

PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective cell killing

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Apoptin (apoptosis-inducing protein) harbors tumor-selective characteristics making it a potential safe and effective anticancer agent. Apoptin becomes phosphorylated and induces apoptosis in a large panel of human tumor but not normal cells. Here, we used an *in vitro* oncogenic transformation assay to explore minimal cellular factors required for the activation of apoptin. Flag-apoptin was introduced into normal fibroblasts together with the transforming SV40 large T antigen (SV40 LT) and SV40 small t antigen (SV40 ST) antigens. We found that nuclear expression of SV40 ST in normal cells was sufficient to induce phosphorylation of apoptin. Mutational analysis showed that mutations disrupting the binding of ST to protein phosphatase 2A (PP2A) counteracted this effect. Knockdown of the ST-interacting PP2A-B56 γ subunit in normal fibroblasts mimicked the effect of nuclear ST expression, resulting in induction of apoptin phosphorylation. The same effect was observed upon downregulation of the PP2A-B56 δ subunit, which is targeted by protein kinase A (PKA). Apoptin interacts with the PKA-associating protein BCA3/AKIP1, and inhibition of PKA in tumor cells by treatment with H89 increased the phosphorylation of apoptin, whereas the PKA activator cAMP partially reduced it. We infer that inactivation of PP2A, in particular, of the B56 γ and B56 δ subunits is a crucial step in triggering apoptin-induced tumor-selective cell death.

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Tumor formation occurs due to a complex set of processes roughly based on enhanced survival and limited cell death activities.¹ Remarkably, a set of viral and cellular proteins has been found to selectively induce cell death in tumor cells.² Among these proteins is the avian virus protein apoptin (apoptosis-inducing protein), which has been shown to induce p53-independent apoptosis in a broad spectrum of human-transformed cells.³ Recent preclinical studies demonstrated the therapeutic potential of apoptin as a safe and efficient anticancer agent.⁴ Therefore, it is of interest to study the mechanisms underlying apoptin-induced apoptosis, particularly the 'switch' responsible for its activation during oncogenic transformation.

SV40-T antigens are known to be involved in oncogenic transformation through interference with many cellular processes.⁵ Distinct domains on LT that bind and inactivate tumor suppressors p53 and Rb (retinoblastoma protein), have long been known to have crucial roles in tumor formation.⁶ The SV40 small t antigen (SV40 ST) protein enforces transformation of normal cells via inhibition of protein phosphatase 2A (PP2A).^{7–9}

This feature is in accordance with the reduced PP2A levels found in various human tumor cell types,¹⁰ and accumulating evidence supporting major tumor suppressive roles for PP2A.¹¹ Another major cellular regulator implicated in carcinogenesis

is protein kinase A (PKA).¹² Its targets include PP2A,¹³ and for instance, the PKA-interacting protein breast cancer-associated gene 3 (BCA3) is known to be highly expressed in breast and prostate cancer cells compared with the normal surrounding tissue.¹⁴

In this study, we investigated the minimal steps leading to the activation of apoptin upon malignant transformation. We previously showed that transient expression of the SV40 large T antigen (SV40 LT) and SV40 ST antigens in normal human fibroblasts results in activation of apoptin, displaying all three of its characteristic features, namely phosphorylation, nuclear localization and apoptosis.¹⁵ This finding provided us with an elegant tool to analyze which domains of the SV40 LT and/or ST antigens are responsible for this activity. In parallel, BCA3 was identified as an apoptin-interacting protein, and the effects of PKA on apoptin phosphorylation were examined. Analysis of both transformation-related pathways revealed that inactivation of PP2A is crucial for the activation of apoptin.

Results

Transient expression of N-terminal SV40 LT136/ST activates apoptin in normal human fibroblasts. Apoptin activity can be induced in normal cells by transient

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Abbreviations: AKIP1, A-kinase-interacting protein; BCA3, breast cancer-associated gene 3; DAPI, 2,4-diamidino-2-phenylindole; NLS, nuclear localization signal; PKA, protein kinase A; PP2A, protein phosphatase 2A; Rb, retinoblastoma protein; RNAi, RNA interference; RSV, Rous sarcoma virus; SV40 LT, SV40 large T antigen; SV40 ST, SV40 small t antigen

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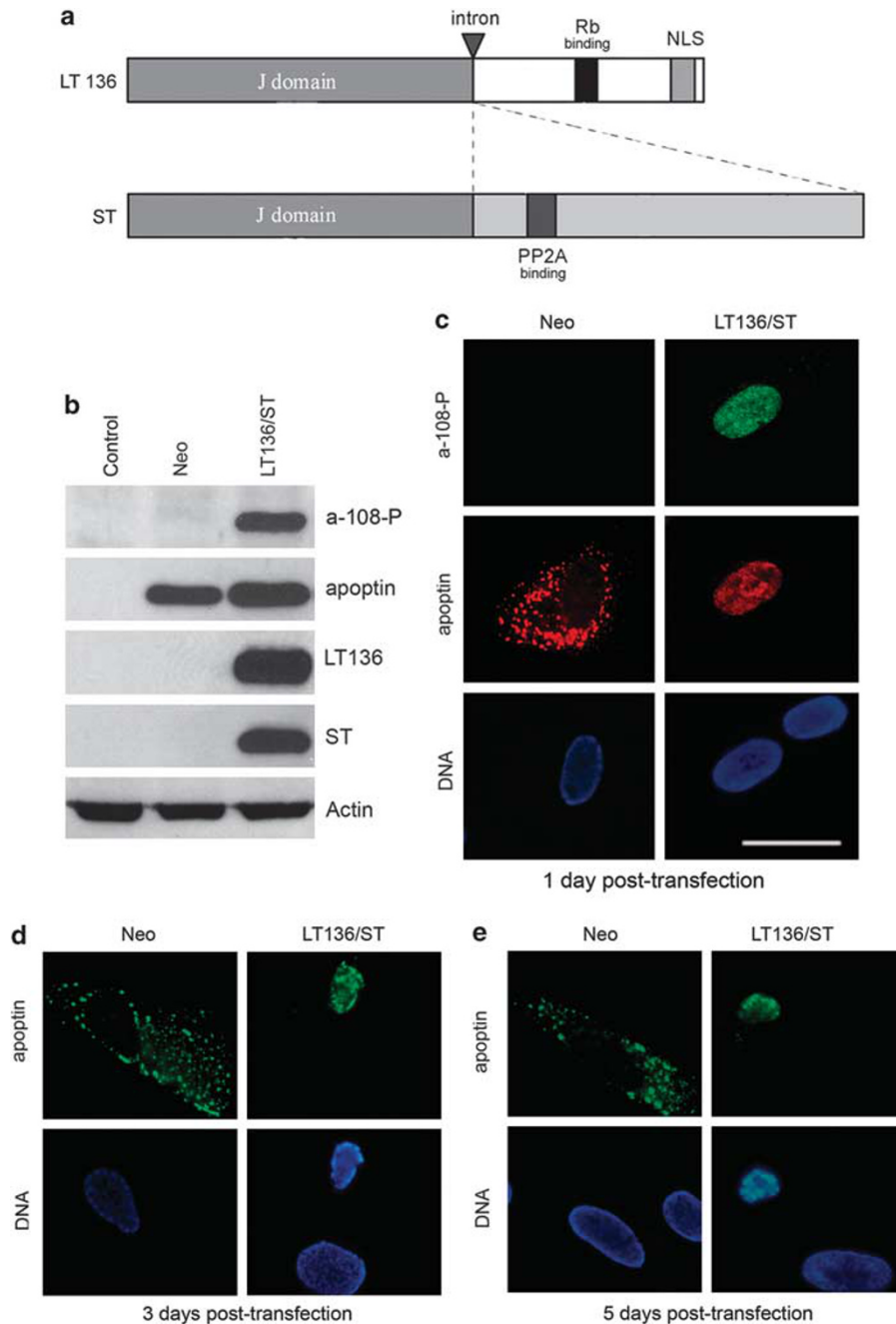


Figure 1 Transient expression of N-terminal determinants of SV40 T antigens activates apoptin. **(a)** Schematic representation of the domains in SV40 LT136/ST. The J domain (amino acids 1–82) is identical in both LT136 and ST. The Rb-binding domain and NLS in LT136 are also shown. ST is expressed by differential splicing and has a unique C-terminus, which contains the PP2A-binding domain. **(b)** Human fibroblasts (F9) were co-transfected with plasmids pcDNA-Flag-apoptin and pcDNA-LT136/ST (LT136/ST) or pcDNA-neo (neo) by AMAXA nucleofactor transfection. Twenty-four hours post transfection, cells were lysed for western blot analysis with the indicated antibodies. Mock-transfected F9 cells were used as control. Antibody α -108-P specifically recognizes phospho-apoptin at its Thr108. Flag-apoptin, LT and ST show the respective total protein amounts in the transfected cells. Actin was used as loading control. **(c–e)** Cells were fixed for immunofluorescence analysis at each given time-point after transfection and then stained with the indicated antibodies by indirect immunofluorescence assay. Scale bar = 20 μ m

expression of transforming SV40 antigens.¹⁵ Hence, we examined the minimal SV40 domains responsible for this effect. Expression of the SV40 LT136/ST DNA sequence results, due to alternative splicing, in two proteins: LT136, comprising the N-terminal 136 amino acids of LT, and the entire ST protein, consisting of 174 amino acids (Figure 1a).¹⁶

LT136 contains a DNA J domain, an Rb-binding site and a nuclear localization signal (NLS). ST shares its DNA J domain with LT136, but has a unique C-terminal domain encompassing a PP2A-binding site.⁵

In normal human foreskin fibroblasts, co-transfection of the SV40 LT136 and ST proteins with Flag-tagged apoptin

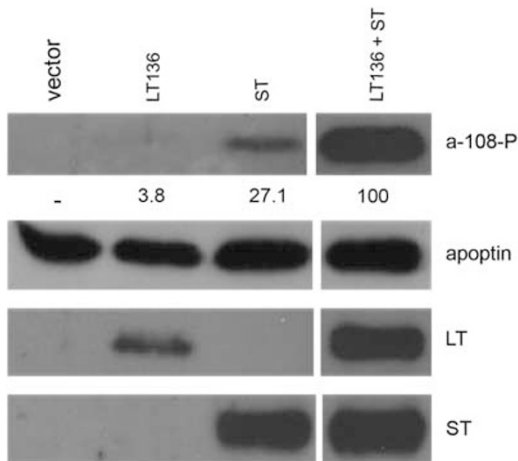


Figure 2 Nuclear targeting of SV40 ST activates apoptin. In normal human fibroblasts, apoptin was co-transfected with vector only (Neo), LT136, ST or both LT136 and ST. Twenty-four hours post transfection, cells were lysed for western blot analysis with the antibodies indicated. The relative amount of phosphorylated apoptin (as compared with the total amount of apoptin) was quantified and is indicated below the a-108-P panel. The LT136 + ST sample was set at 100

resulted in the latter's phosphorylation (Figure 1b). Immunofluorescence analysis of F9 cells expressing all three proteins showed that Flag-apoptin became nuclear already 1 day after transfection. Nuclear Flag-apoptin was also shown to be phosphorylated (Figure 1c), and 3 and 5 days post transfection, apoptin was shown to induce apoptosis (Figures 1d and e). In contrast, F9 fibroblasts expressing apoptin alone contained mainly cytoplasmic Flag-apoptin, which was, as expected, not phosphorylated^{17,18} and did not induce apoptosis (Figures 1c–e).

Our results clearly reveal that transient expression of LT136 and ST proteins in human F9 fibroblasts results in tumor-selective activation of apoptin, providing us with the possibility to explore which domains and respective cellular targets of LT136/ST were responsible for this activity.

Nuclear ST triggers apoptin activation. Next, we examined whether expression of either LT136 or ST protein alone was sufficient to activate apoptin phosphorylation. F9 fibroblasts were co-transfected with plasmids encoding Flag-tagged apoptin and either one of the following: (a) pcDNA-LT136, encoding LT136, (b) pcDNA-ST, encoding ST, (c) pcDNA-LT136/ST, where both LT136 and ST are produced via alternative splicing, or (d) pcDNA-neo, as a negative control. In each experiment, activation of apoptin was assayed by its phosphorylation at position T108, assessed by means of western blotting. Expression of LT136 alone did not trigger apoptin phosphorylation, whereas expression of ST clearly induced apoptin phosphorylation, albeit at a low level (Figure 2). Co-transfection of Flag-tagged apoptin with ST fused to an artificial nuclear localization site (NLS-ST) increased the level of apoptin phosphorylation significantly (Figure 3a).

C103S and P101A point mutations within the PP2A-binding domain of ST disable activation of apoptin. Besides its J domain, shared with LT136, ST contains a unique site for the binding and inactivation of PP2A. This domain has

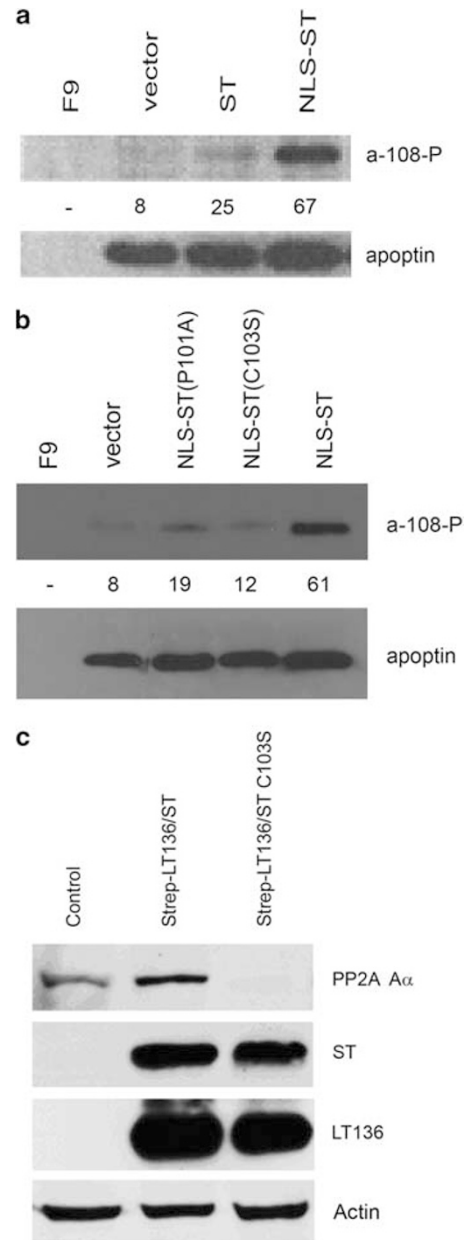


Figure 3 Apoptin activation via ST-mediated inhibition of PP2A. (a) pcDNA-Flag-apoptin was co-transfected with plasmids encoding the indicated proteins or vector DNA into F9 primary fibroblasts. Western blot assays were performed with the indicated antibodies at 24 h post transfection. (b) F9 primary cells were co-transfected with pcDNA-Flag-apoptin and plasmids encoding NLS-ST, NLS-ST(P101A), NLS-ST(C103S) or vector DNA as indicated. Western blot assays were performed with the indicated antibodies at 24 h post transfection. In panels a and b the relative percentage of phosphorylated apoptin in the various samples was quantified and is indicated below the a-108-P panel c. PP2A binding to ST is abolished by C103S mutation. LT136 and ST with or without the C103S mutation (LT136/ST or LT136/ST(C103S)) were fused with a Strep-tag at their N terminus. Cell lysates were prepared at 24 h post transfection for protein–protein interaction assays as indicated in Materials and Methods. The final elutions were analyzed by western blot with antibodies against PP2A α subunit, LT and ST, respectively. Actin was taken as equal loading control. The first lane (input control) indicated total amount of endogenous proteins in cell lysates

been shown to contribute to cellular transformation.⁵ A single amino-acid mutation C103S within the ST protein drastically diminishes the interaction of ST with PP2A (Figure 3c) and its

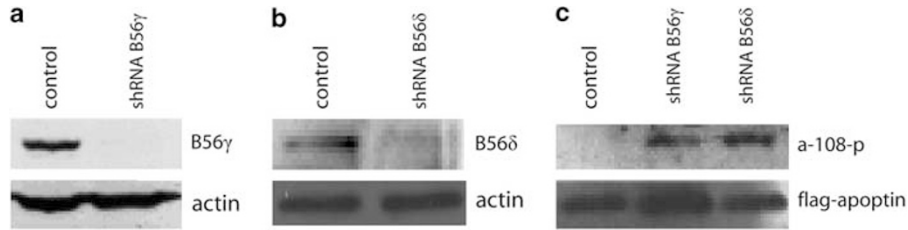


Figure 4 Knockdown of PP2A–B56 γ and B56 δ subunits triggers apoptin phosphorylation in normal cells. (a) Downregulation of PP2A–B56 γ subunit by shRNA. HeLa cells were co-transfected with pCEP-4HA-B56 γ expressing 4HA-tagged B56 γ , and either shB56 γ or control pSuper vector and lysed 48 h post transfection, followed by western blotting analysis with the indicated antibodies. (b) Downregulation of PP2A–B56 δ subunit by shRNA. F9 cells were transfected with pSuper vector encoding shRNA directed against PP2A–B56 δ or pSuper vector control; 24 h post transfection, cell lysates were prepared and subsequently analyzed by western blot. (c) F9 cells were co-transfected with Flag-apoptin and either pSuper vector encoding shRNA directed against PP2A–B56 γ , δ or control; 24–48 h post transfection, cell lysates were prepared and subsequently analyzed by western blot

transforming capacity.¹⁹ Therefore, we studied the effect of the C103S mutation within the PP2A-binding site on the activation of apoptin by (nuclear) ST in normal human cells.

F9 cells were analyzed for phosphorylation of Flag-apoptin upon co-expression with ST, NLS-ST or NLS-ST(C103S) protein. Figure 3b shows that expression of NLS-ST clearly induced apoptin phosphorylation. Introduction of the NLS-ST(C103S) mutation abolished this induction, although apoptin protein was expressed at a similar level. Similar results were obtained with the NLS-ST-(P101A) mutant (Figure 3b). The P101A point mutation within ST is also known to disturb the ST–PP2A interaction and transforming capacity of ST.¹⁹ These results suggest that ST interaction with PP2A is crucial to apoptin activation, and that inactivation of PP2A by ST might be sufficient to activate apoptin.

Knockdown of PP2A B56 γ via RNA interference (RNAi) activates apoptin phosphorylation in normal human fibroblasts. Two independent studies reported that ST interaction with PP2A resulted in the inhibition of the B56 γ regulatory subunit, resulting in cellular transformation.^{8,9} Therefore, we examined whether downregulation of B56 γ via RNAi could trigger phosphorylation of apoptin in normal cells. Our shRNA sequence was verified to reduce ectopic expression of B56 γ (Figure 4a). Normal F9 fibroblasts co-expressing both apoptin and shRNA directed against B56 γ mRNA manifested a clear level of phosphorylated apoptin in comparison with the cells transfected with apoptin and the RNAi control vector (Figure 4c). Our data thus indicate that inhibition of the PP2A–B56 γ subunit is a crucial and sufficient step for apoptin activation.

Overexpression of PKA-interacting protein BCA3 stimulates apoptin activity in tumor cells. Analogous to the enhancing effect of ST on apoptin phosphorylation in normal cells, we observed an enhancement of apoptin phosphorylation in tumor cells by BCA3. BCA3 was identified as an apoptin-interacting protein by means of a yeast two-hybrid assay, and interacts with apoptin in a human cellular background (Figure 5a). Co-expression of BCA3 and Flag-apoptin in human Saos-2 tumor cells resulted in a significant increase in the apoptosis activity of apoptin (Figure 5b). In fact, as early as 6 h after transfection, phosphorylated apoptin could readily be detected in Saos-2 cells expressing

both apoptin and BCA3, whereas in cells expressing apoptin alone (control) apoptin phosphorylation was not yet visible at this early time-point (Figure 5c).

As BCA3¹¹ has been shown to interact with the catalytic subunit of PKA,²⁰ the involvement of PKA in apoptin phosphorylation was investigated. Treatment of Saos-2 cells with H89, a known PKA inhibitor,²¹ enhanced apoptin phosphorylation. In contrast, addition of the PKA activator cAMP²² diminished apoptin phosphorylation (Figure 5d). These results suggest that interference with PKA activity favors activation of apoptin. By taking into account the fact that the B56 δ subunit of PP2A has been shown to be targeted by PKA,²³ we proved that this is also the case in our cellular system. Indeed, H89 treatment of Saos-2 cells decreased the level of active serine-phosphorylated B56 δ , whereas cAMP enhanced the amount of active B56 δ (Figures 5e–g).

Downregulation of PP2A–B56 δ subunit activates apoptin phosphorylation in normal cells. The experiments described above indicate that in cancer cells an inverse relation exists between the activity of PP2A–B56 δ , as judged by its phosphorylation,²³ and the potential of apoptin to become phosphorylated in the same cells. For this reason, it was interesting to examine whether inhibition of the expression of the PP2A–B56 δ subunit in human F9 cells would positively affect the phosphorylation of apoptin. Downregulation of B56 δ protein expression through RNAi was confirmed in normal F9 fibroblasts (Figure 4b). Co-expression of shRNA targeting B56 δ mRNA together with apoptin in normal F9 cells clearly resulted in the activation of apoptin phosphorylation, as compared with F9 cells transfected with apoptin and the RNAi control vector (Figure 4c).

In conclusion, our results imply that PP2A complexes containing the regulatory subunits B56 δ and γ are essential for maintaining a normal cell environment, as the loss of either one of these subunits results in the activation of apoptin.

Discussion

Activation of tumor-selective apoptosis-inducing proteins is an intriguing phenomenon and revealing the molecular switch behind this process could allow important insights for developing anticancer therapies. Apoptin was the first protein known to harbor apoptosis activity selectively in transformed

human cells.² However, the molecular mechanisms underlying apoptin's tumor-selective activity are largely unknown.⁴ Our studies revealed that interference with the normal function of PP2A is sufficient to activate apoptin in a tumor-selective fashion. This is inferred from two independent lines of research.

In one study, we examined the effect of specific domains within the transforming SV40 proteins LT136 and ST on the activation of apoptin. The C-terminal PP2A-binding and transformation domain of ST,^{5,24} when targeted to the nucleus of normal cells, turned out to be crucial for the tumor-characteristic activation of apoptin. We showed that both C103S and P101A point mutations within the ST PP2A-

binding site²⁵ abrogated the phosphorylation of apoptin induced by NLS-ST in normal human fibroblasts. RNAi studies confirmed that inactivation of the B56 γ protein promotes phosphorylation of apoptin in human fibroblasts. We have previously shown¹⁵ that the N-terminal J domain, which is common to both LT and ST (see Figure 1), is involved in apoptin activation. Interestingly, since the completion of this work two independent groups have resolved the molecular structure of the SV40 ST complex with PP2A and shown that both the C-terminal PP2A-binding domain and the N-terminal J domain are in direct contact with the A subunit of PP2A.^{8,9} The structure provides a mechanism how binding of ST to the A subunit of PP2A displaces the B56 γ subunit and as such inactivates PP2A. The results described in Supplementary Figure S1 (Supplementary Information) indicate that also the J domain (either present on LT or ST) needs to be functional in order to activate apoptin in combination with a functional C-terminal PP2A-binding site on ST. The data strongly suggest that the J domain of LT can also contribute to the displacement of B56 γ and the activation of apoptin in concert with our previous conclusions. However, the contribution of ST, which has not been analyzed in detail in the previous study, is essential for activating apoptin (Figure 2).

A second line of research indicated that interference with the PP2A-B56 δ subunit also led to the activation of apoptin.

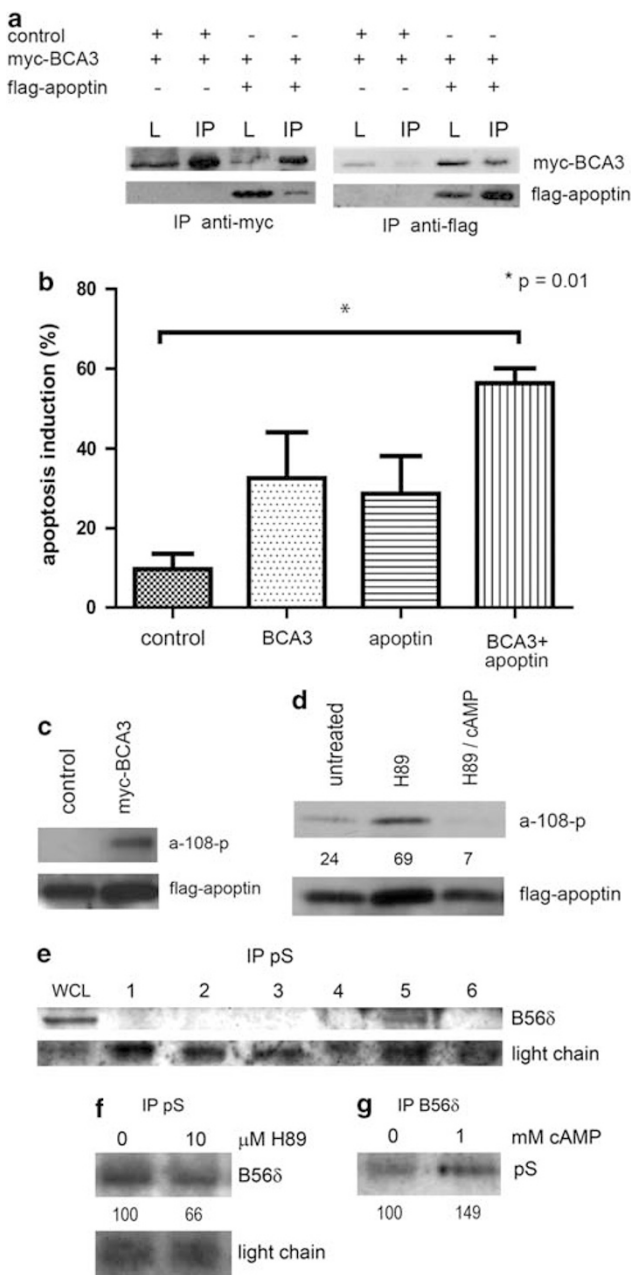


Figure 5 BCA3 interacts with apoptin and stimulates its activity. (a) Apoptin interacts with BCA3 in a cellular background. Normal human foreskin fibroblasts were transfected with plasmids encoding myc-tagged BCA3 and Flag-tagged apoptin, or control plasmid in the indicated combinations. Total lysates (L) or protein complexes immunoprecipitated (IP) with antibody against the myc- (left panel) or Flag-tags (right panel) were separated by SDS-PAGE and analyzed by western blot. (b) Expression of myc-BCA3 together with Flag-apoptin results in increased induction of apoptosis. Human Saos-2 tumor cells were transfected with plasmids encoding Flag-tagged apoptin and myc-tagged BCA3, or vector control in the indicated combinations and grown on glass coverslips. Forty-eight hours post transfection, slides were fixed and stained with appropriate antibodies for immunofluorescence analysis. pMaxGFP (Amara) was used as a negative control. Apoptosis was assessed according to characteristic morphological changes³⁵ following DAPI staining as shown in figure 1. Data are representative of three independent experiments, in which at least 100 cells were scored. (c) Co-expression of myc-BCA3 enhances apoptin phosphorylation. Saos-2 cells transfected with BCA3 and apoptin, or apoptin alone, were lysed 6 h post transfection and analyzed for apoptin phosphorylation by western blot analysis. (d) Inhibition of PKA results in increased apoptin phosphorylation. Saos-2 cells were transfected with Flag-tagged apoptin, and treated with the PKA inhibitor H89 (1 h, 10 μ M) or activator cAMP (30 min, 10 μ M H89, followed by 30 min 1 mM cAMP) 24 h post transfection. Cells were then lysed and their contents analyzed by western blot using indicated antibodies. The relative percentage of phosphorylated apoptin in the various samples was quantified and is indicated below the a-108-P panel. (e) Effect of PKA inhibitors/inducers on PP2A-B56 δ phosphorylation. In a first instance, phosphorylated proteins were immunoprecipitated from Saos-2 whole-cell lysate (WCL) with six different antibodies directed against phosphoserines, as described (Materials and Methods). Western blot was performed with antibodies against B56 δ . The light chain signal shows equal assay conditions. Note that only antibody 5 detects phospho-B56 δ . (f) Saos-2 cells were treated with PKA inhibitor H89 or mock-treated and immunoprecipitated with the phosphoserine antibody 5 (see panel e). The amount of pulled-down B56 δ was determined by western blot using an antibody directed against B56 δ and quantified. The amount of phospho B56 δ in the mock-treated cells was set at 100. (g) Saos-2 cells were treated with PKA-stimulator cAMP or mock-treated and immunoprecipitated with antibodies against B56 δ . The amount of phosphorylated B56 δ was determined by western blot using the phosphoserine antibody 5 (panel e) and quantified. The amount of phosphoB56 δ in the mock-treated cells was set at 100

This was achieved by treating human cancer cells with either PKA activator or inhibitor. PKA is known to phosphorylate the B56 δ subunit of PP2A leading to increased PP2A activity.²³ Ectopic expression of the apoptin- and PKA-interacting protein BCA3, or inhibition of PKA by treatment with H89 both resulted in enhanced phosphorylation and apoptosis activity of apoptin in human cancer cells. Stimulation of PKA by cAMP resulted in enhanced phosphorylation of PP2A–B56 δ and a lower level of phosphorylated apoptin in Saos-2 tumor cells. On the contrary, H89 decreased the level of phosphorylated B56 δ in tumor cells and enhanced apoptin phosphorylation. These data indicate that derailed PP2A activity is crucial for activating apoptin in accordance with the data obtained in our SV40 transformation assay. This vision was further highlighted by RNAi studies, showing that inhibition of the expression of PP2A–B56 δ activated apoptin even in normal human cells.

Our studies reveal that apoptin senses PP2A inactivation during malignant transformation. Interestingly, the δ and γ subunits are the only nuclear PP2A–B56 subunits,²⁵ and nuclear localization is important for both SV40-T antigen-induced cell transformation⁵ and apoptin-induced tumor-selective apoptosis.³ PP2A complexes containing B56 δ domains prevent entry of cells into mitosis upon DNA damage.²⁶ B56 γ is also known to mediate dephosphorylation and stabilization of the tumor suppressor protein p53 upon DNA damage, inhibiting cellular proliferation and transformation.²⁷ Evidence has been provided that derailment of B56 γ results in aberrancies in functioning of, for example, cell cycle and tumor suppressor proteins resulting in cell transformation.^{10,25} Many of these features are likely due to the fact that PP2A–B56 subunits have an essential role in the stabilization of chromosome–spindle interactions during normal cell division.²⁸ In this respect, it is interesting to mention that inhibition of Bub1, another mitotic regulator, results in nuclear translocation of apoptin in normal cells.²⁹ Apparently, apoptin can sense aberrant mitosis and the ensuing genetic instability³⁰ or DNA-damage response signaling.²⁹

PP2A has also a role in the activation of other tumor-selective apoptosis-inducing proteins. Besides apoptin, the adenovirus E4orf4 protein has been demonstrated to selectively induce apoptosis in human cancer cells. Direct interaction of E4orf4 with PP2A regulatory B domains is essential for the tumor-selective apoptosis activity of E4orf4. Interaction of E4orf4 with PP2A–B55 results in downregulation of myc oncogene expression.³¹ In addition to SV40 ST, other viral-transforming proteins also interact with PP2A, further accentuating its relevance in cellular transformation.³²

Further steps within the development of tumorigenic cells seem at least not critical for apoptin's tumor-selective apoptosis characteristics. These conclusions are in accordance with the observations by others and ourselves that apoptin is able to induce apoptosis in a very broad panel of tumor types.^{3,4,33} If one assumes that tumor cells arise by a wide variety of mechanisms, all the while sharing a limited number of key characteristics,³⁴ then apoptin simply needs to recognize one (or a subset) of these characteristics.

In summary, our results show that inactivation of the nuclear PP2A–B56 δ and/or γ subunits are sufficient to trigger apoptin's tumor-selective apoptosis activity. PP2A provides

a central phosphatase activity affecting many cellular signaling pathways. As derailment of PP2A activity is increasingly linked to oncogenic transformation, the sensing of such a central regulator by apoptin might provide a rationale for its efficient killing of tumor cells arising from a wide range of different origins.

Materials and Methods

Cells and cell culture. Human diploid foreskin F9 fibroblasts, isolated from neonatal foreskin, were obtained in the late 1980s from Dr. M Ponc (Department of Dermatology, Leiden University Medical Center). Cells were batch-frozen after careful morphological inspection. At subsequent passages cells were regularly screened for their typical fibroblast-like morphological appearance. All cells used were below passage 15 and cultured in Dulbecco's modified Eagle's medium containing 10% newborn calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Breda, The Netherlands). The human Saos-2 osteosarcoma and the HeLa cervical carcinoma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in the same medium as mentioned above. Cultures were regularly tested to ensure the absence of *Mycoplasma* infection. Cell morphology was regularly monitored to control the absence of cross-contamination. The sensitivity to apoptin is characteristic of the various cell types used and is regularly assessed (see below).

DNA plasmids. The DNA sequence encoding apoptin was synthesized by BaseClear (Leiden, The Netherlands) according to the apoptin sequence used by Danen-Van Oorschot *et al.*,³⁵ and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). The oligonucleotide fragment encoding the Flag-tag (Invitrogen) was inserted to create the pcDNA-Flag-apoptin plasmid encoding apoptin fused with a Flag-tag at its N terminus.

Plasmid pRSV-TN136 encoding the first 136 N-terminal amino acids of SV40 LT, including the region coding for SV40 ST, was a kind gift from Dr. JM Pipas (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA). From pRSV-TN136, we generated the pcDNA-LT136/ST plasmid, which encodes the LT136 (the N-terminally truncated LT fragment containing the first 1–136 amino acids) and full-length ST. pcDNA-LT136, encoding LT136 only, was constructed by introducing an intron deletion disabling ST expression.³⁶

pcDNA expression vectors encoding only ST sequences were derived from pRSV-ST, pcDNA-ST encodes for ST; pcDNA-NLS-ST contains an ST fused to a NLS-ST and pcDNA-NLS-ST(C103S) and NLS-ST(P101A) mutants encode the N-terminal NLS-ST fusion protein containing, respectively, the C103S or P101A mutation within the PP2A-binding site.³⁷

The sequences encoding the N-terminal 136 amino acids of LT together with either full-length ST or the C103S ST-mutant were cloned into pEXPR-IBA105 vector to generate pEXPR-IBA105-LT136/ST and pEXPR-IBA105-LT136/STC103S, respectively. These plasmids expressed Strep-tagged LT136/ST or LT136/ST-C103S proteins enabling interaction studies with PP2A (see below).³⁸ pCEP-4HA-B56 γ , encoding 4HA-tagged B56 γ , was a kind gift from Dr. M Mumby (University of Texas, TX, USA).

Apoptin-interacting partners were obtained by yeast two-hybrid screening and verified by immunoprecipitation assays in mammalian cells, as previously described by Danen-Van Oorschot *et al.*³⁵ Positive clones from the yeast two-hybrid screen were digested with XhoI to generate cDNA fragments, and subcloned into pMT2SM-myc to provide the fragments with an in-frame N-terminal myc-tag. The cDNA fragment encoding BCA3, including the myc-tag, was subsequently cloned into pcDNA.

Transfection methods. We used transfection reagent DOTAP (*N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium methylsulfate)³⁵ or AMAXA nucleofection technology in conjunction with cell-type-specific NucleofectorTM solution (Lonza, Cologne, Germany)³⁹ for DNA delivery into cells. When co-transfection or triple-transfection was performed, the ratio of each plasmid was 1 : 1 or 1 : 1 : 1 (in micrograms). In addition to the analysis by western blot, the cells were seeded on several glass coverslips to allow parallel analysis at the single-cell level by immunofluorescence assay.

Western blot analysis. Cells were lysed directly in Laemmli buffer (2% SDS, 10% glycerol, 60 mM Tris-Cl (pH 6.8), 2% β -mercaptoethanol, 0.002% bromophenol blue). Cell lysates were separated by SDS-polyacrylamide

gel electrophoresis (PAGE), and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). Blots were then incubated with antibodies against phosphorylated apoptin (α -108-P;¹⁵), Flag-apoptin (α -Flag M2, Sigma-Aldrich, St. Louis, MO, USA), SV40 LT (PAb416, Pab419, Calbiochem/Merck, Darmstadt, Germany), SV40 ST (Pab280, Calbiochem/Merck), PP2A α (C-20, Santa Cruz, Heidelberg, Germany), PP2A-B56 γ (α -B56 γ , kind gift from Dr. Marc Mumby, Health Science Center, University of Texas, TX, USA), PP2A-B56 δ (α -B56 δ , Santa Cruz), myc-tagged BCA3 (α -myc, BD Biosciences, Franklin Lakes, NJ, USA) and actin (α -actin, Santa Cruz). Antibodies directed against phosphorylated serine were 1C8 (1), 4A3 (2), 4A9 (3), 4H4 (4), 7F12 (5) and 16B4 (6); they recognize phosphoserine with various efficiencies depending on the surrounding amino-acid motif (Enzo Life Sciences, Antwerp, Belgium). Horseradish peroxidase-conjugated goat antibody against rabbit or mouse immunoglobulin G, or rabbit antibody against goat immunoglobulin G (Sigma-Aldrich) was used as secondary antibody for signal detection by enhanced chemiluminescence. Films were quantified using Quantity One Analysis Software (Bio-Rad).

Protein interaction assays. Detection of a possible interaction of ST or ST-C103S mutant protein with PP2A in human HeLa cells was performed as follows. Twenty-four hours after DNA transfection, cells were washed twice with phosphate-buffered saline and harvested in ice-cold mild lysis buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100, 5 mM NaF, 1 mM Na₃VO₄, 20 mM beta-glycerolphosphate, and Protease Inhibitor Cocktail (Roche, Basel, Switzerland), followed by incubation on ice for 30 min. The supernatant of the lysates was prepared by centrifugation at 13 000 \times g and 4 °C for 30 min. Strep-tagged proteins and their interacting proteins were captured using the One-strep kit (IBA, Göttingen, Germany) according to the manufacturer's protocol, and resolved on SDS-PAGE, followed by western blotting analysis with appropriate antibodies.

RNAi assay. For human PP2A-B56 γ , the target sequence was: 5'-GATGAA CCAACGTTAGAAG-3'; for the PP2A-B56 δ two sequences were targeted (1) 5'-GTGTGTCTCTAGCCCCAT-3' and (2) 5'-GACCATTTTGCATCGCATC-3' (data not shown). The pSUPER vector was designated for shRNA plasmid constructions.⁴⁰ The amplification and purification of plasmids were performed as specified by manufacturer's instruction (GeneService, Cambridge, UK). Cells transfected with shRNA plasmids were lysed at 24–48 h after transfection, and then analyzed by western blot assay as described above.

Immunofluorescence assay. Cells were grown on glass coverslips. At indicated time points after transfection, coverslips were first washed once with phosphate-buffered saline, and subsequently fixed with methanol/acetone (50%/50%) for 5–10 min at room temperature. After air-drying, the slides were used for immunocytochemical staining or stored at –20 °C for further analysis. Immunocytochemical staining was carried out as described by Danen-Van Oorschot *et al.*³⁵ The following antibodies were used: α -108-P, a rabbit polyclonal antibody recognizing phosphorylated apoptin at T108 and α -Flag, a mouse monoclonal antibody recognizing Flag-apoptin. SV40 proteins were visualized with PAb416, a mouse monoclonal antibody recognizing the epitope residing in amino acids 83–128 of LT and non-reactive with ST or PAb280, a mouse monoclonal antibody against the C terminus of ST. The fluorescein isothiocyanate or rhodamine-conjugated goat antibodies (Jackson ImmunoResearch Laboratories, Newmarket, UK) were used as secondary antibodies. Nuclei were stained with 2,4-diamidino-2-phenylindole (DAPI) and apoptosis was assessed according to characteristic morphological changes.³⁵

PKA inhibition and stimulation. Saos-2 cells were transfected with pcDNA-Flag-apoptin and incubated 24 h post transfection with 10 μ M H89 (Merck Millipore, Billerica, MA, USA) for 1 h (PKA inhibition), or 30 min with H89 followed by incubation with 1 mM cAMP (Sigma-Aldrich) for another 30 min (PKA stimulation). Cells were then lysed in Laemmli buffer and the cell lysates analyzed for apoptin phosphorylation by western blotting. To determine the phosphorylation status of B56 δ , cells were lysed after treatment in mild lysis buffer as described for the protein interaction assay with addition of PhosSTOP (Roche). After 30 min of incubation, cell lysate was obtained by 15 min of centrifugation at 16 100 rcf at 4 °C. Whole-cell lysate was incubated 1.5 h with antibody either against B56 δ or against phosphorylated serines. μ MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were added for 1 h and proteins were

pulled down using the μ MACS column system according to the manufacturer's protocol. Elution was performed using Laemmli buffer, and proteins were resolved on SDS-PAGE, followed by western blotting analysis with appropriate antibodies.

Conflict of interest

The authors declare no conflict of interest.

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