

pp32/PHAPI determines the apoptosis response of non-small-cell lung cancer

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During malignant transformation, cancer cells have to evade cell-intrinsic tumor suppressor mechanisms including apoptosis, thus acquiring a phenotype that is relatively resistant to clinically applied anticancer therapies. Molecular characterization of apoptotic signal transduction defects may help to identify prognostic markers and to develop novel therapeutic strategies. To this end we have undertaken functional analyses of drug-induced apoptosis in human non-small cell-lung cancer (NSCLC) cells. We found that primary drug resistance correlated with defects in apoptosome-dependent caspase activation *in vitro*. While cytochrome *c*-induced apoptosome formation was maintained, the subsequent activation of caspase-9 and -3 was abolished in resistant NSCLC. The addition of recombinant pp32/putative human HLA class II-associated protein (pp32/PHAPI), described as a putative tumor suppressor in prostate cancer, successfully restored defective cytochrome *c*-induced caspase activation *in vitro*. Conditional expression of pp32/PHAPI sensitized NSCLC cells to apoptosis *in vitro* and in a murine tumor model *in vivo*. Immunohistochemical analyses of tumor samples from NSCLC patients revealed that the expression of pp32/PHAPI correlated with an improved outcome following chemotherapy. These results identify pp32/PHAPI as regulator of the apoptosis response of cancer cells *in vitro* and *in vivo*, and as a predictor of survival following chemotherapy for advanced NSCLC.

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During cancer initiation and progression, malignant cells have to acquire strategies to evade the various tumor suppressor mechanisms, which guard genomic integrity at the cellular and organismal levels.¹ Apoptosis, a genetically predetermined cell death program essential for the development and homeostasis of complex organisms, is an important tumor suppressive mechanism.² 'Minus signals' such as the lack of survival factors, as well as 'plus signals' such as the deregulated activation of proto-oncogenes or the accumulation of DNA strand breaks equally trigger apoptosis through activation of an evolutionary conserved family of enzymes called caspases. Apoptotic caspases can be assigned to two categories: those with long prodomains, such as caspases-8 and -9, contain caspase recruitment (CARD) or death-effector domains (DED) preceding their catalytic domain. They act upstream of those caspases with short prodomains, including caspase-3, -6 and -7, which execute the death program by cleaving their cellular substrates. Hence, caspase activation proceeds in a two-step process, which is initiated by the formation of complexes to recruit and activate caspases with long prodomains.³ Examples are the 'death-inducing signaling complex' (DISC) composed of ligand-activated 'death receptors' and the adapter FADD to activate caspase-8, and the 'apoptosome' complex, which is formed by cytochrome *c* and the adapter Apaf-1 to activate caspase-9. Activated caspase-8 and -9 cleave the zymogen forms of effector

caspases to unleash active enzymes.⁴ Current understanding places the two pathways upstream of the formation of activator complexes. The 'extrinsic' pathway is initiated by the interaction of 'death receptors' of the tumor necrosis factor receptor family with their respective ligands, and triggers DISC formation. Activation of the 'intrinsic' pathway occurs in response to cellular stresses and is regulated by the BCL-2 family of proteins, which guard the integrity of the mitochondrial outer membrane (MOM). Following permeabilization of the MOM, cytochrome *c* is released into the cytoplasm to induce a conformational change of the adapter Apaf-1 that is essential for apoptosome formation, and which may provide another level of regulation. Crosstalk between these two pathways is provided through the activating cleavage of the BH3-only protein BID by DISC-activated caspase-8, which leads to permeabilization of the MOM and apoptosome formation through the 'extrinsic' pathway.⁵

Biochemical evidence and studies using gene-targeted mice place the 'intrinsic' pathway of caspase activation downstream of the p53 tumor suppressor gene, which is an important inhibitor of cancer initiation and progression, that is inactivated by mutation in approximately half of all human cancers.⁶ Loss of p53 not only facilitates cancer development, but also confers resistance to cytotoxic anticancer drugs *in vitro* and *in vivo*, and these effects are phenocopied by genetic inhibition of the 'intrinsic' pathway of caspase

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Abbreviations: IRS, immunoreactivity score; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; PHAPI, putative human HLA class II-associated protein; SET, endoplasmic reticulum-associated complex; Xiap, X-linked Inhibitor of apoptosis

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activation.^{7,8} Accordingly, defects in the apoptotic program downstream of p53 promote cancer initiation and progression, and result in resistance to anticancer therapies. In fact, genetic alterations of BCL-2 family members have been described in various human cancers, such as follicular lymphoma, breast cancer, colorectal cancer or melanoma.⁹ The expression of BCL-2 proteins correlates with prognosis in some studies of non-small-cell-lung cancer (NSCLC) patients.¹⁰ In contrast, loss of caspase-9 or the adapter Apaf-1 failed to accelerate Myc-induced lymphomagenesis in mice,¹¹ and inactivating mutations or loss of expression of these factors are rarely observed in human cancers.^{12,13} A correlation of nuclear localization of Apaf-1 or caspase expression has been reported to correlate with prognosis in resected NSCLC,^{14,15} and studies in cancer cell lines and human leukemias point at a contribution of defective caspase activation to drug resistance by a yet undefined mechanism.^{16,17}

To this end we have analyzed the role of caspase activation defects in constitutive drug resistance of human NSCLC. By combined biochemical and genetic studies, we have identified hypofunction of pp32/putative HLA class II-associated protein I (pp32/PHAPI) as resistance mechanism to apoptosis

induced by cancer therapy and additional stresses *in vitro* and *in vivo*. We further show that the absence of immunohistochemically detectable pp32/PHAPI expression correlates with poor survival following chemotherapy for NSCLC.

Results

Defects in apoptosome-dependent caspase activation in drug-resistant NSCLC. As a model of drug-resistant cancer, we have used cell lines derived from patients suffering from NSCLC, which were either proficient (A549, NCI-H460) or deficient (NCI-H1299, Calu-6) for the p53 tumor suppressor protein. Treating these cells with apoptotic stimuli, such as staurosporine, paclitaxel, etoposide, actinomycin D, UV-C and γ -radiation, we observed a similar pattern of sensitivity and resistance, which was largely independent of the respective inducer of apoptosis (Figure 1a, and not shown). As these agents trigger signals via the 'intrinsic' pathway of caspase activation, we reasoned whether apoptosis sensitivity of NSCLC cells correlated with the capability to activate caspases in an apoptosome-dependent

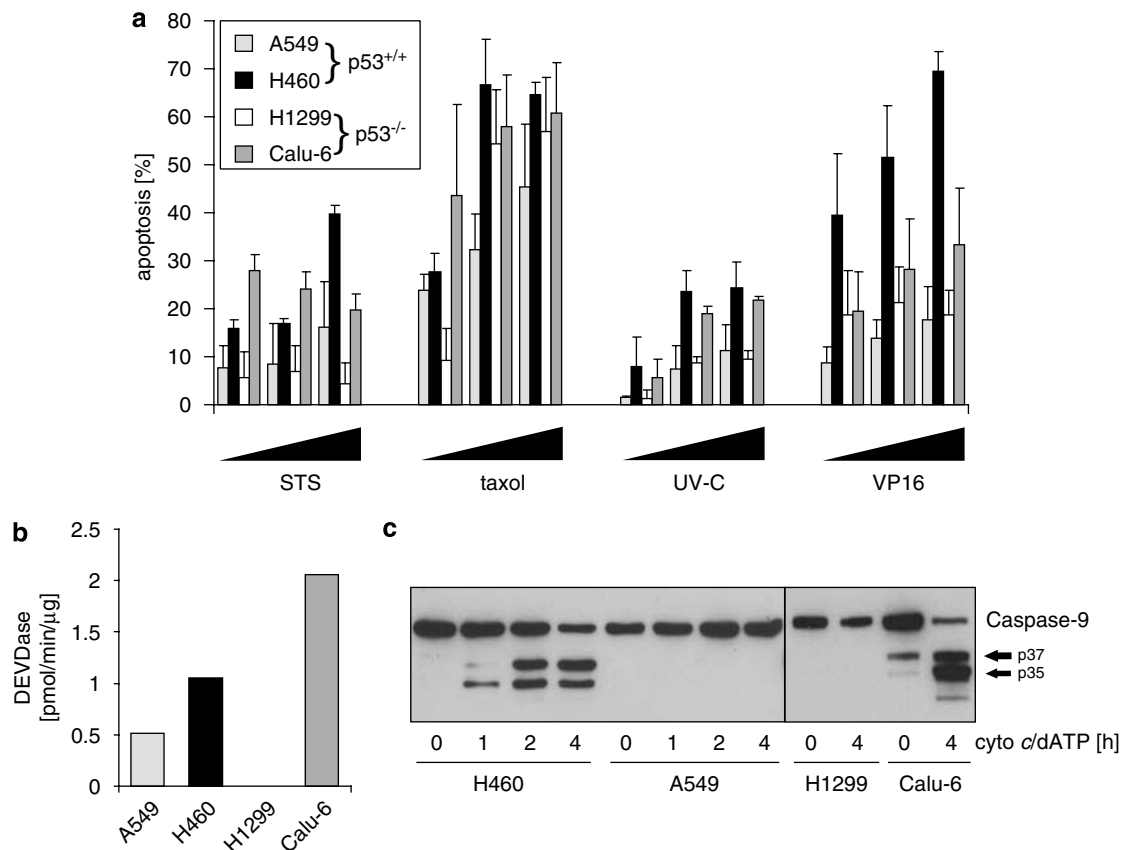


Figure 1 Caspase activation defects in drug-resistant NSCLC. **(a)** NSCLC cell lines with wild-type p53 (A549, light gray bars; NCI-H460, black bars) and p53 deficiency (NCI-H1299, white bars; Calu-6, dark gray bars) were treated with staurosporine (STS; 125, 250 and 500 nM), paclitaxel (taxol; 5, 50 and 500 nM), UV-C radiation (5, 25 and 50 mJ/cm²) or etoposide (VP16; 12.5, 25 and 50 μ M). Apoptosis was measured by flow cytometric detection of cells with subdiploid DNA content (mean values + S.D. of three independent experiments). **(b)** Cellular extracts prepared from the indicated NSCLC cell lines were incubated at 37°C in the presence of cytochrome c and dATP for 4 h. The caspase-3-like protease activity was measured kinetically using a colorimetric assay. Representative results of at least three independent experiments are shown. **(c)** Cellular extracts were treated as in **(b)** and subjected to SDS-PAGE followed by immunoblot detection of caspase-9. Note the occurrence of the caspase-9 cleavage products p37 (resulting from cleavage by activated caspase-3) and p35 (resulting from autocatalytic processing of caspase-9) in drug-sensitive cell lines

manner. To this end, extracts were prepared from relatively resistant and sensitive NSCLC cells and were incubated with cytochrome *c* and dATP. Minimal requirements for cytochrome *c*/dATP-induced caspase activity *in vitro* are the adapter Apaf-1, the activator caspase-9 and the effector caspase-3.¹⁸ This system recapitulates apoptosome-dependent caspase activation, and thus serves as a functional readout for the 'post-mitochondrial' step of the apoptotic signal transduction cascade via the 'intrinsic' pathway. The presence of essential factors was confirmed by immunoblotting analysis of extracts derived from sensitive and resistant NSCLC lines, and the extent of protein expression apparently failed to correlate with drug sensitivity (Supplementary Figure 1A). Cytochrome *c*/dATP readily induced caspase activation in extracts derived from apoptosis-sensitive NSCLC cells or control extracts from hematopoietic cells, as demonstrated by cleavage of a consensus caspase substrate as well as by the generation of caspase cleavage products indicative of activation (Figure 1b and c). In contrast, extracts prepared from drug-resistant NSCLC cells exhibited a blunted response to cytochrome *c*/dATP (Figure 1b and c).

For activation caspase-9 has to be recruited into the apoptosome complex, which is formed by seven molecules of the adapter Apaf-1 upon an ATP/dATP-driven conformational change.¹⁹ Hence, caspase activation defects in NSCLC

could result from failure to assemble the Apaf-1/caspase-9 complex. To study cytochrome *c*/dATP-induced apoptosome formation, cellular extracts were subjected to gel-filtration chromatography followed by immunoblot analysis of the resulting size fractions. Incubating sensitive and resistant cellular extracts with cytochrome *c*/dATP we found that Apaf-1 and caspase-9 equally shifted into the high-molecular-weight apoptosome complex (Figure 2a and b). However, extracts prepared from resistant cell lines failed to exhibit specific free cleavage products of caspase-9 (p37) and caspase-3 (p17), which are indicative of effector caspase activation (Figure 2a and b, and not shown). This was consistent with a functional defect at the level of caspase-9 or caspase-3 in drug-resistant A549 and NCI-H1299 NSCLC cells, which occurs downstream of apoptosome formation and recruitment of caspase-9. Caspase-9 and -3 protein expression was detectable (Supplementary Figure 1A) and wild-type DNA sequences were confirmed (not shown) in all NSCLC cell lines analyzed in this study, thus arguing for the presence of inhibitory factor(s) or the absence of stimulatory factor(s).

pp32/PHAPI restores caspase activation in NSCLC. Recently, pp32/PHAPI was identified as an important cofactor of caspase activation downstream of apoptosome formation.^{20,21} Moreover, pp32/PHAPI was found in the endoplasmic reticulum-associated complex (SET) complex, which is implied in the

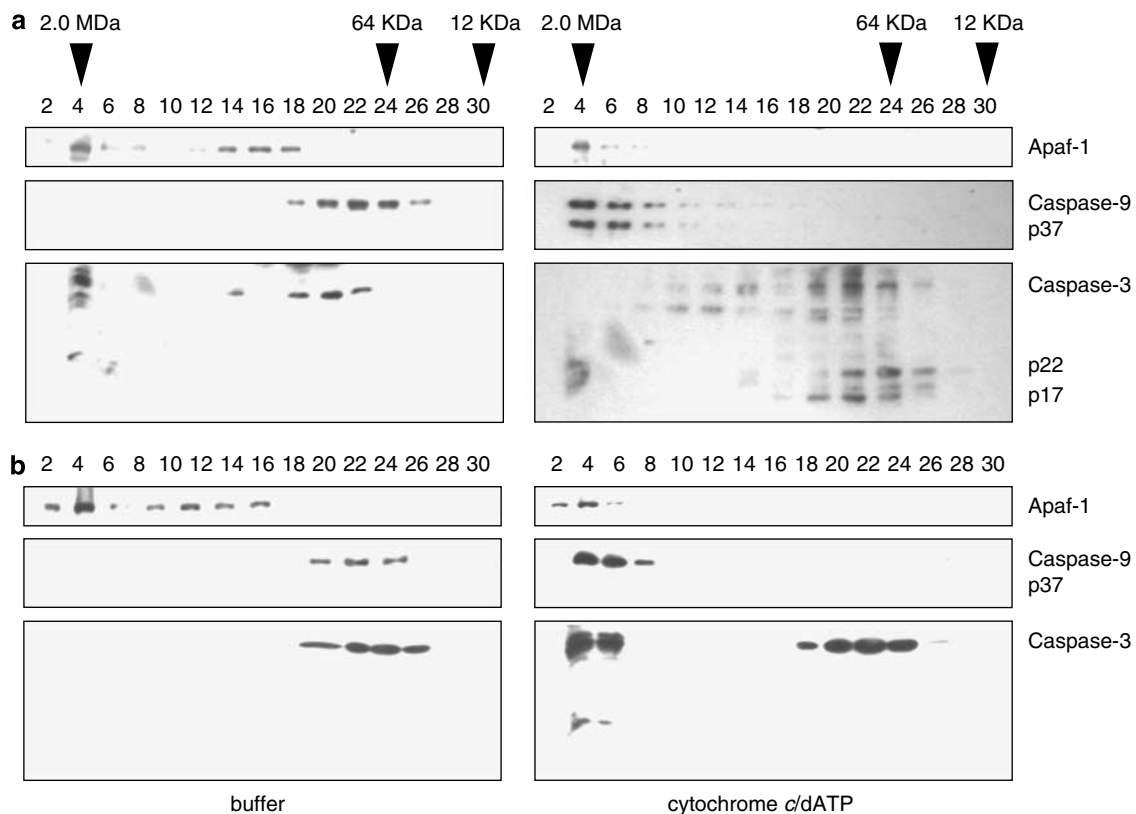


Figure 2 Apoptosome formation is maintained in drug-resistant NSCLC. Extracts prepared from the caspase activation-proficient K562 (a) and caspase activation-defective A549 (b) cell lines were incubated in the absence (buffer) or presence of cytochrome *c* and dATP at 37°C for 4 h followed by gel-filtration chromatography. The resulting size fractions were analyzed by immunoblotting using the indicated primary antibodies. Size markers are indicated by arrowheads. Note the shift of Apaf-1 and caspase-9 into fractions 4–8 in response to cytochrome *c*/dATP. Cleavage products of caspase-9 and -3 are absent in the activation-defective extract (b)

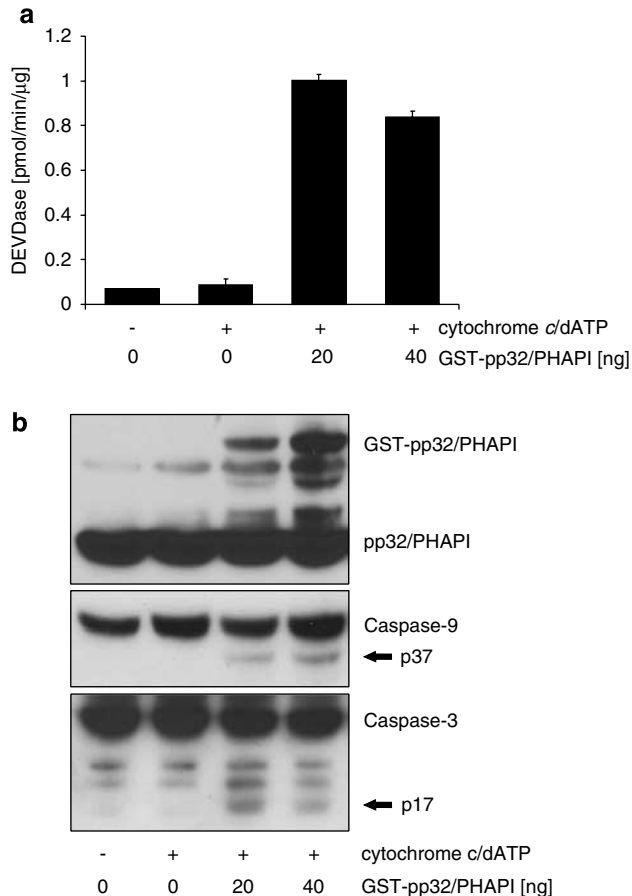


Figure 3 Sensitization to cytochrome *c*/dATP-induced caspase activation by recombinant pp32/PHAPI. Hyporesponsive cellular extracts (A549) were incubated with or without cytochrome *c*/dATP and recombinant human pp32/PHAPI. (a) Caspase-3-like activity was measured kinetically for 24 min using a colorimetric assay (mean values + S.D. of three repetitions). (b) Immunoblot analysis to detect exogenous (GST-pp32/PHAPI) and endogenous pp32/PHAPI, as well as the p37 cleavage product of caspase-9 and the p17 cleavage product of caspase-3 indicative of effector caspase activation only in the presence of GST-pp32/PHAPI

regulation of nuclear DNA fragmentation by the cytotoxic T lymphocyte protease granzyme A,²² supporting its role as regulator of death-inducing proteases. To study whether pp32/PHAPI could overcome the caspase activation defect observed in resistant NSCLC cells, cellular extracts were supplemented with recombinant human pp32/PHAPI followed by incubation with cytochrome *c*/dATP (Figure 3a). Indeed, the addition of pp32/PHAPI facilitated early caspase activation in drug-resistant NSCLC cells, which are hyporesponsive to cytochrome *c*/dATP *in vitro* (Figure 3a and b). Exogenously added pp32/PHAPI also accelerated cytochrome *c*/dATP-induced caspase activation in drug-sensitive NCI-H460 NSCLC cells, which are fully capable of apoptosome-dependent caspase activation (Figure 1b and c, and not shown). This suggests that beyond a certain threshold, pp32/PHAPI is a general enhancer of apoptosome-dependent caspase activation.

As all NSCLC cell lines endogenously expressed comparable pp32/PHAPI protein levels, we reasoned whether posttranslational regulation of pp32/PHAPI stability could impact on sensitivity to cytochrome *c*/dATP. To this end the

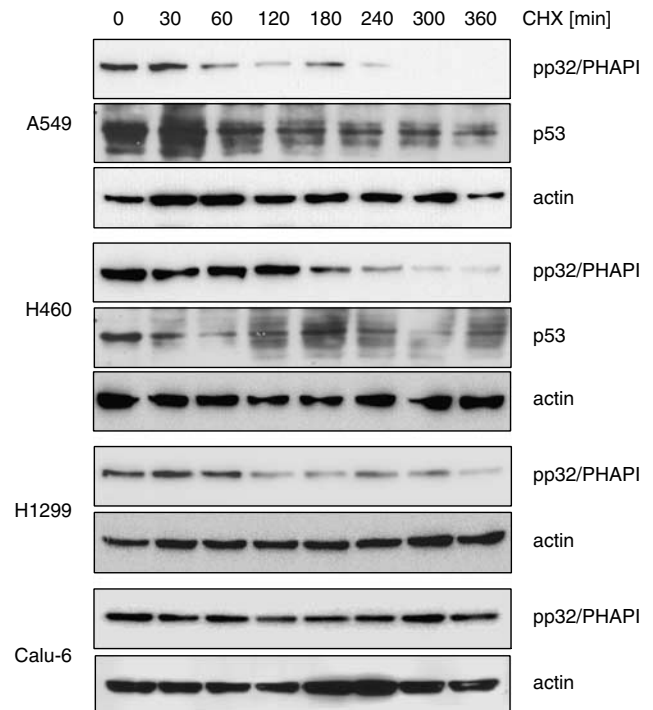


Figure 4 Decreased stability of endogenous pp32/PHAPI in drug-resistant NSCLC. The indicated NSCLC cells were cultured in the presence and absence of the translation inhibitor CHX (50 μg/ml) for the indicated periods. Immunoblot analysis of endogenous pp32/PHAPI protein levels. Actin served as a control protein with long half-life, p53 was detected in p53-proficient cell lines (A549, NCI-H460) as a control protein with short half-life. Note the decrease in pp32/PHAPI protein levels after 30 and 60 min in drug-resistant A549 and NCI-H1299 cells, respectively

expression of endogenous pp32/PHAPI was studied in NSCLC cells treated with the translation inhibitor cycloheximide (CHX) and the proteasome inhibitor bortezomib, respectively. Following CHX treatment, apoptosis-sensitive NCI-H460 and Calu-6 cells exhibited enhanced pp32/PHAPI stability, as compared with apoptosis-resistant A549 and NCI-H1299 cells (Figure 4). In contrast, bortezomib given at concentrations that are biologically active in NSCLC cells had no effect on pp32/PHAPI levels *in vitro* (not shown). Hence, proteasome-independent post translational mechanisms might impact on pp32/PHAPI protein stability.

In order to assess whether increasing the availability of caspases could alleviate the requirement of the cofactor pp32/PHAPI, purified bioactive human caspases-9 and -3 (Figure 5a) were added to cellular extracts in the presence or absence of cytochrome *c*/dATP. Neither the addition of caspase-9 nor caspase-3 overcame the hyporesponsiveness to cytochrome *c*/dATP observed in extracts prepared from drug-resistant NSCLC cells (Figure 5b and c), thus supporting the requirement of sufficiently high levels of pp32/PHAPI.

The X-linked inhibitor of apoptosis (XIAP) was described as an inhibitor of caspase activation, which in turn is repressed by the second mitochondria-derived activator of apoptosis (Smac, or DIABLO in mice).^{23,24} Genetic or pharmacologic mimetics of Smac were shown to facilitate caspase activation

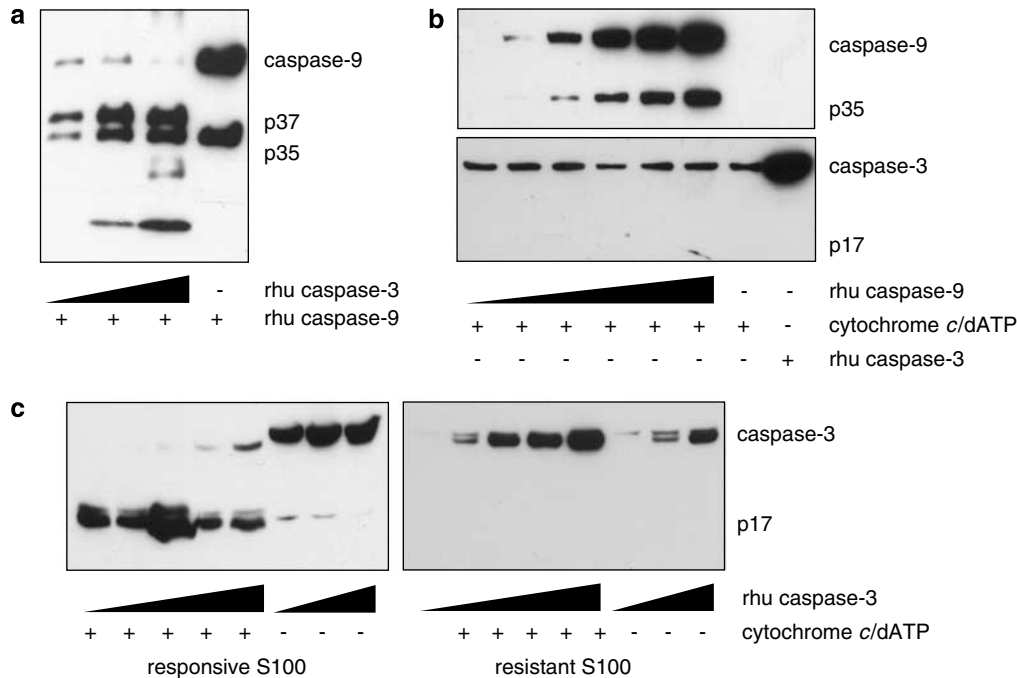


Figure 5 Addition of exogenous caspases fails to reverse hyporesponsiveness to cytochrome *c*/dATP. (a) Immunoblot analysis of purified recombinant human (rhu) caspase-9 following co-incubation with increasing amounts of purified rhu caspase-3. The autocatalytic p35 cleavage product indicates activity of caspase-9; the loss of full-length caspase-9 and the occurrence of the p37 cleavage product indicate cleavage of caspase-9 by successfully activated rhu caspase-3. (b) Extracts from drug-resistant lung cancer cells were incubated with increasing amounts of purified recombinant caspase-9 in the presence of cytochrome *c*/dATP followed by immunoblot analysis of caspases-9 and -3. While the p35 cleavage product of rhu caspase-9 indicates autocatalytic activity, cleavage and activation of endogenous caspase-3 cannot be detected. Rhu caspase-3 was run in the last lane as a size control. (c) Extracts (S100) from responsive (left panel) and resistant (right panel) cancer cells were incubated with increasing amounts of purified rhu caspase-3 in the presence or absence of cytochrome *c*/dATP followed by immunoblot analysis. Note the cytochrome *c*/dATP-dependent complete processing and activation of exogenous caspase-3 in the responsive extract

and apoptosis in several preclinical tumor models.^{25,26} While constitutive expression of XIAP and Smac was confirmed in all NSCLC cell lines analyzed in this study, protein expression levels did not correlate with drug sensitivity or responsiveness to cytochrome *c*/dATP *in vitro* (Supplementary Figure 1A). In contrast to reports from different experimental settings,^{21,27} XIAP failed to co-elute with the apoptosome complex in extracts derived from cytochrome *c*/dATP-sensitive and -resistant cell lines, making unlikely that XIAP inhibits caspase-9 at the level of the apoptosome in this system (Supplementary Figure 2A). Survivin is another IAP protein that was reported to inhibit caspase activation in cancer cells despite its physiological role as a chromosomal passenger protein regulating mitosis.²⁸ In gel-filtration analysis of drug-resistant NSCLC cells, Survivin failed to co-elute with caspase-9 in the apoptosome fraction, thus arguing against a role for Survivin in the caspase activation defect observed in our experimental system (Supplementary Figure 2A). By means of immunoblot analysis of cytochrome *c*/dATP-sensitive and resistant cellular extracts following treatment with a crosslinking agent, we were able to detect dimers of caspase-3, but no additional species of caspase-3 bound to a potential inhibitory factor (Supplementary Figure 2B). Moreover, conditional expression (Supplementary Figure 1B) of full-length or amino-terminally truncated Smac (resulting in its cytoplasmic targeting) as well as addition of a peptide-based Smac mimetic²⁵ failed to sensitize NSCLC cells to apoptosis (Supplementary Figure

2C, and not shown) or to suppress spontaneous or staurosporine-inhibited clonogenic survival (Supplementary Figure 1C).

These results are in support of pp32/PHAPI protein stability as an important determinant of caspase activation. They argue against a role for insufficient caspase expression, or inhibition of caspases by XIAP and alternative Smac-sensitive factors in apoptosis resistance of NSCLC.

Expression of pp32/PHAPI correlates with improved outcome following chemotherapy for NSCLC. Against this background, we reasoned whether the expression of pp32/PHAPI in tumor cells correlated with the clinical course of NSCLC patients treated with cytotoxic chemotherapy. To this end we analyzed tumor samples from 58 patients chemotherapy-naïve patients suffering from stage IV NSCLC, who were treated within a randomized trial receiving either gemcitabine/vinorelbine/cisplatin or gemcitabine/vinorelbine followed by paclitaxel or paclitaxel/cisplatin. Immunohistochemical staining of paraffin-embedded tumor samples revealed cytoplasmic staining with an immunoreactivity score (IRS) of at least 3²⁹ for pp32/PHAPI in 24 patients (41%), whereas 34 cases (59%) were scored negative (Figure 6a). Cross-table analysis (two-sided Pearson χ^2 -test) showed no association of pp32/PHAPI staining with sex, age (dichotomized <70 versus \geq 70 years) and ECOG performance status. Assessing overall survival, Kaplan–Meier plots (Figure 6b) indicated a survival

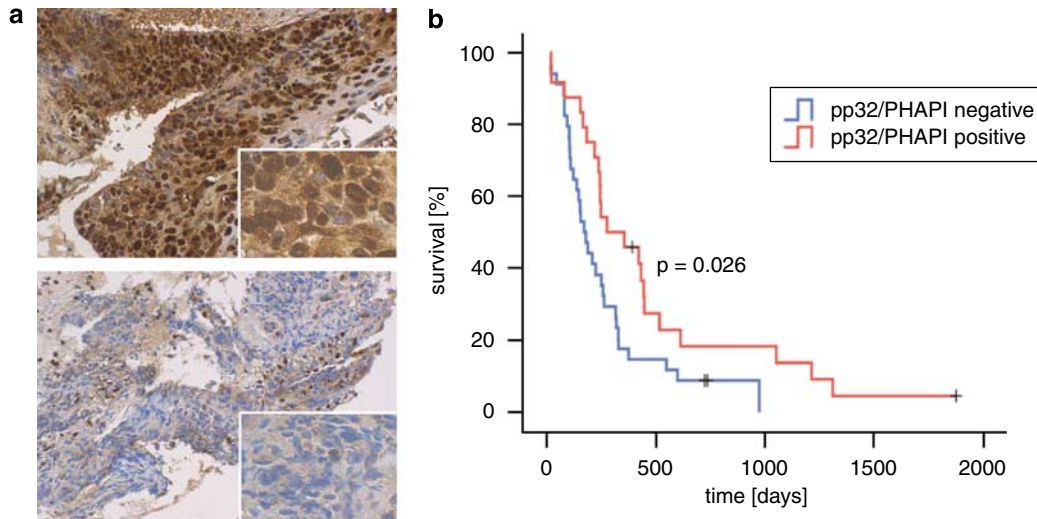


Figure 6 Expression of pp32/PHAPI correlates with improved survival following chemotherapy for lung cancer. (a) Photomicrographs of representative sections of a pp32/PHAPI-positive (upper panel) and a pp32/PHAPI-negative NSCLC (lower panel). The peroxidase-based detection system results in a brown nuclear and cytoplasmic staining of pp32/PHAPI-positive cells; hematoxylin counterstained nuclei appear blue. (b) Kaplan-Maier plot of the actuarial survival of 24 NSCLC patients with positive cytoplasmic immunoreactivity for pp32/PHAPI (red line) and 34 NSCLC patients with negative cytoplasmic pp32/PHAPI staining (blue line)

advantage for pp32/PHAPI-positive patients ($P = 0.026$, log rank). Median survival was 179 and 276 days for pp32/PHAPI-negative and pp32/PHAPI-positive patients, respectively. Likewise, the survival rate at 1 year differed substantially between the pp32/PHAPI-negative (17.6%) and the pp32/PHAPI-positive group (45.8%). Hence, in this prospectively treated cohort of advanced NSCLC patients, the cytoplasmic expression of pp32/PHAPI was a strong predictor of outcome following chemotherapy.

Conditional expression of pp32/PHAPI sensitizes NSCLC cells to apoptosis. A moderate increase of pp32/PHAPI protein levels facilitated cytochrome *c*/dATP-induced caspase activation *in vitro* (Figure 3). To study this effect at a cellular level, we generated a vector for doxycycline-induced conditional expression of the human pp32/PHAPI cDNA in NSCLC cells (Figure 7a). Increasing the availability of cellular pp32/PHAPI resulted in a profound suppression of clonogenic survival of drug-resistant NSCLC cells (Figure 7a and b). This was consistent with a tumor suppressive activity of pp32/PHAPI, which had been reported in previous studies.³⁰ Interestingly, conditional expression of pp32/PHAPI-sensitized resistant NSCLC cells to caspase activation and apoptosis induced by several proapoptotic stimuli (Figure 7b–d).

This tumor suppressive activity of conditionally expressed pp32/PHAPI could be confirmed *in vivo* using NSCLC cells xenografted into immune-deficient non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Figure 8a). Importantly, when exponentially growing lung cancers were established in mice, delayed conditional expression of pp32/PHAPI not only halted further tumor growth, but also resulted in a pronounced reduction in tumor size (Figure 8b). Ex-planted tumors from mice with doxycycline-induced pp32/PHAPI expression exhibited large central regions of cells with strong staining for fragmented DNA, whereas the pool of

mitotic (i.e., proliferating) cells located in glandular structures within the tumor cortex remained unaffected (Figure 8c and d). The central apoptotic region, which was also observed to a much lesser extent in large xenografts in the absence of conditional pp32/PHAPI expression (Supplementary Figure 1D), most likely resulted from reduced supply with survival factors due to an insufficient tumor vasculature. However, conditional expression of pp32/PHAPI dramatically enlarged these central apoptotic regions (Supplementary Figure 1D), which can be interpreted as pp32/PHAPI sensitizing cancer cells to apoptosis under limiting survival factor conditions *in vivo*.

Discussion

Many tumor suppressor proteins and cancer therapies utilize common intracellular effector mechanisms such as apoptosis, cell-cycle arrest and senescence, to exert their biological activity. Accordingly, defects in tumor suppressor pathways, which are invariably acquired during tumor initiation and progression, may also impair the efficacy of cancer therapy. This apparent dilemma can, however, be converted into an advantage by identifying molecular targets at the converging points of important antitumor effector pathways. Therapeutic modulation of such targets could equally restore tumor suppressor functions as well as sensitivity to anticancer drugs, and thus may serve the development of novel strategies for cancer treatment. This approach requires a detailed molecular understanding of functional deregulations of the underlying pathways in human cancers.

Against this background, we have analyzed apoptotic caspase activation defects in human NSCLC models. We have focused on ‘post-mitochondrial’ apoptosome-dependent caspase activation, as this step is the converging point of the ‘intrinsic’ pathway and the ‘extrinsic pathway’ (at least in ‘type II cells’ requiring mitochondrial amplification of death receptor-

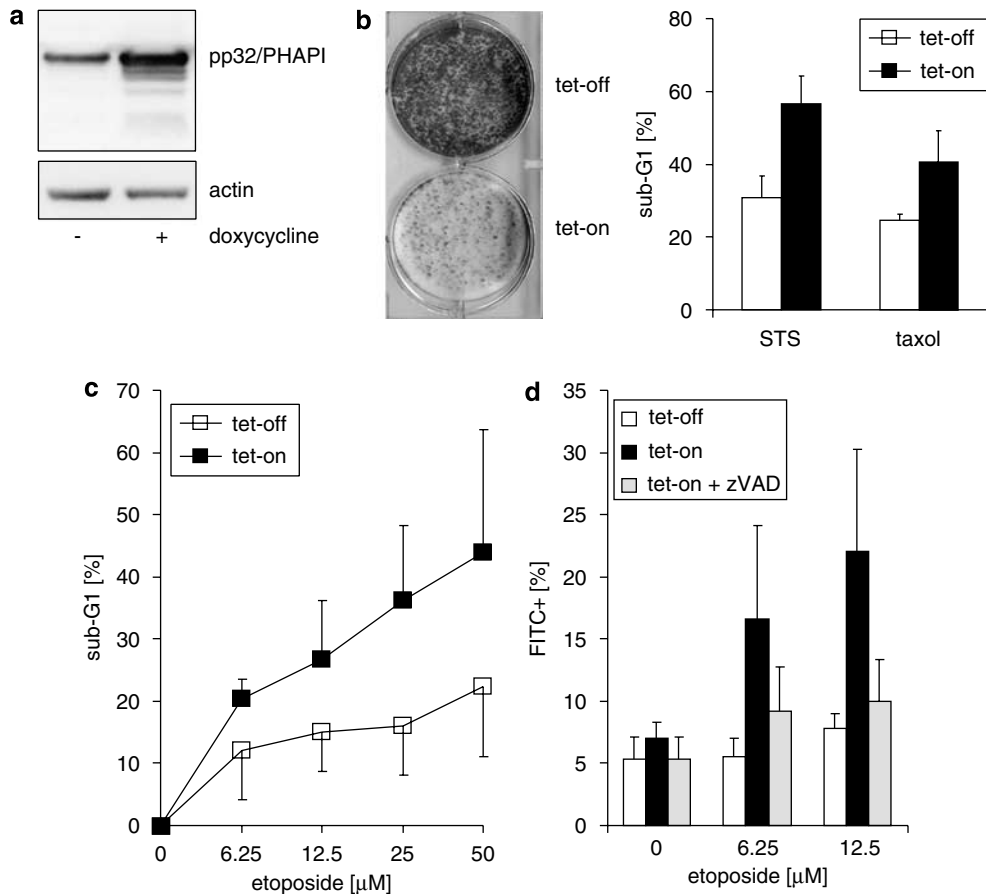


Figure 7 Conditional expression of pp32/PHAPI in drug-resistant NSCLC cells inhibits clonogenic survival and sensitizes to apoptosis. (a) Immunoblot analysis of endogenous and conditional expression of pp32/PHAPI in drug-resistant A549 lung cancer cells. (b) Drug-resistant A549 lung cancer cells were grown in the absence or presence of doxycycline to induce conditional pp32/PHAPI expression, and the resulting colonies were stained after 7 days with brilliant blue. Representative photograph of at least five independent experiments (left panel). Conditional A549 cells were treated with STS (500 nM) or paclitaxel (taxol, 5 μ M) in the absence (open bars) or presence (closed bars) of doxycycline, and apoptosis was quantified by flow cytometric detection of cells with subdiploid DNA content (mean values + S.D. of three independent experiments; right panel). (c) Drug-resistant A549 lung cancer cells were treated with increasing doses of etoposide in the absence (tet-off, open boxes) or presence (tet-on, closed boxes) of doxycycline to induce conditional pp32/PHAPI expression. Apoptosis was quantified by flow cytometric detection of cells with subdiploid DNA content; mean values \pm S.D. of three independent experiments. (d) Drug-resistant A549 lung cancer cells were treated with increasing doses of etoposide in the absence (tet-off, open bars) or presence (tet-on, closed bars) of doxycycline to induce conditional pp32/PHAPI expression, or in the presence of doxycycline and the caspase inhibitor *N*-benzyloxycarbonyl-VAD-fluoromethylketon (zVAD-fmk) (gray bars). Cells with active caspase were detected by flow cytometry following staining with the fluorochromic caspase substrate FITC-VAD-fmk; mean values + S.D. of FITC-positive cells of three independent experiments

induced caspase activation), and depends on a small number of genetically and functionally non-redundant molecules. Biochemical and genetic evidence has confirmed that Apaf-1, caspase-9 and (under most circumstances) cytochrome *c* are necessary and sufficient for apoptosome-mediated activation of effector caspases.^{18,31–34} This compares favorably to the ‘pre-mitochondrial’ step of MOM permeabilization, which is regulated by a complex rheostat of more than 20 partially redundant members of the BCL-2 family of proteins.

Interestingly, we found that drug-resistant NSCLC cells exhibit hyporesponsiveness to cytochrome *c*/dATP-induced caspase activation despite the presence of Apaf-1, caspases-9 and -3. This hyporesponsiveness could be overcome by a moderate increase in pp32/PHAPI *in vitro*. pp32/PHAPI, also known as acidic nuclear phosphoprotein 32A or leucine-rich acidic nuclear protein, belongs to the leucine-rich family of proteins, which is implied in a variety of pathways such as

chromatin remodeling, cytoskeletal dynamics, signal transduction or protein degradation.³⁵ Recently, pp32/PHAPI and additional PHAP proteins were biochemically characterized as cofactors of caspase activation in HeLa cell extracts. In this study, pp32/PHAPI facilitated recruitment and accelerated activation of caspase-9 downstream of apoptosome formation.²⁰ This is consistent with the effects of pp32/PHAPI observed in our model, which overcomes hyporesponsiveness to cytochrome *c*/dATP that occurs despite successful apoptosome formation. In another study, recombinant pp32/PHAPI enhanced the proteolytic activity of Jurkat cellular extracts and of apoptosomes isolated from these extracts in a Xiap-independent manner. In contrast to the previous work,²⁰ pp32/PHAPI also enhanced the activity of recombinant caspase-3, thus adding another possible level of apoptosis regulation by pp32/PHAPI.²¹ Interestingly, pp32/PHAPI was also found in the SET complex together with the DNase

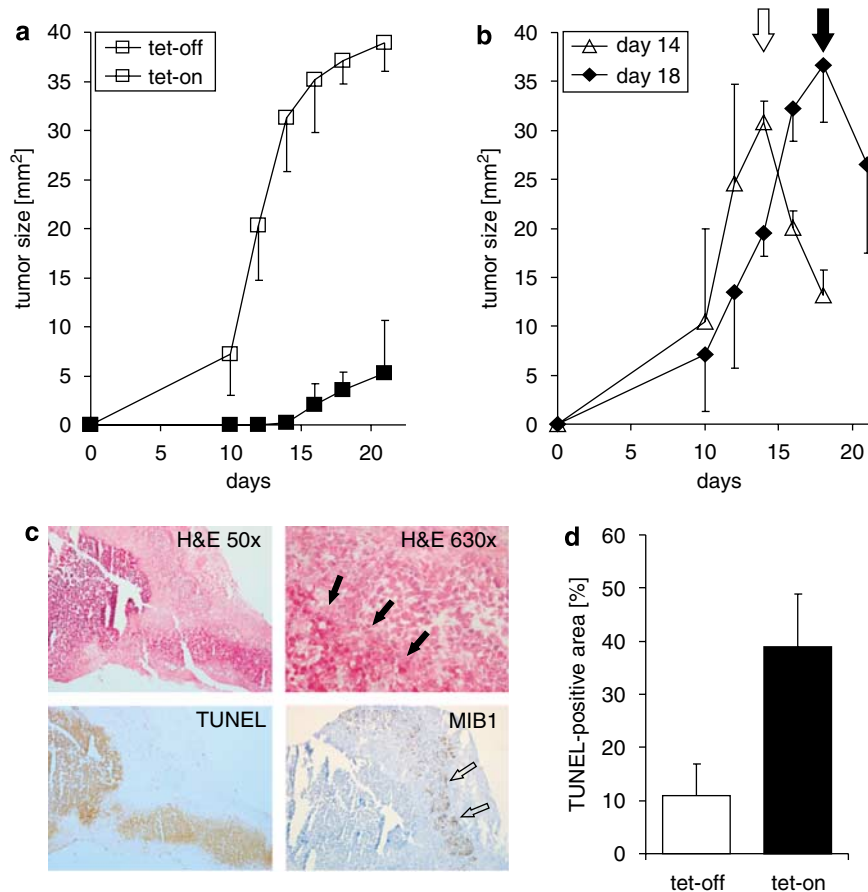


Figure 8 Tumor suppression and induction of apoptosis in drug-resistant NSCLC by conditional expression of pp32/PHAPI *in vivo*. (a) Growth of A549 xenograft tumors in NOD/SCID mice in relation to conditional pp32/PHAPI expression. *In vivo* transgene expression (tet-on, closed boxes) was achieved by feeding the mice with doxycycline-supplemented drinking water. Mean tumor sizes \pm S.D. of four mice per group. (b) Shrinkage of established A549 tumors by delayed induction of conditional pp32/PHAPI expression. The arrows indicate the time points (open arrow and triangles, day 14; closed arrow and diamonds, day 18) of doxycycline treatment of the tumor-bearing NOD/SCID mice. Mean tumor sizes \pm S.D. of four mice per group. (c) Photomicrographs of stained cryosections of a representative A549 tumor explanted following induction of pp32/PHAPI expression. Apoptotic nuclei with fragmented DNA were detected by TUNEL staining, the Ki-67 antigen of mitotic cells was detected using the antibody MIB1. The closed arrows indicate the border between viable and apoptotic tumor cells; the open arrows indicate glandular tumor formations with high proliferative activity. (d) Area of TUNEL-positive apoptotic tumor cells in relation to full tumor size in cryosections from A549 xenografts without (tet-off, open bar) or with (tet-on, closed bar) conditional pp32/PHAPI expression *in vivo*. Mean values \pm S.D. of at least three tumors per group

NM23-H1. The protease granzyme A secreted from cytotoxic T lymphocytes cleaves SET to release and activate NM23-H1, which executes caspase-independent DNA fragmentation. A regulatory function of pp32/PHAPI in this pathway is not yet defined, but it has been implied in the nuclear translocation of granzyme A.²² All these observations are in line with a role for PHAPI in the regulation of cellular enzymes involved in death processes.

Recently, the generation of pp32/PHAPI-deficient mice has been reported, and extensive analyses of their general and neuronal development failed to detect any abnormalities.³⁶ While apoptosis regulation has not been specifically addressed in this study, the knockdown of pp32/PHAPI in HeLa cells by RNAi technology has also been reported as inconclusive.²⁰ These negative reports were explained by possible overlapping functions of the multiple forms of PHAP proteins, some of which have probably arisen by gene duplication.

Overexpression of pp32/PHAPI was shown to suppress oncogenic transformation,³⁰ and pp32/PHAPI and homologs

located on different chromosomes were implied in the regulation of human prostate cancer.³⁷ In our study, conditional pp32/PHAPI inhibited clonogenic survival and sensitized intact NSCLC cells to caspase activation by apoptotic stimuli *in vitro*. Moreover, increasing the level of pp32/PHAPI lowered the resistance of NSCLC cells to the depletion of survival factors *in vivo*, as demonstrated by the dramatic increase in spontaneous apoptosis in the undersupplied central regions of lung cancer xenografts. Most importantly, our analysis of tumor samples from patients with NSCLC revealed a strong correlation between the absence of immunohistochemically detectable cytoplasmic pp32/PHAPI expression and poor outcome following chemotherapy. Collectively, these findings suggest that pp32/PHAPI controls the drug response of NSCLC cells, and is a prognostic marker in NSCLC patients treated with anticancer chemotherapy. A recent study in breast cancer cells reported that higher cellular levels of pp32/PHAPI correlated with increased sensitivity to apoptosome-dependent caspase activation,³⁸ thus sup-

porting our observation that the level of pp32/PHAPI determines the responsiveness to cytochrome *c*/dATP. Here, we observed comparable expression levels but a decreased stability of endogenous pp32/PHAPI in drug-resistant NSCLC cells, which might contribute to their phenotype.

In conclusion, we have characterized pp32/PHAPI as an important cofactor of apoptosome-dependent caspase activation in NSCLC, which determines the sensitivity to apoptosis occurring under various conditions *in vitro* and *in vivo*. Immunohistochemical detection of cytoplasmic pp32/PHAPI in tumor cells defines a subgroup of advanced NSCLC patients, which exhibit improved survival following cytotoxic chemotherapy. The level and/or stability of pp32/PHAPI might impact on the responsiveness of organs and tissues to physiological and pathological death stimuli. Moreover, pp32/PHAPI seems to be involved in tumor suppression as well as responsiveness to anticancer therapies, thus making it a promising target for cancer treatment.

Materials and Methods

Cell culture, apoptosis assays and retroviral transductions. Unless otherwise indicated, cell lines were maintained in full DMEM or RPMI1640 supplemented with fetal bovine serum (FBS, PAA), L-glutamine, penicillin and streptomycin (Gibco). Conditional transgene expression was achieved in cells growing in medium supplemented with tetracycline-free FBS (Clontech) by addition of doxycycline (1 μ g/ml; Sigma). The Phoenix packaging cell line (provided by Dr. GP Nolan) was used for generation of retroviruses following a standard calcium phosphate transfection protocol. Transduction efficiencies were monitored in parallel experiments by flow cytometric detection of enhanced green fluorescent protein expression. Cell-cycle analysis and detection of apoptosis were carried out by flow cytometric determination of DNA content following propidium iodide (Sigma) staining of permeabilized cells, or by detection of FITC-positive cells following staining with fluorochromic caspase substrate FITC-VAD-fmk (Oncogene).

Plasmids, antibodies and reagents. The human pp32/PHAPI cDNA was amplified (sense primer 5'-AGAGATGGAGATGGGCAGAC-3', antisense primer 5'-AGTCATCATCTTCTCCCTCA-3') and cloned into the retroviral vector pRevTRE (Clontech) to allow conditional expression controlled by doxycycline and rt-TA.³⁹ Polymerase chain reaction-generated cDNA-encoding full-length and amino-terminally truncated human Smac fused to a carboxy-terminal FLAG tag were also cloned into pRevTRE. All vector inserts were confirmed by sequencing. Prokaryotic expression vectors for human caspase-9, caspase-3 and PHAPI were generous gifts from Drs. BB Wolf, GS Salvesen and X Wang. The following primary antibodies were used for immunoblotting analysis: actin (C4; ICN), FLAG (M2; Sigma), Apaf-1 (MAB868; R&D Systems), caspase-3 (ab7850; Abcam), caspase-9 (MAB4609; Chemicon), Survivin (NB500-201; Novus), Xiap (AF822; R&D Systems). Smac and pp32 were detected by antisera and antibodies generously provided by Drs. X Wang, J Lieberman and DC Pallas. Doxycycline, staurosporine, bortezomib and anticancer drugs were purchased from Sigma or the Hospital Pharmacy of the Johannes Gutenberg University.

Cell-free caspase activation and gel-filtration chromatography. For preparation of extracts, cell pellets were resuspended in CEB (20 mM Hepes-KOH pH 7.5, 10 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 0.25 M sucrose, 2 mM DTT, 10 μ M Cytochalasin B, Complete™ protease inhibitors; Roche) and vigorously broken using a dounce homogenizer. For caspase activation, extracts (protein concentration \geq 10 mg/ml) were incubated with 10 μ M cytochrome *c* with or without 1 mM dATP (Sigma) at 37°C. The kinetics of the resulting caspase-3-like activity were measured colorimetrically using DEVD-p-nitroaniline (Calbiochem) as substrate. Gel-filtration chromatography was performed using a XK 16 \times 60 column (Pharmacia) packed with Toyopearl HW-55F (Tosoh Biosep). Following ultracentrifugation at 100 000 \times g, S100 extracts were loaded on the column and size fractions were eluted with EB (20 mM Hepes pH 7.0, 50 mM NaCl, 5% saccharose, 5 mM DTT, 0.1% CHAPS).

Expression and purification of recombinant proteins. Polyhistidine-tagged human caspase-9 and -3 (vectors provided by Drs. GS Salvesen and BB Wolf) and pp32/PHAPI (vector provided by Dr. X Wang) were expressed in the BL21 strain of *Escherichia coli*, and purified under native conditions using Ni-NTA agarose (Qiagen) with different imidazole concentrations in the wash and elution steps. Recombinant GST-tagged pp32/PHAPI was purchased from Calbiochem.

Analysis of tumor samples. Data were analyzed from 85 chemotherapy-naïve patients suffering from stage IV NSCLC, who were treated in the AIO/AASLC 03 trial at our institution, and who had provided written informed consent for scientific analyses of surplus tumor samples. Sufficient tumor material was available from 58 patients who were included in the analysis. The median age of this cohort was 60 years (range 36–75 years); the Eastern Cooperative Oncology Group (ECOG) performance score was 0 in 16% and 1 in 84% of patients, respectively. Slides from paraffin-embedded tumor biopsies were stained using a computer-controlled autostainer (Dako TechMate 500; Dako) and the Dako EnVision system using a monoclonal anti-human acidic nuclear phosphoprotein 32A (clone RJ1) antibody (Alexis Biochemicals, Grünberg, Germany), and a peroxidase-labeled secondary antibody (Dako EnVision). The staining reaction was visualized by adding DAB substrate (Dako EnVision), and sections were counterstained in hematoxylin. For quality control and reliability, staining was repeated twice. The slides were evaluated using the BX40 microscope (Olympus) connected to a digital imaging system. Classification according to the IRS immunoreactivity score²⁹ was independently performed by two investigators (S Biesterfeld and R Wiewrodt). An IRS \geq 3 was considered positive. Statistical analysis was performed using SPSS V13 software. Kaplan–Meier plots were analyzed using the following tests: Log-rank (Mantel Cox), Breslow (generalized Wilcoxon) and Tarone-Ware. All patients had provided written informed consent, and studies were approved by the Ethics Committee of the Landesärztekammer Rheinland-Pfalz.

Animal model. Irradiated (150 rad) NOD/SCID mice received single subcutaneous flank injections of 10×10^6 transgenic lung cancer cells diluted in 200 μ l saline. Tumor growth was monitored by bidimensional measurements using a calliper. For induction of conditional pp32/PHAPI expression *in vivo*, the drinking water was supplemented with doxycycline (1 mg/ml). All animal studies were conducted in compliance with institutional guidelines and were approved by the responsible regulatory authority (SGD Süd Rheinland-Pfalz).

Microscopy and histochemical analysis. Cells with apoptotic nuclei were visualized in cryosections by means of the TUNEL assay (*In Situ* Cell Death Detection kit; Roche) following the manufacturer's instructions. Mitotic cells were detected by standard immunohistochemistry using anti-Ki-67 (MIB1; Dianova) as the primary antibody. Photomicrographs were taken on a Leica DMR microscope connected to a Leica DFC480 digital camera and a computer system. Image analysis including quantitation of tumor areas was performed using the Leica IM500 software.

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1. Hanahan D, Weinberg RA. The Hallmarks of Cancer. *Cell* 2000; **100**: 57–70.
2. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature* 2004; **432**: 307–315.
3. Adrain C, Brumatti G, Martin SJ. Apoptosomes: protease activation platforms to die from. *Trends Biochem Sci* 2006; **31**: 243–247.
4. Salvesen GS, Adams JM. Caspase activation – stepping on the gas or releasing the brakes? Lessons from humans and flies. *Oncogene* 2004; **23**: 2774–2784.
5. Green DR. Apoptotic pathways: ten minutes to dead. *Cell* 2005; **121**: 671–674.

6. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002; **2**: 594–604.
7. Strasser A, Harris AW, Bath ML, Cory S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* 1990; **348**: 331–333.
8. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions *in vivo*. *Cancer Cell* 2002; **1**: 289–298.
9. Kaufmann SH, Vaux DL. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene* 2003; **22**: 7414–7430.
10. Pezzella F, Turley H, Kuzu I, Tunjekar MF, Dunnhill MS, Pierce CB *et al*. bcl-2 protein in non-small-cell lung carcinoma. *N Engl J Med* 1993; **329**: 690–694.
11. Scott CL, Schuler M, Marsden VS, Egle A, Pellegrini M, Nestic D *et al*. Apaf-1 and caspase-9 do not act as tumor suppressors in myc-induced lymphomagenesis or mouse embryo fibroblast transformation. *J Cell Biol* 2004; **164**: 89–96.
12. Soung YH, Lee JW, Kim SY, Park WS, Nam SW, Lee JY *et al*. Mutational analysis of proapoptotic caspase-9 gene in common human carcinomas. *APMIS* 2006; **114**: 292–297.
13. Peltenburg LTC, de Bruin EC, Meersma D, Smit NP, Schrier PI, Medema JP. Expression and function of the apoptosis effector Apaf-1 in melanoma. *Cell Death Differ* 2005; **12**: 678–679.
14. Besse B, Candé C, Spano JP, Martin A, Khayat D, Le Chevalier T *et al*. Nuclear localization of apoptosis protease activating factor-1 predicts survival after tumor resection in early-stage non-small cell lung cancer. *Clin Cancer Res* 2004; **10**: 5665–5669.
15. Takata T, Tanaka F, Yamada T, Yanagihara K, Otake Y, Kawano Y *et al*. Clinical significance of caspase-3 expression in pathologic-stage I, nonsmall-cell lung cancer. *Int J Cancer* 2001; **96**: 54–60.
16. Wolf BB, Schuler M, Li W, Eggers-Sedlet B, Lee W, Taylor P *et al*. Defective cytochrome c-dependent caspase activation in ovarian cancer cell lines due to diminished or absent APAF-1 activity. *J Biol Chem* 2001; **276**: 34244–34251.
17. Schimmer AD, Munk Pedersen I, Kitada S, Eksioglu-Demiralp E, Minden MD, Pinto R *et al*. Functional blocks in caspase activation pathways are common in leukemia and predict patient response to induction chemotherapy. *Cancer Res* 2003; **63**: 1242–1248.
18. Kim H-E, Du F, Fang M, Wang X. Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. *Proc Natl Acad Sci USA* 2005; **102**: 17545–17550.
19. Riedel SJ, Li W, Chao Y, Schwarzenbacher R, Shi Y. Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* 2005; **434**: 926–933.
20. Jiang X, Kim H-E, Shu H, Zhao Y, Zhang H, Kofron J *et al*. Distinctive roles of PHAP proteins and prothymosin- α in a death regulatory pathway. *Science* 2003; **299**: 223–226.
21. Hill MM, Adrain C, Duriez PJ, Creagh EM, Martin SJ. Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *EMBO J* 2004; **23**: 2134–2145.
22. Fan Z, Beresford P, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* 2003; **112**: 659–672.
23. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; **102**: 33–42.
24. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE *et al*. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; **102**: 43–53.
25. Fulda S, Wick W, Weller M, Debatin K-M. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. *Nat Med* 2002; **8**: 808–815.
26. Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau M-J *et al*. Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* 2004; **5**: 25–35.
27. Bratton SB, Walker G, Srinivasula SM, Sun XM, Butterworth M, Alnemri ES *et al*. Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *EMBO J* 2001; **20**: 998–1009.
28. Ambrosini G, Adida C, Altieri DC. A novel antiapoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997; **3**: 917–921.
29. Remmele W, Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. *Pathologe* 1987; **8**: 138–140.
30. Brody JR, Kadkol SS, Mahmoud MA, Rebel JMJ, Pasternack GR. Identification of sequences required for inhibition of oncogene-mediated transformation by pp32. *J Biol Chem* 1999; **274**: 20053–20055.
31. Li K, Li Y, Shelton JM, Richardson JA, Spencer E, Chen ZJ *et al*. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* 2000; **101**: 389–399.
32. Hao Z, Duncan GS, Chang CC, Elia A, Fang M, Wakeham A *et al*. Specific ablation of the apoptotic functions of cytochrome c reveals a differential requirement for cytochrome c and Apaf-1 in apoptosis. *Cell* 2005; **121**: 579–591.
33. Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA, Gruss P. Apaf-1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* 1998; **94**: 727–737.
34. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS *et al*. Differential requirement for caspase 9 in apoptotic pathways *in vivo*. *Cell* 1998; **94**: 339–352.
35. Santa-Coloma TA. Anp32e (Cpd1) and related protein phosphatase 2 inhibitors. *Cerebellum* 2003; **2**: 310–320.
36. Opal P, Garcia JJ, McCall AE, Xu B, Weeber EJ, Sweatt JD *et al*. Generation and characterization of LANP/pp32 null mice. *Mol Cell Biol* 2004; **24**: 3140–3149.
37. Kadkol SS, Brody JR, Pevsner J, Bai J, Pasternack GR. Modulation of oncogenic potential by alternative gene use in human prostate cancer. *Nat Med* 1999; **5**: 275–279.
38. Schafer ZT, Parrish AB, Wright KM, Margolis SS, Marks JR, Deshmukh M *et al*. Enhanced sensitivity to cytochrome c-induced apoptosis mediated by PHAPI in breast cancer cells. *Cancer Res* 2006; **66**: 2210–2218.
39. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci USA* 2000; **97**: 7963–7968.

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