposide. Note the decrease of cytosolic Bax and the increase of cytosolic Cytochrome $c$ following 8 h of treatment. (**c**) (Upper panel) MEF-expressing RNA oligonucleotides interfering with Noxa (open diamonds, light bars) or irrelevant control genes (closed squares, open triangles, dark bars) were treated with etoposide (VP16), UV radiation (UV), staurosporine (STS) or actinomycin D (actD). Apoptosis was measured by flow cytometry (sub-G$_1$ fraction) 24 h following treatment. (Lower panel) Expression of endogenous p53, Bax and Noxa in parental wt MEF (mock), and MEF expressing RNA oligonucleotides interfering with Noxa (noxa) and two control oligonucleotides (C1, C2)

## Discussion

Depending on the cellular context, the p53 tumor suppressor protein engages several stress response pathways including cell cycle arrest, cellular senescence and apoptosis.[1,2] The molecular mechanism whereby p53 signals to the apoptotic machinery, however, is still not completely understood.[3] Using genetically defined murine fibroblasts, which were sensitized to apoptosis by the expression of oncogenes, we identified conditions, under which stress-induced cell death is dependent on p53, the proapoptotic Bcl-2 family protein Bax and caspase activation. In this system, apoptotic p53 signaling triggers the 'mitochondrial' pathway, as indicated by the release of Cytochrome *c* into the cytoplasm in the absence of zVAD-inhibitable caspase activation. Mitochondrial Cytochrome *c* release is mediated by proapoptotic Bcl-2 family proteins, such as Bax or Bak,[57] and was prevented in bax$^{-/-}$ MEF under our experimental conditions.

In cancer cell lines, proapoptotic stimuli result in a redistribution of the Bcl-2 family proteins Bax[36–40] and Bim[42] from the cytoplasm or microtubules to the mitochondria. This relocalization appears to be a prerequisite for caspase activation via mitochondrial Cytochrome *c* release. In the present study, we show that p53 activation leads to a translocation of Bax from the cytoplasm to the mitochondria. Bax translocation was inhibitable by Mdm-2, but not by Bcl-x$_L$ or by the broad-spectrum caspase inhibitor zVAD-fmk. A similar observation was recently reported using Myc-transformed fibroblasts expressing a temperature-sensitive p53 mutant.[77] In this study, p53-dependent transcriptional activation of the zinc finger gene *Peg3/Pw1* was implicated to mediate activation and mitochondrial translocation of Bax. Alternatively, p53-regulated BH3-only proteins,[78] such as PUMA and Noxa, might act as activators of the proapoptotic 'multidomain' Bcl-2 proteins Bax and Bak.[76,79] By quantitative real-time RT-PCR analysis, we found a p53-dependent induction of *Bax* and *Noxa* RNA following etoposide treatment of oncogene-expressing MEF. RNA expression of *PUMA* and *Peg3/Pw1* was also significantly induced in wt MEF, however, to a much lesser extent than the former genes. Comparing the timing of gene induction with the order of events at the protein level, the expression of the BH3-only protein Noxa preceded Bax translocation and Cytochrome *c* release, as detected by cell fractionation and immunoblotting. Using an RNAi approach,[74] we demonstrated that endogenous Noxa is necessary for p53-dependent apoptosis induced by DNA damage in oncogene-expressing fibroblasts. These results are consistent with a model, in which p53 signals apoptosis through the BH3-protein Noxa, which then activates the 'mitochondrial' pathway of caspase activation through Bax in oncogene-expressing MEF, or additional proapoptotic 'multidomain' Bcl-2 proteins in other experimental systems.[72,79]

Our results using quantitative RT-PCR analysis are suggestive of a mechanism involving p53-dependent transcriptional activation of the *Noxa* gene as the most upstream event in this cascade. However, alternative mechanisms of activation of BH3-proteins have been described.[32,42] Moreover, stress-induced p53-dependent apoptosis can proceed independent of transcription or translation in some experimental systems.[80–82] In addition, Bax-independent activation

of stress-induced apoptosis by certain cytotoxic agents has been reported.[53] Thus, it is possible that p53 can activate several proapoptotic Bcl-2 family members through alternative or parallel pathways leading to mitochondrial Cytochrome *c* release and apoptosis. This complexity, however, may explain the discrepant results obtained in various experimental systems studying the molecular events of p53-dependent apoptosis.

## Materials and Methods

### Cell culture

Fibroblasts from 2-week-old p53$^{-/-}$ murine embryos and their wild-type littermates were prepared according to standard techniques. Early-passage MEF from bax$^{-/-}$ mice were generously provided by Dr. SJ Korsmeyer.[50] Cells were expanded in DMEM containing 15% fetal bovine serum (FBS), L-glutamine, antibiotics and $\beta$-mercaptoethanol, and were transduced at passage 4 using high-titer retroviral vectors as previously described.[51,83] The HeLa cervical carcinoma cell line was obtained from ATCC (Manassas, VA, USA), and was maintained in DMEM containing 10% FBS, L-glutamine and antibiotics.

### Vectors, antibodies and reagents

Retroviral vectors expressing bcl-x$_L$ and DN-FADD were generated by subcloning the respective cDNA into pBabePuro or pBabeHygro. The mdm-2 cDNA was subcloned into pMxIG (a gift from Dr. T Kitamura). Retroviral vectors expressing E1A, H-rasG12 V and Myc were kindly provided by Drs. SW Lowe and GJ Hannon. Adenoviral p53 expression vectors[43] and a plasmid expressing a GFP-Cytochrome *c* fusion protein[67] were used as previously described. The pEGFP-bax expression plasmid was a gift from Dr. RJ Youle.[68]

Detection of protein expression was performed by standard immunoblotting techniques using primary antibodies against p53 (PAb 122 and PAb 240, Pharmingen), Bcl-x (rabbit polyclonal antiserum, Pharmingen), Bax and Bak (rabbit polyclonal IgG, Upstate Biotechnology), Noxa (goat polyclonal antiserum, Santa Cruz), Cytochrome *c* (clone 7H8.2C12, Pharmingen), PARP (N20, goat polyclonal antiserum, Santa Cruz), caspase 3 (rabbit polyclonal antiserum, Pharmingen) and Actin (clone C4, ICN Pharmaceuticals).

Anticancer drugs were purchased from Sigma, the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) from Enzyme System Products, and the mitochondrial stain tetramethyl-Rhodamine-ethylester (TMRE) was obtained from Molecular Probes.

### Cell death assays

Cell death was assessed by dual color flow cytometry after staining with annexin-V-FITC (Clontech) and propidium iodide (PI), and by flow cytometry and cell cycle analysis following permeabilization and staining with PI.[56]

### Cell fractionation

Following treatment, adherent and floating cells were harvested and washed with PBS. Cell pellets were dissolved in 2 volumes of mitochondria buffer[43] supplemented with 100 $\mu$g/ml digitonin (Sigma), and were incubated on ice for 10 min or until <10% of cells excluded trypan blue.

Alternatively, cells were broken using a Dounce homogenizer, as described previously.[43] Cytosolic supernatants and mitochondria-containing pellets were obtained by centrifugation at 13 000 × g. The pellets were resuspended in lysis buffer, freeze–thawed, vortexed and again centrifuged at 13 000 × g. The resulting supernatants were normalized for protein concentration, boiled in Laemmli buffer, and analyzed by SDS-PAGE and immunoblotting.

## Clonogenicity assays

Early-passage MEF were retrovirally transduced to express Myc and H-rasG12 V. The transduction efficacy consistently ranged from 80 to 90% of cells in parallel experiments using a GFP-expressing vector. Cells were plated at 10⁴/well in 35 mm dishes, and were cultured for 7 days in selection media. After washing with PBS, cells were fixed with 70% ethanol for 1 h, and were stained with methylene blue for enumeration.

## Fluorescence microscopy

Cells were plated overnight in cell culture dishes containing sterilized coverslips. The next day transductions were performed as described.[43] After a 24 h incubation in the presence or absence of the caspase inhibitor zVAD-fmk, the culture dishes were placed in a heating chamber and images were taken in 10 or 15 min intervals using an MRC 1024ES laser scanning confocal microscope (BioRad) as described previously.[67] The 488 nm line was attenuated 91 and 94% to image the GFP-Cytochrome c and the GFP-Bax fusion proteins, respectively.

For the detection of the activated conformation of endogenous Bax, oncogene-expressing MEF growing in LabTek chamber slides were treated with various apoptotic stimuli. After 12–18 h, cells were fixed with 4% paraformaldehyde in PBS, and were washed 6 times with blocking buffer (4% BSA, 0.05% saponin in PBS). The slides were incubated overnight at 4°C with a rabbit polyclonal antiserum against the N-terminus of Bax (BAX-NT, Upstate Biotechnology) and/or a monoclonal anti-Cytochrome c antibody (Pharmingen). Texas Red-conjugated anti-rabbit Ig and FITC-conjugated anti-mouse Ig (Santa Cruz) were used as secondary antibodies, and nuclei were counterstained with Hoechst 33342 (Sigma). Images were taken on an Olympus IX-70 inverted microscope at 550, 488 and 390 nm excitation frequency using a digital imaging system, and BAX-NT-positive cells were enumerated.

## Reverse transcription and quantitative real-time PCR analysis

Total RNA (tRNA) was isolated from wt and p53⁻/⁻ MEF by means of the QIAgen RNeasy kit following the manufacturer's instructions. Per time point 2 μg of tRNA was transcribed into cDNA using the Omniscript reverse transcriptase (QIAgen). Real-Time PCR™ was performed using AmpliTaq Gold™ polymerase in a PE Biosystems 5700 Thermocycler using the SyBr Green™ detection protocol as per the manufacturer's instructions. Briefly, 12 ng of total cDNA, 50 nM of each primer and 1X SyBr Green mix were used in a total volume of 25 μl. The primer sequences were murine BCL2 (sense 5′-ACT TCG CAG AGA TGT CCA GTC A-3′ and antisense 5′-TGG CAA AGC GTC CCC TC-3′), murine BAK (sense 5′-GTC ACA AAG TCG CGA GGT TTC-3′ and antisense 5′-ATG GTG GTG GAT GCC GC-3′), murine NOXA (sense 5′-AGG AAG GAA GTT CCG CCG -3′ and antisense 5′-AGC GTT TCT CTC ATC ACA TCA CA-3′), murine BAX (sense 5′-GGA GCA GCT TGG GAG CG-3′ and antisense 5′-AAA AGG CCC CTG TCT TCA TGA-3′), murine PUMA

(sense 5′-GCC TCC TTT CTC ACC GAG C-3′ and antisense 5′-GGC CGC AAT CTT TGC A-3′), murine p21/WAF1 (sense 5′-CCG TTG TCT CTT CGG TCC C-3′ and antisense 5′-CAT GAG CGC ATC GCA ATC-3′), murine Peg3 (sense 5′-GCC TCC TTT CTC ACC GAG C-3′ and antisense 5′-TGG CCG CAA TCT TTG CA-3′), 18 S RNA (sense 5′-ATG GTA GTC GCC GTG CCT AC-3′ and antisense 5′-CCG GAA TCG AAC CCTGAT T-3′), and murine beta-actin (sense 5′-TTCGTTGCCGGTCCACA-3′ and antisense 5′-ACCAGCGCAGCGATATCG-3′). All samples were read in triplicate, and values were normalized for baseline expression and for expression of β-actin RNA.

## RNA interference

Hairpin oligonucleotides were designed using an Internet tool (oligoengine.com), and were ligated into the pSUPER.retro vector.[75] The murine Noxa oligos were NOXA216_S 5′-GAT CCC GGA CGA GTG TGC TCA ACT CTT CAA GAG AGA GTT GAG CAC ACT CGT CCT TTT TGG AAA-3′ and NOXA216_AS 5′-AGC TTT TCC AAA AAG GAC GAG TGT GCT CAA CTC TCT CTT GAA GAG TTC AGC ACA CTC GTC CGG-3′. Retroviral vectors were generated and oncogene-expressing (hygromycin-selected) wt MEF were transduced as described above. Following selection with puromycin, MEF populations expressing oligonucleotides interfering with Noxa or control oligonucleotides were analyzed for protein expression by immunoblotting as described above.

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