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GRAMD4 mimics p53 and mediates the apoptotic function of p73 at mitochondria

K John[1], V Alla[1,2], C Meier[1,2] and BM Pützer*[,1]

p73, a member of the p53 family, shares high sequence homology with p53 and shows many p53-like properties: it binds to p53-DNA target sites, transactivates p53-responsive genes and induces cell cycle arrest and apoptosis. Apart from this transcription-dependent effect, less is known about the downstream mechanism(s) by which p73 controls cell fate at the mitochondria. We have previously identified GRAMD4 (alias KIAA0767 or Death-Inducing-Protein) as a novel p53-independent pro-apoptotic target of E2F1, which localizes to mitochondria. In this study, we found that p73-induced apoptosis is mediated by GRAMD4 expression and translocation to the mitochondria. We showed that this protein physically interacts with Bcl-2, promotes Bax mitochondrial relocalization and oligomerization, and is highly efficient in inducing mitochondrial membrane permeabilization with release of cytochrome *c* and Smac. Overexpression of p73α and p73β isoforms, but not p53, leads to direct *GRAMD4* promoter transactivation. In addition, GRAMD4 induces changes in Bcl-2 and Bax protein levels. *GRAMD4* transcription is activated in response to cisplatin (cDDP) in a manner dependent on endogenous p73. Using solid tumor xenografts, ectopic expression of GRAMD4 together with cDDP resulted in enhanced cancer killing. Our findings demonstrate that p73 is able to trigger apoptosis via the mitochondrial pathway by a new mechanism using pro-apoptotic GRAMD4 as mediator, and strongly support its p53-like function.
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The induction of apoptosis is central to the tumor-suppressive activity of p53 and its homologous p73.[1] Once accumulated both proteins mediate their biological response by transcriptional activation of pro-apoptotic target genes including those encoding Bax, PUMA, and the death receptor CD95 (FAS, TNFRSF6/APO-1).[2–6] Apart from this transcription-dependent mechanism, several studies indicated that p53-induced apoptosis also occurs in a transcription-independent manner.[7,8] It was shown that in response to death stimuli such as DNA damage or hypoxia, cytoplasmic p53 protein rapidly translocates to mitochondria in primary and transformed cells.[8–10] Mitochondrial p53 precedes loss of mitochondrial membrane potential, cytochrome *c* release, and caspase activation by regulating the activity of Bcl-2 family members. Several pieces of evidence demonstrated that it physically interacts with antiapoptotic Bcl-xL/Bcl-2 proteins, thereby neutralizing their inhibitory effects on pro-apoptotic Bcl-2 family proteins.[8,11] In addition, p53 directly interacts with Bak, which causes its release from Mcl-1 and Bak oligomerization.[12] Although a direct interaction between p53 and Bax could not be detected, previous data indicated that p53 also promotes Bax oligomerization.[13]

Considering the important role of p73 in apoptosis induction, much less is known about the executing mechanism(s) by which the p53 homolog controls cell fate. We have previously identified the GRAM domain containing four gene encoding the death inducing protein GRAMD4 (alias DIP or KIAA0767) as a p53-independent target of the cellular transcription factor E2F1, and showed that upregulation of endogenous GRAMD4 by E2F1 activation or GRAMD4 overexpression leads to enhanced apoptosis in various tumor cells and karyotypically normal fibroblasts.[14] Structural analysis of the GRAMD4 protein (Q6IC98_HUMAN) revealed a nuclear localization signal in the *N*-terminal region comprising amino acids 110–121, two transmembrane regions encompassing amino acids 246–268 and 345–367, and a GRAM domain at the *C*-terminus often found in membrane-associated proteins. Our data demonstrated that GRAMD4 localizes to the mitochondria in response to E2F1 stimulation and is capable of activating caspase-3 and cleavage of poly(ADP-ribose)-polymerase.

In this study, we provide evidence that *GRAMD4* is a p73, but not p53, primary-response gene. We show that in cells undergoing p73-dependent apoptosis, GRAMD4 translocates from the nucleus to mitochondria. It interacts directly with Bcl-2 and stimulates Bax mitochondrial relocalization and oligomerization, thereby causing disruption of mitochondrial membrane integrity and release of cytochrome *c* and Smac.

[1]Department of Vectorology and Experimental Gene Therapy, Biomedical Research Center, University of Rostock, Rostock, Germany
*Corresponding author: BM Pützer, Department of Vectorology and Experimental Gene Therapy, Biomedical Research Center, University of Rostock, Schillingallee 69, Rostock D-18057, Germany. Tel: +49 381 4945066; Fax: +49 381 4945062; E-mail: brigitte.puetzer@med.uni-rostock.de
[2]These authors contributed equally to this work.
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Abbreviations: GRAMD4, GRAM domain containing 4; Ad, adenovirus; GFP, green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; MOI, multiplicity of infection; mRNA, messenger RNA; 4-OHT, 4-hydroxytamoxifen; ER, estrogen receptor; CHX, cycloheximide; BH domain, Bcl-2 homology domain; BMH, 1,6-bismale-imidohexane; CCCP, carbonyl cyanide M-chlorophenylhydrazone; 5-FU, 5-fluorouracil; cDDP, cisplatin; ChIP, Chromatin Immunoprecipitation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBP, TATA box binding protein; Ni-NTA, nickel-nitrilotriacetic acid; DMSO, Dimethylsulfoxide; DAPI, 4′6-diamidino-2-phenylindole; TMRE, tetramethyl rhodamine ethyl ester
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Furthermore, GRAMD4 is, like p73, activated in response to DNA damage leading to increased tumor killing *in vivo*. This suggests a p53-like function of the GRAMD4 protein, which enables p73 to execute its apoptotic capacity at the mitochondria.

Results

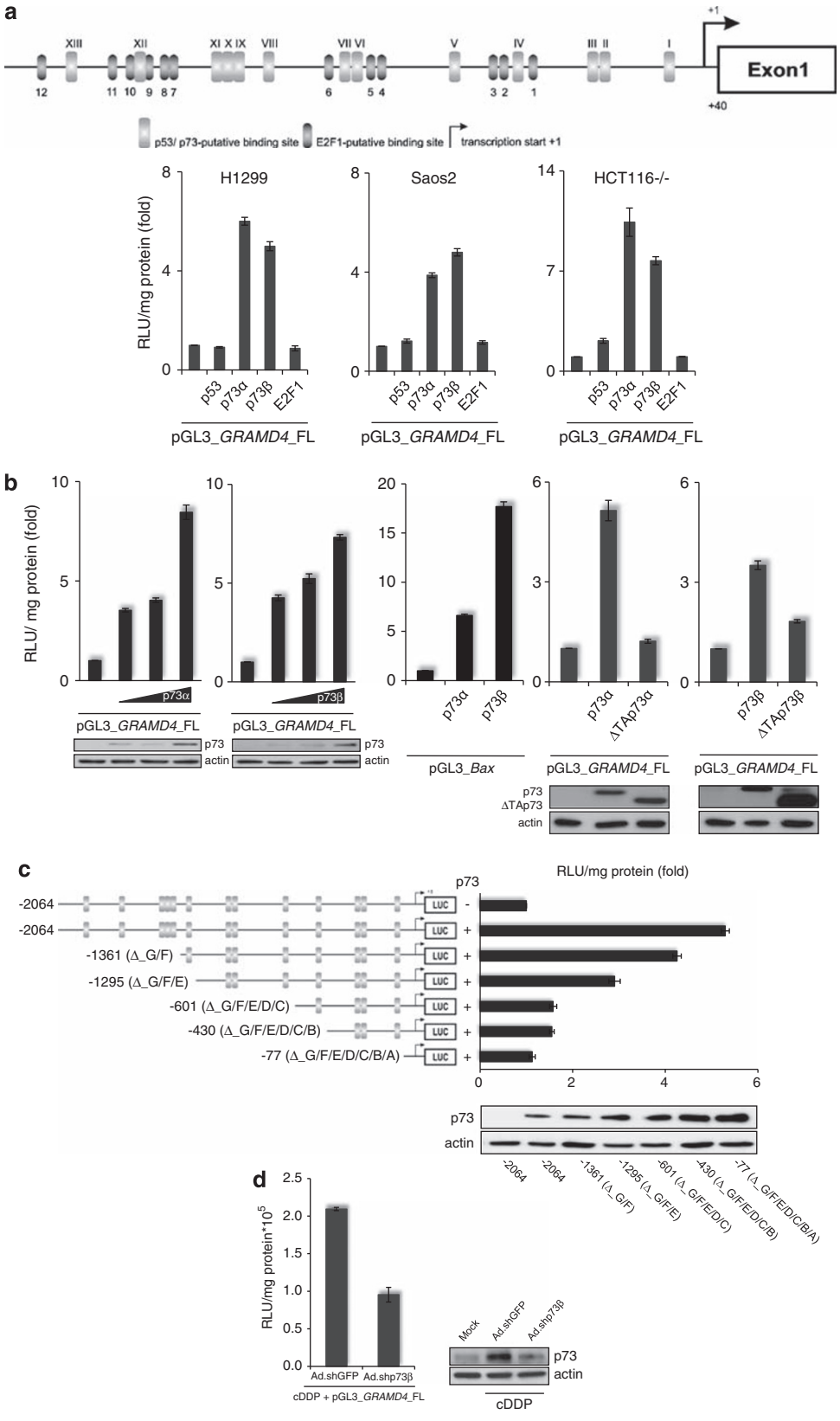
GRAMD4 is directly regulated by the p53-homolog p73 but not by p53. We have previously shown that GRAMD4 functions as a mediator of E2F1-induced apoptosis regardless of p53, but the molecular mechanism through which GRAMD4 can be activated is so far unknown. Our current knowledge of how E2F1 promotes cell death in a p53-independent manner includes direct transcriptional activation of pro-apoptotic genes.[15] To discriminate between *GRAMD4* being a direct or indirect E2F1 target, we took advantage of Saos-2 and H1299 cell lines stably expressing estrogen (ER)-E2F1. Activation of ER-E2F1 by 4-hydroxytamoxifen (4-OHT) in presence of cycloheximide (CHX) did not lead to the upregulation of GRAMD4 messengerRNA (mRNA) (data not shown), suggesting that an indirect mechanism is involved. To ascertain transcription factor binding sites in the human *GRAMD4* promoter, we cloned a genomic fragment encompassing 40 base pairs from exon 1 of *GRAMD4*. Sequence analysis revealed several putative binding motifs for E2F1 and p53 family proteins in the region between -2064 to $+40$ upstream of the transcription initiation site (Figure 1a). Transfection of p53-null H1299, Saos-2, and HCT116 cell lines with the complete *GRAMD4*_FL-2064 promoter fragment demonstrated a substantial induction of luciferase activity by co-transfection of the p73 isoforms α (4- to 11-fold) and β (5- to 7-fold), whereas neither E2F1 or wild-type p53 itself had an enhancing effect (Figure 1a). Promoter upregulation in response to p73 occurred in a dose-dependent manner, reaching levels comparable to the p53/p73-driven *Bax* promoter (Figure 1b). In contrast to wild-type p73 isoforms, dominant-negative p73 mutants (ΔTAp73α and β) lacking the transactivation domain, were unable to stimulate *GRAMD4*, implicating that this domain is required for the stimulatory effect. Testing of 5′-deletion mutants showed the main loss of p73-induced promoter activity with the constructs Δ_G/F (-2064 to -1361), Δ_G/F/E (-1361 to -1295), and Δ_G/F/E/D/C (-1295 to -601) (Figure 1c). Further deletion of the 5′-end (Δ_G/F/E/D/C/B) gradually affected responsiveness to p73, which was completely abrogated in the Δ_G/F/E/D/C/B/A construct lacking all p53/p73 sites. This indicates that the region between -2064 and -601 (sites V–XIII) mediates maximal stimulation through p73. A clear activation of the *GRAMD4* promoter by endogenous p73 was detectable following cisplatin treatment (cDDP) (Figure 1d). Chromatin Immunoprecipitation (ChIP) assays revealed a direct interaction between p73 and the motifs located in the regions B, C, D, E, and F (Figure 2a). Consistent with the luciferase reporter data, only weak or no binding of p73 was detectable for fragment A and G, and in non-infected cells or cells immunoprecipitated with IgG. Strong increase in p73 binding to the *GRAMD4* promoter fragment F was also evident under stress conditions, which was not seen with fragment A serving as a negative control (Figure 2b).

p73 induces *GRAMD4* transactivation and apoptosis via its translocation from nucleus to mitochondria. Overexpression of p73α and p73β resulted in a significant increase of endogenous GRAMD4 transcript levels after 8 and 16 h, respectively, compared with uninfected cells, whereas p53 was essentially incapable to induce GRAMD4 mRNA expression (Figure 3a, upper panel). GRAMD4 upregulation by p73 was independent of *de novo* protein synthesis (Figure 3a, bottom left). E2F1 activation in H1299.ER-E2F1 cells caused a substantial increase of the p73 transcript level followed by GRAMD4 upregulation, whereas exposure of cells to infection with Ad.shp73 clearly abolished GRAMD4 expression (Figure 3a, bottom right). Implication of p73 in GRAMD4 protein regulation was further supported by the observation that its enhanced activity in p53-negative tumor cells leads to a strong induction of GRAMD4 staining (Figure 3b, top panel). Compared with H1299 cells transduced with control plasmid (I), confocal microscopy revealed that endogenous GRAMD4 protein increased in the cytoplasm of a significant amount of p73β overexpressing cells (upper panel, V, *green fluorescence*) showing a completely punctuate distribution (V-A) co-localizing with mitochondria (VI-A and VIII-A). Single other cells show a more clumped or diffuse green staining that also is Mito-Tracker positive, and most likely are apoptotic-condensed cells as samples were taken at 72 h after p73 upregulation. As shown in Figure 3b (bottom), GRAMD4 protein rose in the nucleus after 24 h followed by translocation to the cytosol, and significantly accumulated in the mitochondrial fraction at 72 h after infection (starting at 48 h) as compared with untreated cells (Figure 3b, bottom).

To investigate whether GRAMD4 functions as a mediator of p73-induced cell death, we examined its apoptotic function in GRAMD4 defective cells. Hoechst staining of H1299 cells co-infected with Ad.p73 and Ad.shGRAMD4[16] revealed a significant 50% reduction of p73-related cell killing when GRAMD4 was knocked down, whereas the viability of cells treated with control Ad.shGFP was unaffected (Figure 4). Identical results were obtained by flow cytometry (data not shown).

GRAMD4 influences Bax and Bcl-2 protein expression. p53 and p73 elicit apoptosis via the mitochondrial pathway using pro-apoptotic Bax as a direct transcriptional mediator,[17,18] and in case of p53 through transcriptional repression of antiapoptotic Bcl-2.[19] To elucidate the possible involvement of Bcl-2 family proteins in GRAMD4-induced apoptosis, we first monitored protein expression of Bax, Bak, and Bcl-2 in H1299, p53$-/-$ and p53$+/+$ HCT116 cells at different time points after infection with Ad.GRAMD4 *versus* Ad.GFP control. Figure 5 shows that the level of Bax protein was considerably induced in all cell lines independently of their p53 status by GRAMD4, whereas Bax was undetectable in uninfected cells. The increase of Bax occurred in a time-dependent manner with a maximum at 24 h in H1299 cells and 72 h in colon cancer cells. No changes were observed for the Bak protein, which

was already expressed at higher levels in non-infected controls. Furthermore, we found a significant reduction in Bcl-2 levels at 72 h after transduction with GRAMD4. This, in keeping with our data on apoptosis (Figure 4),[16] result in a decreased Bcl-2/Bax ratio as an index of the apoptosis signature. The mechanism of Bax and Bcl-2 alterations by



GRAMD4 is, however, unknown. As GRAMD4, consistent with the lack of a DNA binding and transactivation domain,[16] induced its p53-like effect on Bax and Bcl-2 expression without any significant change in the promoter activities (data not shown), our data suggest that GRAMD4 is functioning through indirect perhaps post translational mechanisms.

GRAMD4 induces Bax mitochondrial relocalization. Transcriptional-independent induction of apoptosis by p53 requires translocation of Bax from cytosol to mitochondria.[14] In non-apoptotic cells, Bax exists as inactive monomer in the cytosol, but inserts into the mitochondrial membrane upon death signals.[20,21] As shown by confocal microscopy, Bax moved from a diffuse peripheral spreading (Figure 6a, III,

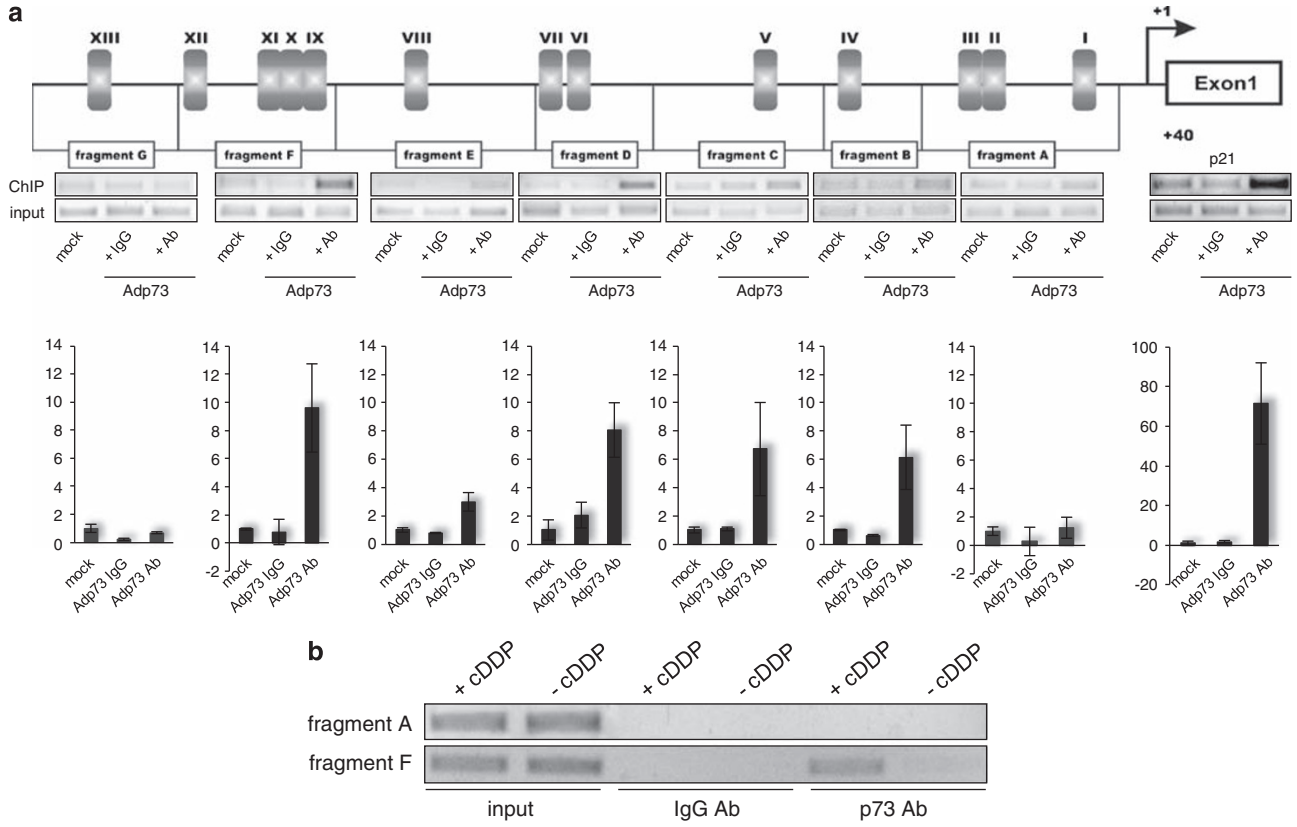


Figure 2 Binding of p73 to the *GRAMD4* promoter *in vivo*. Chromatin immunoprecipitation was performed on H1299 cells at 24 h after infection with Ad.p73 (**a**) or cDDP (30 μM) treatment, (**b**) using a control IgG antibody or antibody against p73. PCR primers were designed to amplify the individual fragments A-G of the full-length *GRAMD4* promoter. Primers for the p21 promoter were used as positive control. Input lane, 10% of total chromatin used in chromatin immunoprecipitation assay. Representative bands are indicated. Bar graphs show results from three independent experiments as relative software units cleared for background signals and normalized to input bands. Data represent the mean ± S.D.

Figure 1 Activation of the *GRAMD4* promoter depends on p73 but not p53. (**a**) Schematic diagram of putative binding sites for p53/p73 (light gray, Roman numerals I–XIII) and E2F1 (dark gray, Arabic numerals 1–12) within 2064 bp upstream of exon 1 of the human *GRAMD4* promoter. Positions are labeled relative to the first nucleotide of the published *GRAMD4* cDNA (accession number NM_015124), which is considered as nt $+1$. p53-negative H1299, Saos-2, and HCT116 cells were co-transfected with 0.5 μg of *GRAMD4* promoter–reporter construct (-2064 to $+1$) and 1 μg of indicated expression plasmids or empty vector. Cells were harvested for luciferase assay at 24 h after transfection. Promoter activities (RLU) are shown relative to pGL3_GRAMD4_FL set as 1.0 and normalized to total protein concentration in the cell extract. Error bars indicate 1 S.D. of the mean from three separate experiments. (**b**) Luciferase activity of pGL3_GRAMD4_FL (0.5 μg) was measured in H1299 cells co-transfected with increasing amounts (0.25 μg, 0.5 μg, 0.75 μg) of expression plasmid encoding p73α and p73β, or 0.5 μg of the transactivation-defective p73 isoforms ΔTAp73α and ΔTAp73β. A measure of 0.5 μg of the p73 responsive human *Bax* promoter construct was used as positive control. Expression of p73 after transfection is shown using actin as loading control (bottom). The histograms show means ± S.D. of three different experiments, each performed in duplicate. (**c**) The 5′-deletion mutants of the human *GRAMD4* promoter cloned upstream of the firefly *luciferase* gene are indicated by nucleotide positions: $-2062/+8$, $-1361/+8$, $-1295/+8$, $-601/+8$, $-430/+8$, $-77/+8$. Locations of putative p73 binding sites are light gray. The reporter constructs of 0.5 μg were co-transfected with 0.5 μg of p73 expression plasmid in H1299 cells. Luciferase activities are expressed as the fold activation relative to co-transfection of the entire *GRAMD4* promoter construct with empty vector. Error bars indicate 1 S.D. of the mean. Protein expression levels in p73-transfected cells are indicated (bottom panel). Actin was used for equal loading. (**d**) *GRAMD4* promoter activity in cDDP (30 μM) treated H1299 cells at 24 h after infection with Ad.shp73 or Ad.shGFP (left). Error bars, S.D. of three independent measurements. p73 knockdown after chemotherapy of Ad.shp73 infected H1299 cells compared with controls was analyzed by Immunoblot (right). At 48 h after infection, cells were lysed and extracts were probed with anti-p73 (ER-15) antibody. β-actin was used for equal loading

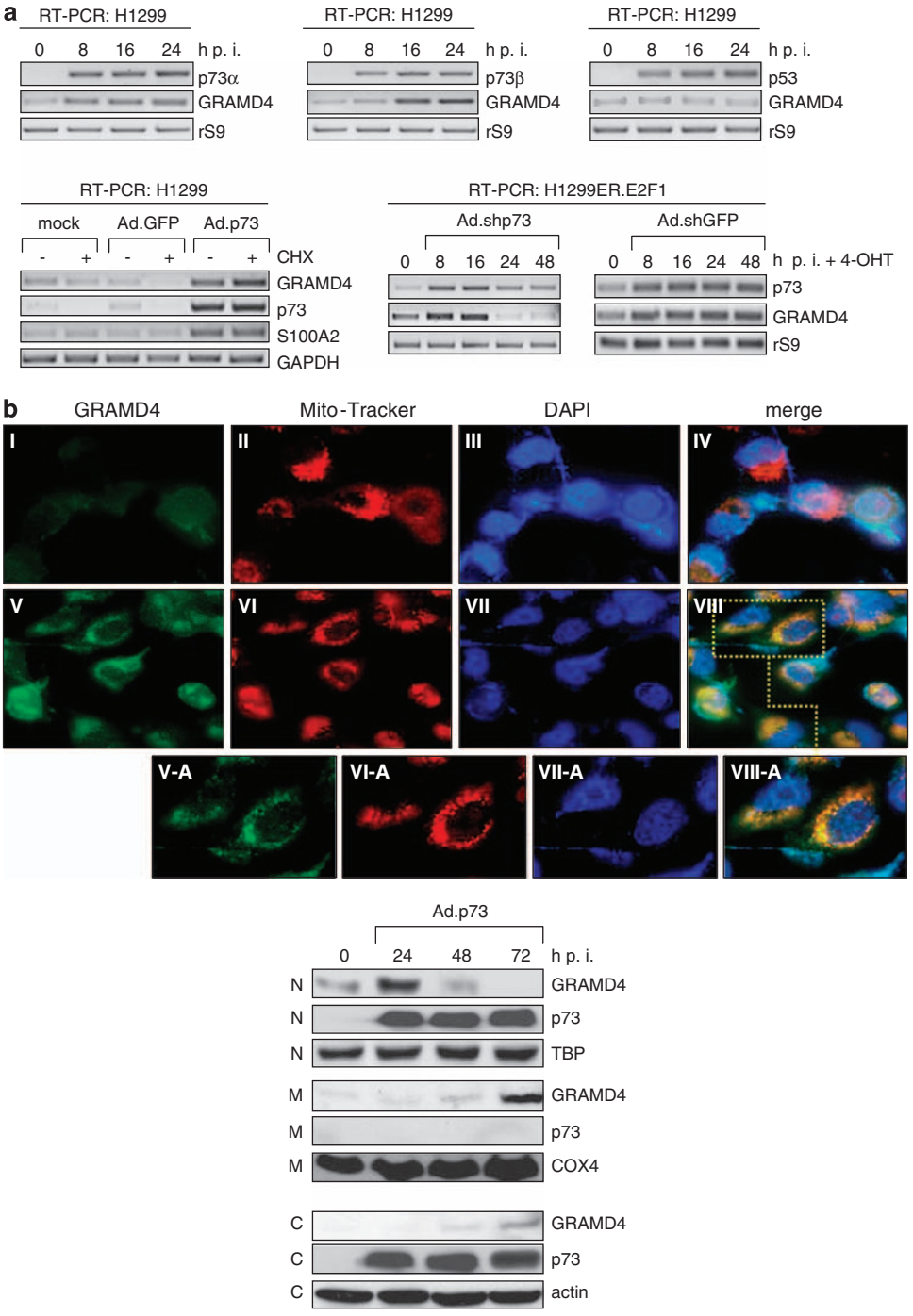


Figure 3 p73 induces *GRAMD4* transactivation and mitochondrial translocation. (**a**) Upper panels: semi-quantitative RT-PCR analysis on total RNA for endogenous GRAMD4 expression in H1299 cells at 8, 16 and 24 h following infection with Ad.p73α (left), Ad.p73β (middle), and Ad.p53 (right). Data were normalized to rS9 values. Bottom panel, left: GRAMD4 mRNA levels were assessed in parental H1299 cells infected with Ad.p73β and Ad.GFP control virus in the absence or presence of CHX by semi-quantitative RT-PCR and normalized to GAPDH expression. S100A2 is shown as a control for a direct *p73* target gene. p73 expression is as indicated. Bottom panel, right: upregulation of GRAMD4 by endogenous p73 in H1299.ER-E2F1 cells at indicated time points after E2F1 activation in response to 4-OHT addition. GRAMD4 and p73 transcript levels were detected in Ad.shp73 and Ad.shGFP infected compared with untreated cells using RT-PCR. Data were normalized to rS9 values. (**b**) Upper panel: endogenous GRAMD4 protein expression in H1299 cells at 72 h after transfection with p73β expression plasmid (V–VIII) or control pcDNA (I–IV) was detected using anti-GRAMD4 and Alexa Fluor 488 (anti-rabbit). Mito-Tracker and DAPI were used for visualization of mitochondrial and nuclear staining by laser scanning microscopy. GRAMD4, green; Mito-Tracker, red; DAPI, blue. Magnification $\times$ 40 (I–VIII), $\times$ 60 (V–VIII-A). Bottom panel: subcellular localization of endogenous GRAMD4 and ectopically expressed p73 protein shown by western blot at 24, 48, and 72 h after infection of H1299 cells with Ad.p73β using anti-GRAMD4 and anti-p73 antibody. N, nuclear fraction; C, cytosolic fraction; M, mitochondrial fraction. Mitochondrial COX4, cytosolic actin and nuclear TBP were used for equal loading

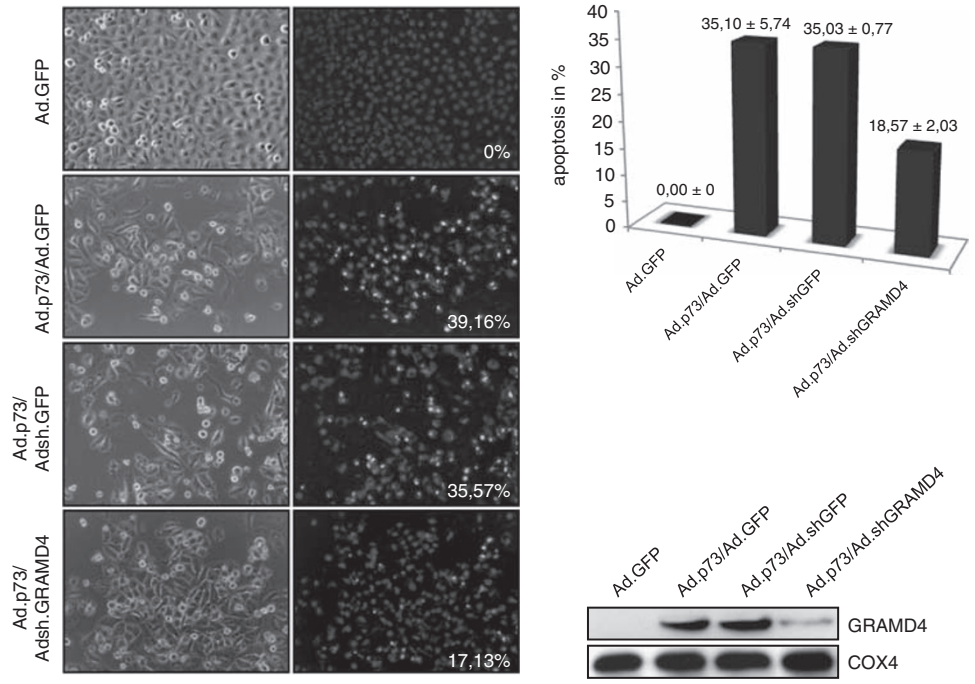


Figure 4 Induction of GRAMD4 by p73 accounts for p73-mediated apoptosis. H1299 cells were co-infected with Ad.p73 and Ad.shGRAMD4, Ad.GFP or Ad.shGFP. Apoptotic cells were measured at 48 h following infection by counting the cells after staining with Hoechst 33342 under fluorescence microscopy (magnification $\times$ 10). A representative experiment is shown (left). Bar graphs indicate the mean ± S.D. of the three performed (right, top). Endogenous GRAMD4 expression in infected cells was analyzed from the mitochondrial fraction by western blot (right, bottom) using COX4 as loading control

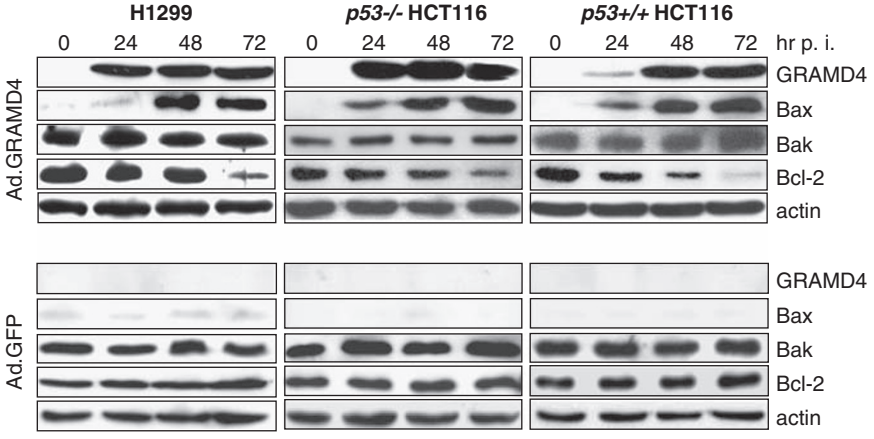


Figure 5 GRAMD4 expression leads to induction of pro-apoptotic Bax and downregulation of antiapoptotic Bcl-2 protein. Western blot for GRAMD4, Bax, Bak, and Bcl-2 expression in H1299, p53$-/-$ and p53$+/+$ HCT116 cell lines infected with adenoviral vector expressing GRAMD4 compared with Ad.GFP control virus. At the indicated times post infection, whole-cell lysates were prepared and immunoblotted with appropriate antibodies. Actin was used as loading control

green fluorescence) to a punctuate mitochondrial localization after GRAMD4 upregulation (IV and VI, *red-yellow fluorescence*). GRAMD4 induced a substantial increase in Bax protein levels in the purified mitochondrial fraction at 24 h after infection, which was accompanied by its decrease in the cytosol (Figure 6b). Mitochondrial relocation of Bax occurred at similar kinetics as GRAMD4 itself was translocated to the mitochondria.

Mitochondrial GRAMD4 physically interacts with Bcl-2, stimulates Bax oligomerization, and is highly efficient in inducing mitochondrial membrane permeabilization. Mitochondrial translocation of p53 results in physical interaction with antiapoptotic Bcl proteins to neutralize their inhibitory effects on pro-apoptotic family members, induces Bax oligomerization, and permeabilizes mitochondrial membranes leading to cytochrome *c* release and caspase

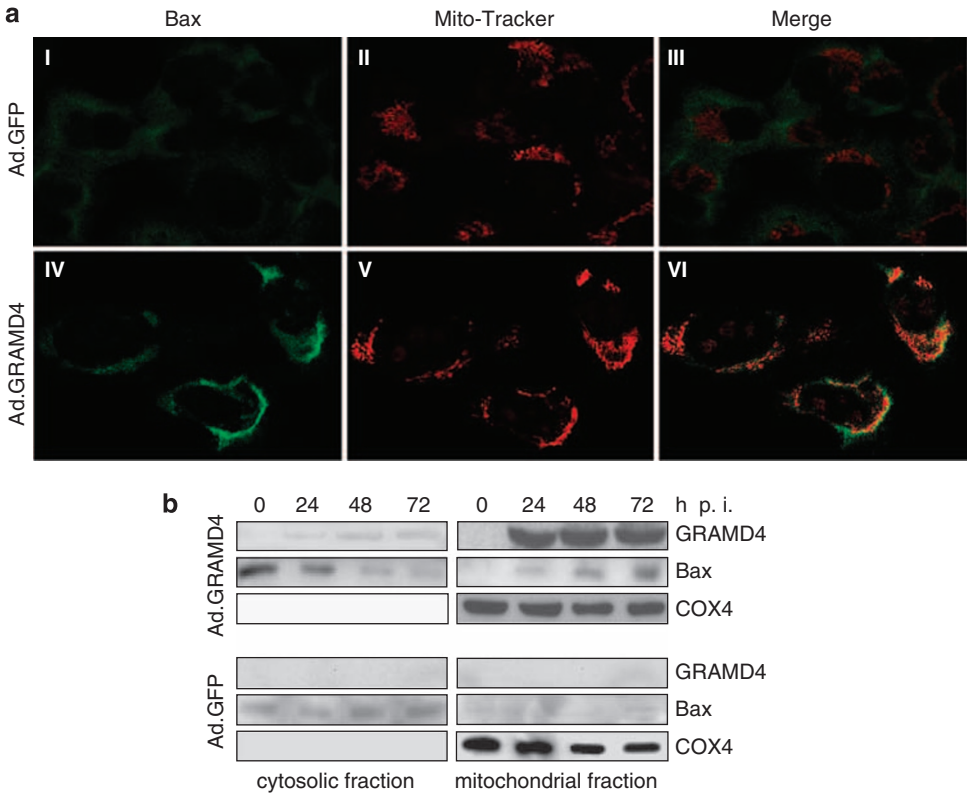


Figure 6 GRAMD4 induces Bax translocation from cytosol to mitochondria. (**a**) H1299 cells were infected with Ad.GRAMD4 for 48 h (IV–VI), and endogenous Bax protein levels were detected by the anti-Bax antibody clone 6A7, which recognizes the activated membrane-bound form of Bax, compared with mock infected controls (I–III). Mitochondria were stained with Mito-Tracker orange CMXRos. A representative experiment of three is shown. Bax immunofluorescence staining (green); Mitochondria labeling (red); merged images (red-yellow). (**b**) Evaluation of GRAMD4 and Bax protein levels in the cytosolic and mitochondrial fraction of H1299 cells at indicated time points upon adenoviral-mediated GRAMD4 overexpression compared with Ad.GFP infected cells. The mitochondrial COX4 protein was used as control

activation.[8,12,13] Considering that GRAMD4 translocates to the mitochondria upon p73 overexpression (Figure 3b), we then examined whether GRAMD4 has the capacity to engage the apoptotic program directly from the mitochondria. Indeed, specific GRAMD4/Bcl-2 complexes were readily detectable from transfected lysates by immunoprecipitating with anti-Bcl-2 antibody and blotting with anti-GRAMD4 (Figure 7a, left, lane 4). Although weaker, the specific endogenous complex of GRAMD4–Bcl-2 was also seen in cells transfected with GRAMD4 plasmid alone (lane 3), because of the fact that H1299 cells express basal Bcl-2 levels. Mapping by SMART database searches to predict putative interaction sites between both proteins revealed a Bcl-like domain for GRAMD4 encompassing amino acids 92–176. As demonstrated in lanes 4 and 5 of Figure 7a (right), deletion of this Bcl-2 homology (BH) domain clearly disrupts the interaction of GRAMD4 with endogenous (lane 2) and ectopically expressed Bcl-2 protein (lane 3).

Bax can be induced to form homodimers and to insert into the outer mitochondrial membrane[22] as the biochemical hallmark of Bax lipid pore activation and mitochondrial membrane permeabilization.[23] Given that GRAMD4 directly interacts with Bax inhibitory Bcl-2 protein, we assayed its ability to induce Bax oligomerization using the chemical cross-linker BMH (1,6-bismale-imidohexane) and Bax antibody 6A7 recognizing the activated membrane-bound form. Bax activator p53 served as a positive control.[23,24] GRAMD4 protein was able to induce Bax-containing oligomers in mitochondria from H1299 cells similar to wild-type p53, but not the GFP control, indicating the specificity of the oligomeric bands (Figure 7b, left). Full Bax oligomerization occurred at 72 h after GRAMD4 overexpression (Figure 7b, right). This suggests that GRAMD4 is able to activate Bax by releasing it from a pre-inhibiting complex with Bcl-2.

To gain insight about the functional consequences of the GRAMD4-Bcl-2 interaction and Bax activation at the mitochondria, we undertook permeabilization studies. Overexpressed GRAMD4 was detected after 24 h in the mitochondrial fraction compared with uninfected cells, whereas protein levels were low in the cytoplasm. Compared with cells infected with the Ad.GFP control virus, GRAMD4 induced the release of cytochrome *c* and Smac/Diablo, two intermembranous soluble apoptotic activators, from mitochondria into the cytosolic fraction (Figure 7c). Strikingly, the appearance of both pro-apoptotic factors in the cytoplasm was time-dependent reaching a maximum at 24 h, indicating that the kinetics of GRAMD4 expression correlate with their release. This function of GRAMD4 protein is comparable to p53[11] and in line with caspase activation previously found in H1299 cells following GRAMD4 overexpression.[16] The loss of mitochondrial membrane potential $\Delta\psi$m was measured by tetramethyl rhodamine ethyl ester (TMRE) fluorescence. After 24 h, loss of $\Delta\psi$m was

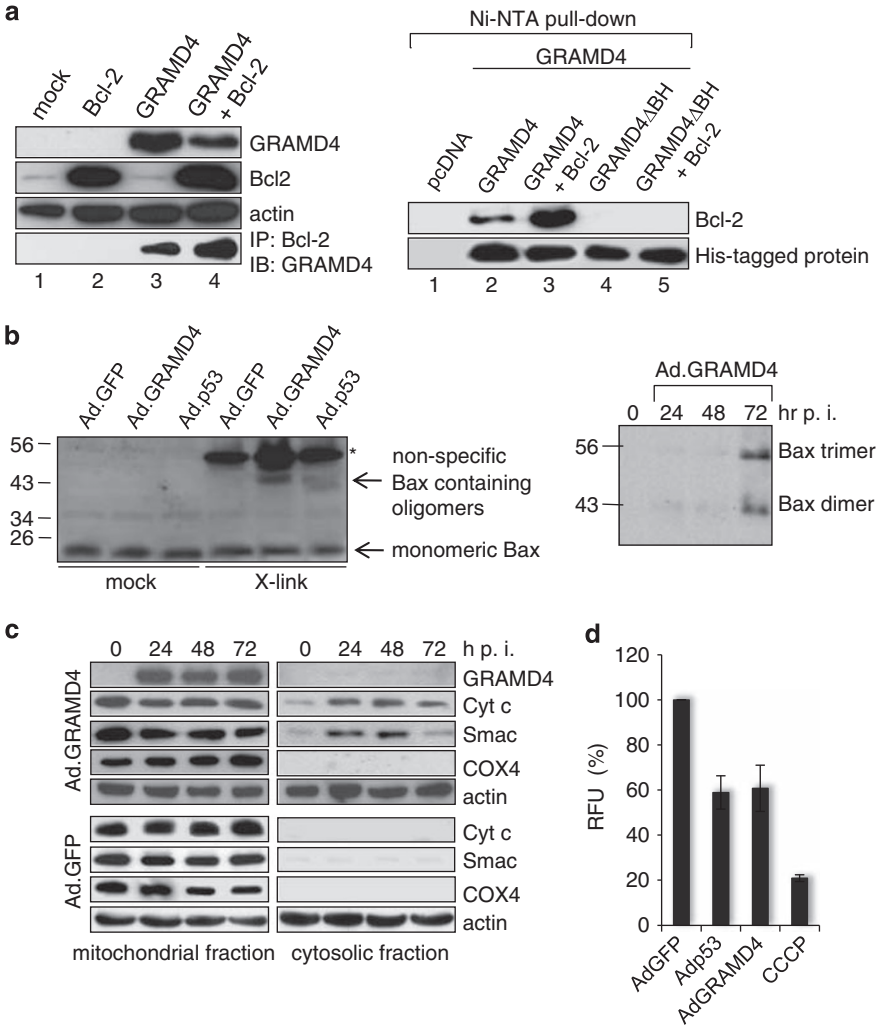


Figure 7 Mitochondrial GRAMD4 directly interacts with Bcl-2 and induces Bax oligomerization and mitochondrial membrane permeabilization. (**a**) GRAMD4 and Bcl-2 expression in untransfected (Mock) H1299, and cells transfected with Bcl-2 and GRAMD4 plasmids were determined by western blot. The total amount of transfected plasmids was kept constant by addition of an appropriate empty vector. The blots were reprobed for actin as loading control. For immunoprecipitation, protein extracts were precipitated with Bcl-2 antibody, and co-precipitated GRAMD4 protein was detected by immunoblotting using anti-GRAMD4 antibody (left). Right: whole-cell lysates from H1299 cells transfected with pcDNA (lane 1), GRAMD4 (lane 2), and GRAMD4Δ mutant encoding plasmid lacking the Bcl-like domain (lane 4) or co-transfected with Bcl-2 expression plasmid (lanes 3 and 5) were subjected to Ni-NTA pull-down experiments. The Ni-NTA pull-down products (lanes 2–5) were resolved by SDS-PAGE and probed with antibodies against Bcl-2 and the PolyHis-tag as indicated. Note that the His-tagged proteins (GRAMD4 and GRAMD4ΔBH) were expressed at similar levels in each set of cells and that their sizes are different, but were aligned to save space. (**b**) GRAMD4 is a potent inducer of Bax oligomerization. Mitochondria from H1299 cells infected for 48 h with GRAMD4 or p53 expressing Ad vector were subjected to cross-linking with BMH or mock treated (DMSO only) and immunoblotted for Bax using anti-Bax N-20 (left). Full Bax oligomerization was detected by Bax 6A7 antibody at 72 h after Ad.GRAMD4 infection (right). (**c**) Subcellular localization of mitochondrial proteins after GRAMD4 overexpression in H1299 cells at indicated times post adenoviral infection compared with control virus infected cells. The released apoptogenic factors cyt *c* und Smac were detected in the cytosolic fraction by immunoblotting, and the retained amount of the indicated factors was detected in the mitochondrial fractions. The level of mitochondrial GRAMD4 and COX4 used as loading control is shown. (**d**) Mitochondrial $\Delta\psi$m was measured in p53-null H1299 cells infected with Ad.GRAMD4, Ad.p53 or Ad.GFP by TMRE fluorescence. H1299 cells exposed to CCCP (50 μM) depolarizing agent were used as control. *RFU*, relative fluorescence unit

significantly increased after transduction of GRAMD4. As shown in Figure 7d, GRAMD4 and p53 have comparable efficiencies in their ability to permeabilize mitochondrial membranes in contrast to cells expressing GFP. Taken together, these data suggest that mitochondrial GRAMD4 is capable of severely disrupting the integrity of mitochondrial membranes subsequent to initiation of apoptosis.

Activation of GRAMD4 transcription in response to DNA damage and antitumoral efficacy *in vivo*. We next investigated endogenous GRAMD4 expression upon various stress signals and analyzed this portion of p73 death signaling for its antitumoral activity *in vivo*. Figure 8a (left panel) shows that the level of GRAMD4 mRNA is significantly upregulated in response to cDDP and, to a lower extent, 5-fluorouracil (5-FU) in HCT116 cells independently of p53. Interestingly, the kinetics of GRAMD4 transcriptional activation in *p53−/−* HCT116 cells after both treatments are identical with p73, implicating that the p73 response to chemotherapy[25] does at least in part rely on p73-mediated stimulation of GRAMD4. This is also supported by p73 knockdown data shown in Figure 8a (right panel),

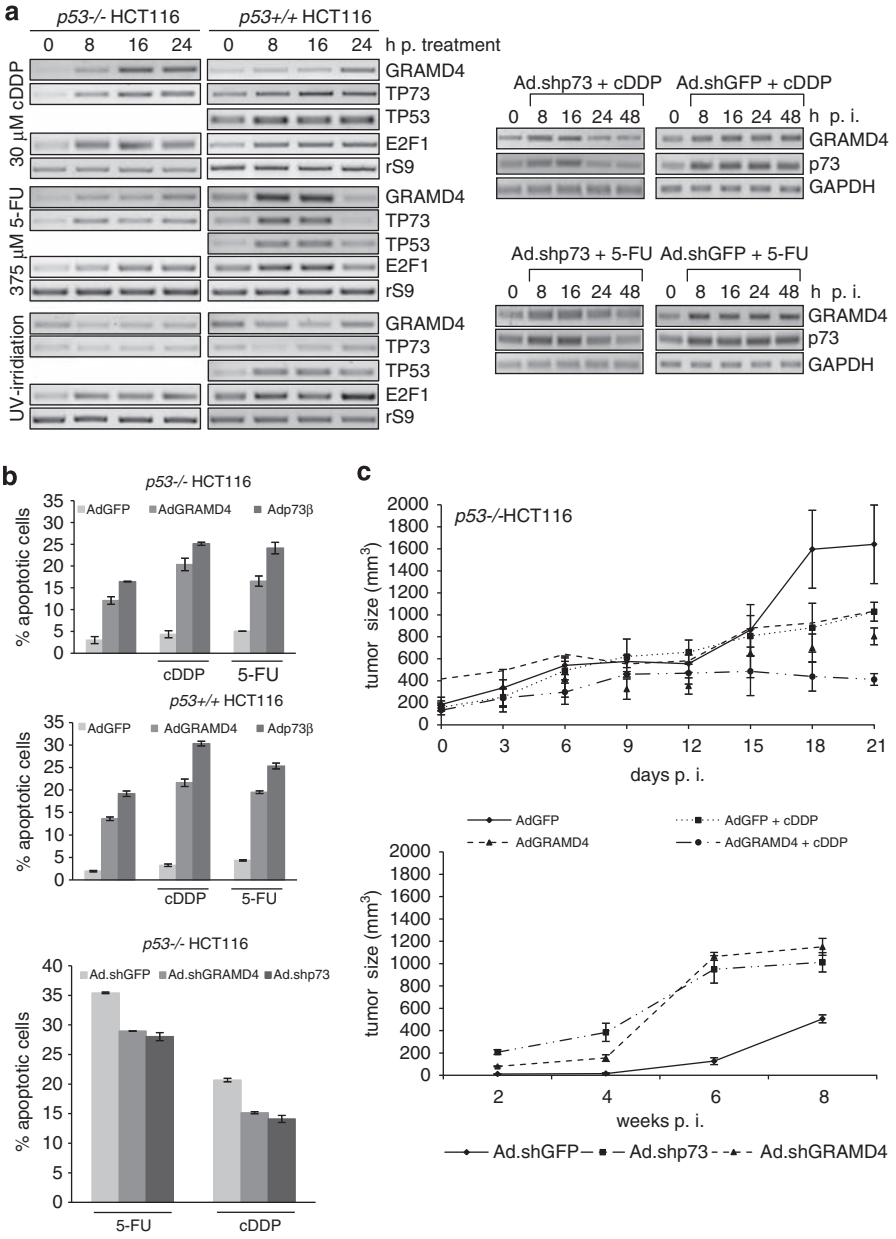


Figure 8 *GRAMD4* transcription and apoptosis is activated in response to drug treatment in a manner dependent on p73 and potentiates tumor growth inhibition. (**a**) p53-negative and wild-type HCT116 cells were treated with cDDP (30 μM), 5-FU (375 μM) or UV irradiation. Semi-quantitative RT-PCR for endogenous *GRAMD4*, *TP73*, *TP53*, and *E2F1* expression at indicated times after drug exposure was carried out under linear amplification conditions (left). Ribosomal S9 (rS9) is shown as control. *GRAMD4* and *TP73* transcript levels in p53−/− HCT116 cells after infection with Ad.shp73 or Ad.shGFP control virus plus chemotherapy (30 μM cDDP, 375 μM 5-FU) are indicated in the right panel using GAPDH as control. (**b**) To determine the mean percentage of apoptotic cells following treatment with Ad.GRAMD4, Ad.p73, and Ad.GFP alone or in combination with cDDP or 5-FU, the percentage of cells with a sub-G1 DNA content was analyzed from three experiments by flow cytometry (upper and middle panel). The mean apoptosis in response to cDDP and 5-FU treatment after GRAMD4 or p73 knockdown is shown at the bottom. (**c**) Tumors established in BALB/c nude mice by subcutaneous injection of 5 × 10e6 HCT116 cells were directly injected with Ad.GRAMD4 or Ad.GFP alone and in combination with cDDP and monitored for tumor volume (top). Ad.GRAMD4 + cDDP *versus* Ad.GRAMD4, Ad.GFP, and Ad.GFP + cDDP at day 21 ($P<0.05$); Ad.GRAMD4 *versus* Ad.GFP and Ad.GFP + cDDP at day 21 ($P<0.05$, paired two-sided *t*-test). Each group, $n=4$. Effect of GRAMD4 and p73 inhibition on tumor growth of p53-negative colon cancer cells infected with Ad.shGRAMD4 or Ad.shp73 in mice exposed to cDDP (bottom). Ad.shGFP infected cells were used as control. Student's *t*-test (two-sided) indicates statistically significant differences between groups 8-weeks post injection (shGRAMD4 *versus* shGFP, $P\leq 0.037$); shp73 *versus* shGFP, $P\leq 0.015$). Three mice per group were studied

which indicate that GRAMD4 expression in response to chemotherapy is modulated through endogenous p73. In contrast, GRAMD4 was like p73 not induced by UV-irradiation (Figure 8a, left). To verify whether GRAMD4 is able to sensitize tumor cells to genotoxic stress, *p53*−/− and *p53*+/+ HCT116 were infected with Ad.GRAMD4 alone and in combination with drugs. As expected, overexpression of GRAMD4 in combination with cDDP and 5-FU resulted in a marked increase of sub-G1 cells at 48 h after infection, at levels comparable to p53 null cells infected

with p73, or p53 positive cells (Figure 8b). Contrariwise, cDDP- and 5-FU-mediated death measured after 72 h was partially prevented by GRAMD4 knockdown similar to Ad.shp73-infected cells, again underscoring a p73-GRAMD4 axis in genotoxic cell death. Finally, in solid tumor xenografts, we used intratumoral delivery of GRAMD4 either alone or together with cDDP to monitor the impact of mitochondrial apoptosis on tumor growth (Figure 8c, upper panel). Reproducibly, a substantial inhibition of tumor growth was found after GRAMD4 overexpression. The efficacy of GRAMD4 was more pronounced over time when combined with cDDP, leading to a prolonged growth retardation. In contrast, both GFP either alone or with drug therapy was ineffective in inhibiting tumor growth. Moreover, when these tumor cells were infected with Ad.shGRAMD4 and subsequently injected into nude mice treated with cDDP, tumor volumes of established tumors increased faster over time compared with cells infected with Ad.shGFP, indicating that the apoptotic response of these cells to chemotherapy is to some extent abrogated if endogenous GRAMD4 is depleted (Figure 8c, bottom panel). A similar effect on tumor growth was observed in cells when p73 was knocked down.

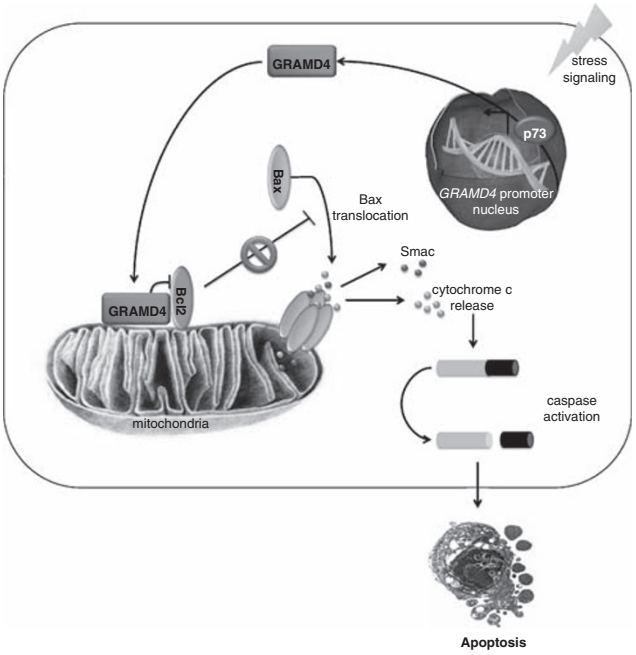


Figure 9 Model of the mitochondrial role of GRAMD4 during p73-mediated apoptosis

Discussion

Although compelling evidence has accumulated, indicating that p53 can directly activate components of the apoptotic machinery and that this involves translocation of p53 to the mitochondria,[8,10,13] hints for a transcription-independent role of p73 in apoptosis induction has been controversial. One report has described the cleavage of p73 by caspases in response to DNA damaging agents and death-receptor activation, along with the localization of full-length p73 and some of its cleaved products to the mitochondria.[26] A transcription-deficient p73 mutant was found to enhance TRAIL-induced cell death, and the addition of recombinant p73 protein induced cytochrome *c* release from isolated mitochondria, supporting that non-nuclear p73 may have functions in apoptosis progression. In contrast to this, an earlier study showed that, in cells undergoing p73-dependent apoptosis, p73 does not itself localize to the mitochondria, and that *BAX* transactivation by p73 is not crucial for Bax subcellular redistribution and the induction of death. Instead, Melino *et al.*[2] indicated that nuclear p73 elicits apoptosis via the mitochondrial pathway through its ability to directly transactivate *PUMA*, which then causes Bax mitochondrial translocation.

We demonstrate in this study that p73 mediates its apoptotic function, at least in part, through direct transactivation of the *GRAMD4* gene via binding sites for TAp73 that are not responsive to p53. p73 specifically recognizes multiple *cis*-regulatory target elements spanning from −2064 to −601 within the promoter region and induces the expression of endogenous GRAMD4 at both mRNA and protein levels. Although it is also in this case unclear whether particular structural features of the response elements, sequence conformation, or as recently shown, the energy of DNA bending contributes to promoter preference between p73 and p53, *GRAMD4* belongs to the p73-specific target genes.[27–31] As an important molecular component of apoptosis, p73 functions in the transduction of drug-induced DNA damage to the apoptotic machinery of the cell.[25] We also found that *GRAMD4* transcription in p53-negative and wild-type p53 cancer cells is activated by genotoxic agents, but not UV irradiation, in a p73-like manner. This suggests that a substantial proportion of the p73 response to chemotherapy is mediated through GRAMD4 as well.

In accordance with the finding that p73 is localized in the nucleus and remains with nuclear protein during the execution of cell death,[2] GRAMD4 protein instead is induced to localize from the nucleus to the mitochondrial surface of cultured p53-deficient tumor cells upon p73 overexpression, in which it promotes mitochondrial translocation and oligomerization of endogenous Bax. Moreover, we demonstrated that p73-responsive GRAMD4 can directly induce permeabilization of the outer mitochondrial membrane by forming inhibitory complexes with protective Bcl-2 protein, resulting in the release of cytochrome *c* and Smac/Diablo as part of the pro-apoptotic function of p73 at the mitochondria. Thus, our data reveal an indirect extranuclear death function of p73 that is accomplished by the stimulation of GRAMD4 protein, which exhibits significant functional similarity with the mitochondrial apoptotic activity of p53 (Figure 9). On translocation to the mitochondria, p53 seems to function analogous to BH3-only proteins. Specifically, p53, Noxa, PUMA, Bim, and Bad all bind antiapoptotic Bcl-2 family proteins and neutralize their inhibitory effects on pro-apoptotic multi-domain BH123 proteins Bax and Bak allowing them to oligomerize and form lipid pores on the mitochondrial outer membrane, and induce mitochondrial outer membrane permeabilization with subsequent cytochrome *c* release and caspase activation. Although translocated BH3-only proteins interact with Bcl-2/XL via their BH3 domain,[32] data from different laboratories indicate that p53 uses conserved residues within the DNA binding

domain.[11,33] Because GRAMD4 lacks the BH3 domain (our data) and has apart from two transmembrane domains of membrane-associated proteins no DNA binding domain,[16] we searched for a putative protein interaction site involved in complex formation. Analog to other pro-apoptotic proteins such as Siva-1 and p54AIP1, which like p53 lack a BH3 domain but bind and inactivate Bcl-2, GRAMD4 protein binds Bcl-2 apparently through its Bcl-like domain encompassing amino acids 92–176. This is supported by the finding that a mutant of GRAMD4 harboring a deletion of the Bcl-like region failed to interact with Bcl-2. It is likely that the interaction between GRAMD4 and apoptosis inhibitory proteins such as Bcl-2 enables GRAMD4, similar to p53, to liberate pro-apoptotic Bax from pre-existing complexes and/or activate monomeric Bax in the cytosol, thereby mediating its translocation to mitochondria.

In addition to the direct apoptogenic role of GRAMD4 at the mitochondria, present data provide evidence for yet another conformity between the GRAMD4 and p53 tumor suppressor proteins. It has been known that p53 as a transcriptional regulator directly activates Bax expression,[4] whereas the antiapoptotic *Bcl-2* gene is downregulated either directly or indirectly via a p53-dependent negative response element.[34] Our finding of the ability of ectopically expressed GRAMD4 to upregulate Bax and downregulate Bcl-2 protein levels *in vitro*, resulting in a decreased Bcl-2/Bax ratio parallels that seen with p53. However, as GRAMD4 because of structural features does not display a transcription-dependent apoptotic function in response to p73, any regulation of these proteins is likely indirect. Such indirect activity outside the mitochondria is also fortified by increased GRAMD4 protein levels upon p73 overexpression in the nuclei of p53-deficient tumor cells. As described for other proteins involved in the regulation of cell death in the cytoplasm, such as cIAP1 or the Bcl-2 family member Mcl-1, which exhibit a growth inhibitory function when localized in the nuclear compartment by interacting with cell cycle regulatory proteins,[35,36] it can be speculated that GRAMD4 without DNA binding domain can possibly serve other as yet unknown nuclear functions in addition to its activity at the mitochondria upon p73 stimulation.

In summary, these data provide a mechanistic basis for the regulation of cell death by p73 at the mitochondria. Unlike p53, the mitochondrial apoptogenic activity of p73 seems to be indirect and transcription dependent. We propose that p73 exerts a pro-apoptotic role at the mitochondria through the transcriptional activation and mitochondrial translocation of GRAMD4, which facilitates Bax subcellular relocalization and triggers mechanisms at the mitochondria that ultimately lead to the induction of apoptosis, rather than being extranuclear active itself. As the mitochondrial GRAMD4 activity might contribute to p73-driven death of cancer cells *in vivo* in conjunction with DNA damaging agents, it opens new possibilities for the treatment of cancers with altered death pathways.

Materials and Methods

Cell culture. HCT116 (p53−/−) and HCT116 (p53+/+) were kindly provided by B. Vogelstein (Johns Hopkins Medical Institutions, Baltimore, MD USA). H1299 and Saos-2 cell lines were obtained from ATCC. Cells were generally cultured in Dulbecco's modified Eagle's medium (DMEM; PAA, Pasching, Austria) supplemented with 10% fetal calf serum (FCS, Biochrom; Berlin, Germany), 1% penicillin/streptomycin (PAA, Pasching, Austria) and 0.5% amphotericin (PAA) in a humidified atmosphere of 5% CO_2 at 37°C. Media for maintenance of H1299.ER-E2F1 cells, constitutively expressing E2F1 fused to the binding domain of the murine ER receptor, contained puromycin (Sigma, Steinheim, Germany) at a concentration of 1 μg/ml. E2F1 activity in H1299.ER-E2F1 cells was induced by (4-OHT) at final concentration of 1 μM. For drug treatment, cells were exposed to cDDP and 5-FU at concentrations of 30 and 375 μM, respectively.

Adenoviral vectors. The recombinant adenoviral vectors expressing human p73α, p73β, GRAMD4, GFP, and shRNA against GFP, GRAMD4 or p73 have been described previously.[16,37] Ad vectors were propagated in 293 cells, purified by CsCl buoyant density centrifugation, and titrated by standard plaque assay. Adenoviral infections were carried out at multiplicities of infection (MOI) that allow 100% transduction of each cell line (MOI = 10 for H1299 and H1299.ER-E2F1, 40 for HCT116 cells).

Chromatin Immunoprecipitation (ChIP). ChIP assay was performed as described earlier.[38] Briefly, H1299 cells were infected with Ad.p73β or treated with DNA damaging agent cisplatin (30 μM). Proteins bound to DNA were cross-linked using formaldehyde at a final concentration of 1.42% for 15 min at room temperature. The reaction was stopped by adding glycine to a final concentration of 125 mM. Following sonication, protein–DNA complexes were immunoprecipitated using primary antibody for anti-p73 (BD Bioscience, Heidelberg, Germany) or appropriate control IgG and placed on a rotating platform at 4°C overnight. Immunoprecipitated chromatin was eluted from the beads in 10% Chelex100 and boiled for 10 min. The resulting DNAs were subjected to semi-quantitative PCRs using the following primer pairs: region +8 to −370: 5′-TGAGAACCCTGGCTTCC TTCT-3′ (GRAMD4_P_fragA-sense) and 5′-ACATGACGCTCGGGTTCCCT-3′ (GRAMD4_P_fragA-antisense); region −350 to −558: 5′-AGAATCAACCGAGG CATCCCTA-3′ (GRAMD4_P_fragB-sense) and 5′-AGAAGGAAGCCAGGGTTCT CA-3′ (GRAMD4_P_fragB-antisense); region −547 to −835: 5′-GTGTGACCAGGTT TCTGTGTTGA-3′ (GRAMD4_P_fragC-sense) and 5′-TAGGGATGCCTCGGTTGA TTCT-3′ (GRAMD4_P_fragC-antisense); region −813 to −1004: 5′-TCCTGTGG GCCACTGATCCT-3′ (GRAMD4_P_fragD-sense) and 5′-TCAACACAGAAACCTG GTCACAC-3′ (GRAMD4_P_fragD-antisense); region −985 to −1369: 5′-CTGACC TTGCTCTCAGTGCT-3′ (GRAMD4_P_fragE-sense) and 5′-AGGATCAGTGGCCC ACAGGA-3′ (GRAMD4_P_fragE-antisense); region −1350 to −1648: 5′-GCCTCC CAAAGTGCTGGGAATTA-3′ (GRAMD4_P_fragF-sense) and 5′-AGCACTGAGAG CAAGGTCAG-3′ (GRAMD4_P_fragF-antisense); region −1627 to −1918: 5′-CCT GTGTCCTGTGGGATTTT-3′ (GRAMD4_P_fragG-sense) and 5′-TAATTCCAGCA CTTTGGGAGGC-3′ (GRAMD4_P_fragG-antisense).

Cloning of *GRAMD4* promoter constructs and GRAMD4ΔBH/V5/His. GRAMD4 promoter fragments were generated by PCR (Elongase Enzyme Mix, Invitrogen, Darmstadt, Germany) using the BAC clone RP-3-439F8 (BACPAC Resources; Oakland, CA, USA) and sequence specific primers containing artificial restriction sites (*Kpn*I and *Bgl*II). The oligonucleotides are based on the genomic DNA sequence of the human *GRAMD4* gene localized on human chromosome 22q13.31. Fragments obtained from PCR were subcloned into the pGEM-T vector (pGEM-T Vector Systems; Promega, Madison, WI, USA) and inserted into the pGL3-basic luciferase reporter vector (Promega) using *Kpn*I and *Bgl*II restriction sites. All promoter constructs terminate at nt +8 of exon 1 where +1 represents the first nt of exon 1. The individual primers used for amplification of the full-length promoter fragment from −2064 to +8 of the *GRAMD4* gene (GRAMD4_FL -2064) and five 5′ deletion mutants (DEL-1361, -1295, -601, -430 and -77) are: full length promoter: 5′-GCCGGGGTACCCCTATATGGGAAGCTTTTCC-3′ (fw), DEL-1361: 5′-GCCGGGGTACCGCTCTCAGTGCTTCCTTG-3′ (fw), DEL-1295: 5′-GCCGG GGTACCGATCACTCCCCGCCCCAT-3′ (fw), DEL-601: 5′-GCCGGGGTACCCGG GACACAAGGCCAGGTCC-3′ (fw), DEL-430: 5′-GCCGGGGTACCTTCCCAAT GCCAGAGCAGC-3′ (fw), DEL-77: 5′-GCCGGGGTACCGTGTGTGGGAGCT GGCTCGT-3′ (fw), and 5′-AAGTCTGGAAGATCTACATGACGCTCGGGTTCC-3′ (rev). GRAMD4Δ/V5/His containing a 255-bp deletion of the Bcl-like domain (amino acids 92–176) was generated by Two-Step-PCR using pcDNA3.1-GRAMD4 and sequence specific oligonucleotides based on the cDNA sequence of GRAMD4 (NM_015124). The individual primers are: P1-s: 5′-GTCATGCTAAGGAGGTTGGA-3′; P2-as: 5′-GCTGTCCCCGCCAGACG-3′ (without stop-codon); P3-s: 5′-GC CTTATTGGAAAAATACGTGGAG GACTTC-3′; and P4-as: 5′-GAAGTCCTCC ACGTATTTTTCCAATAAGGC-3′. PCR product obtained was cloned into

pcDNA3.1 and confirmed by sequencing. Full-length GRAMD4 (1737 bp) expresses a protein of 66 kDa, whereas the 255 bp-deletion mutant expresses a protein of 56 kDa.

***Luciferase* reporter gene assay.** Cells were co-transfected with 0.5 μg *luciferase* reporter gene constructs containing human *GRAMD4* promoter sequences (pGL3_GRAMD4_FL-2064, pGL3_GRAMD4-1361, -1295, -601, -430 and -77) and indicated amounts of the plasmids expressing different p73 isoforms (p73α, p73β, ΔTap73α, and ΔTAp73β), wild-type p53, and E2F1. The total amount of transfected plasmids was kept constant (1.5 μg for a 6-cm plate) by addition of an appropriate empty vector. Transfections were done in 6-cm plates (5×10^5 cells per plate) using Effectene (Qiagen, Hilden, Germany) according to manufacturer's instructions. Luciferase activity was measured at 24 h after transfection using the luciferase reporter assay system (Promega) and normalized to total protein concentration in the cell extract.

Semi-quantitative RT-PCR. RT-PCR was performed on total RNA prepared by Nucleospin RNAII (Macherey–Nagel, Düren, Germany). A measure of 1 μg RNA was reverse transcribed using Omniscript RT (Qiagen) and Oligo-dT. PCR amplification was performed as previously described[38] using the following specific primers: GRAMD4 5′-TCTTACAGGAGGAGCTCCGGA-3′ (fw) and 5′-TTAGTGAC CGGCTTGGCA-3′ (rev); p21 5′-TTCTTATGCCAGAGGCTGG-3′ (fw) and 5′-TGTG GACCTGTCACTGTCTTG-3′ (rev); p73 5′-GACGGAATTCACCACCATCCT-3′ (fw) and 5′-CCAGGCTCTCTTTCAGCTTCA-3′ (rev); p53 5′-CTCAGACTGACATTCTC CAC-3′ (fw) and 5′-ACTCTGTGAGGTAGGTGCAA-3′ (rev); E2F1 5′-GGTGAGGT CCCCAAAGTCAC-3′ (fw) and 5′-GCCACCATAGTGTCACCACC-3′ (rev); rS9 5′-GATGAGAAGGACCCACGGCGT-3′ (fw) and 5′-GAGACAATCCAGCAGCCCA GG-3′ (rev); and GAPDH 5′- CACCACCCTGTTGCTGTA-3′ (fw) and 5′- CACAGTC CATGCCATCAC-3′ (rev).

Western blotting, co-immunoprecipitation studies and analysis of Bax oligomerization. Cells were lysed in RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and total protein concentration was quantified by Bradford assay. Equal amounts of cellular protein were separated by (SDS-PAGE) sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany), and probed with appropriate primary antibodies: custom made anti-GRAMD4 (Eurogentec, Seraing, Belgium) anti-Bax (sc-493; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bak (sc-1035; Santa Cruz Biotechnology), anti-Bcl-2 (sc-492; Santa Cruz Biotechnology), and anti-actin (clone AC-15; Sigma, Munich, Germany). The corresponding peroxidase-labeled secondary antibody was detected using ECL western blot reagents (Amersham Biosciences). For subcellular fractionation, Apo Alert Cell Fractionation Kit (Clontech, Heidelberg, Germany) was used according to the manufacturer's recommendations. Briefly, cells were harvested, washed twice with PBS, suspended in cell fractionation buffer and homogenized. Cytosolic and mitochondrial extracts were fractionated by differential centrifugation. Proteins samples (50 μg) from both fractions were separated on 12% SDS-PAGE and western immunoblotted with anti-COX4 antibody (Cell Signaling Technology, Danvers, USA), anti-cytochrome *c* (ApoAlert Kit), anti-TATA box binding protein (TBP) (Abcam, Cambridge, UK), anti-Smac/Diablo (Cell Signaling Technology, Danvers, MA, USA), anti-Bax clone 6A7 (BD Pharmingen, Heidelberg, Germany) or N-20 (Santa Cruz Biotechnology), and anti-GRAMD4 antibodies. The preparation of cytoplasmic and nuclear extracts was performed using the Nuclear Extract kit (Active Motif, Rixensart, Belgium) according to the instructions. Supernatants were harvested as cytoplasmic fractions. Pellets were resuspended in 50 μl of complete lysis Buffer and centrifuged at $14\,000 \times g$ for 10 min at 4°C. Supernatants were saved as the nuclear fractions.

For immunoprecipitation experiments, 500 μg of protein lysate from H1299 cells transfected with pcDNA3.1–GRAMD4[15] and pRc/CMVBcl2 alone or in combination were precipitated with 2 μg of rabbit polyclonal anti-Bcl-2 antibody (N-19, Santa Cruz Biotechnology). Protein A/G-Sepharose beads (20 μl, 50% slurry; Amersham) were added and the immune complexes were pulled down over 1 h at 4°C under rotation. Beads were washed extensively with lysis buffer to remove unbound proteins and lysed by boiling in the presence of Laemmli buffer. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose (Amersham Biosciences), and immunoblotted using GRAMD4-antibody. Nickel-nitrilotriacetic acid (Ni-NTA) affinity pull-down experiments were performed by mixing whole-cell lysates with 50 μl Ni-NTA beads (Qiagen). Samples were incubated for 3 h at 4°C, washed five times, and analyzed for His-tagged protein interactions by performing western blot.

For detection of Bax oligomers, mitochondria from H1299 cells were subjected to the crosslinker BMH (1 mM in dimethylsulfoxide (DMSO) for 1 h) or mock treated (DMSO only). The mitochondria were pelleted, dissolved in SDS buffer and loaded on SDS-PAGE.

Immunofluorescence. H1299 cells were grown on coverslips with adenoviral vector expressing GRAMD4 and GFP or transfected with pcDNA3.1-p73β and pcDNA3.1 as control. At 48 h after virus-infection and 72 h after transfection, cells were fixed in 3% paraformaldehyde, permeabilized, and stained with custom-made GRAMD4 antibody or anti-Bax (clone 6A7, 556467; BD Pharmingen). A secondary anti-rabbit conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) and anti-mouse antibody conjugated to Alexa Fluor 633 (Molecular Probes) were used for visualization with a laser-scanning microscope. Cell nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI) (5 μg/ml) and mitochondria using Mito-Tracker orange CMXRos (Molecular Probes, Eugene).

Flow cytometry. For flow cytometry analysis, cells were harvested at indicated times after infection and chemotherapeutic treatment, fixed in 70% ethanol and stained for DNA content with propidium iodide. Analysis was carried out in a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) using CellQuest Software.

Hoechst staining. Hoechst 33342 was added to the cell culture medium at 1 μg/ml at 48 h after infection. Cells were incubated for 20 min at 37°C in 5% CO_2 and subsequently subjected to fluorescence microscopy.

Determination of mitochondrial $\Delta\psi$m. Loss of mitochondrial $\Delta\psi$m indicative of apoptosis was detected using TMRE/TMRM Mitochondrial Membrane Potential Assessment Kit (ImmunoChemistry Technologies, Bloomington, MN, USA) according to manufacturer's instructions. Briefly, infected cells were incubated with TMRE potentiometric dye for 20 min at 37°C 24 h post transduction and analyzed using a fluorescence plate reader set for 550 nm excitation and 580 nm emission. Cells exposed to 50 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP; provided by the manufacturer) depolarizing agent for 45 min at 37°C were used as apoptotic control.

Animal experiments. Athymic BALB/c *nu/nu* mice of 6- to 8-week-old (Central Animal Facility, University of Essen, Essen, Germany) were subcutaneously injected into the right flank with 5×10^6 HCT116 (p53−/−) and HCT116 (p53+/+) tumor cells. Mice with visible and palpable tumors received a single direct intratumoral injection of 5×10^9 pfu Ad.GFP or Ad.GRAMD4 followed by intraperitoneal injections of cDDP (6 mg/kg) on day 7, 10, 13, 16, and 19. Mice were monitored for tumor regression over 21 days. To monitor the effect of GRAMD4 and p73 knockdown on tumor growth in response to cDDP treatment, tumor cells were *ex vivo* infected with shRNA expressing virus and subsequently injected into nude mice. Tumor volume was calculated as follows: V (tumor) = (length × (width^2)/2). Animals were killed when the longest diameter was greater than 15 mm or when any two measurements were greater than 10 mm. Animal experiments were performed in accordance with institutional guidelines set by the University Institutional Animal Care and Use Committee.

Conflict of interest

The authors declare no conflict of interest.

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