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Interaction with Ppil3 leads to the cytoplasmic localization of Apoptin in tumor cells

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ABSTRACT

Apoptin, a small protein encoded by chicken anemia virus (CAV), induces cell death specifically in cancer cells. In normal cells, Apoptin remains in the cytoplasm; whereas in cancerous cells, it migrates into the nucleus and kills the cell. Cellular localization appears to be crucial. Through a yeast two-hybrid screen, we identified human Peptidyl-prolyl isomerase-like 3 (Ppil3) as one of the Apoptin-associated proteins. Ppil3 could bind Apoptin directly, and held Apoptin in cytoplasm even in tumor cells. We then demonstrated that the nuclearcytoplasmic distribution of Apoptin is related to the expression level of intrinsic Ppil3. Moreover, extrinsic modifying of Ppil3 levels also resulted in nuclearcytoplasmic shuffling of Apoptin. The Apoptin P109A mutant, located between the putative nuclear localization and export signals, could significantly impair the function of Ppil3. Our results suggest a new direction for the localization mechanism study of Apoptin in cells.

Apoptin, a 14 kDa protein encoded by VP3 gene of chicken anemia virus (CAV), represents a potential anticancer therapeutic agent, because it induces apoptosis specifically in human transformed or tumor cells, but not in normal cells [1]. The Apoptin mediated apoptosis is p53-independent [2] and can not be blocked by Bcl-2 [3,4]. The molecular mechanism of Apoptin is largely unknown. Several studies have demonstrated that nuclear localization of Apoptin is required for its induction of apoptosis [5,6]. It is well documented that the tumor-specific nuclear accumulation of Apoptin is mediated mainly by its C-terminal fragment, including a bipartite nuclear localization signal (NLS; amino acids 82–88 and 111–121) [8,9] and a nuclear export signal (NES; amino acids 97–105) [9,10]. The phosphorylation of threonine^{108} by an unknown tumor-specific kinase might also contribute to the activation of the NLS and inactivation of the NES in tumor cells [7,9].

Several Apoptin associated proteins, including DEDAF, Nur77, Nmi, Hippi, APC1, and p85 subunit of PI3-K [8,11–15] have been reported to interact with Apoptin and contribute to either its tumor-specific nuclear location or cytotoxicity. Previous screens have mainly focused on the downstream targets that trigger apoptosis, but the cancer selective upstream regulation of Apoptin remains to be determined. We performed a yeast two-hybrid assay and found human peptidyl-prolyl isomerase (cyclophilin)-like 3 (Ppil3) was another protein that interacted with Apoptin and contributed to its tumor-specific nuclear localization. *PPIL3*, also known as CyP J, belongs to the cyclophilin (CyP) family and shows a 46% sequence identity to Cyclophilin A [16,17]. Cyclophilins, also called peptidyl-prolyl isomerases (PPIase), belong to a large family which can specifically catalyze *cis–trans* isomerization of peptidyl-prolyl imidic bonds [18,19] and act as molecular catalysts or chaperones in protein folding, assembly, or repair. Members of this family have been reported to be functional in mitochondrial maintenance, apoptosis, cell cycle progression, T-cell regulation, inflammation, and many other cellular processes [19–23]. Furthermore, the expression and functions of cyclophilins may be correlative with the tumor biology of several types of cancers [19,24–29].

As a PPIase, Ppil3 might regulate the conformation of Apoptin through a direct protein–protein interaction, and contribute to the characteristic of Apoptin in cells. In this work, we confirmed this physical interaction, and found that both intrinsic and extrinsic up-regulation of *PPIL3* correlated with the cytoplasmic localization of Apoptin in hepatocellular carcinoma (HCC) cell lines tested. And the siRNA based knockdown of *PPIL3* was able to recover the nuclear localization ability of Apoptin. Further, we showed that proline^{109} of Apoptin is required for its Ppil3 depended cytoplasmic distribution. Our results suggest a new insight into the physiological regulation of the viral protein Apoptin.

Abbreviations: PPIase, peptidyl-prolyl *cis–trans* isomerase; *PPIL3*, peptidyl-prolyl isomerase-like 3; NLS, nuclear localization signal; NES, nuclear export signal; HCC, hepatocellular carcinoma; *GST*, glutathione-*S*-transferase; *EGFP*, enhanced green fluorescent protein; *HPRT1*, hypoxanthine phosphoribosyltransferase-1; DAPI, 4′,6-diamino-2-phenylindole.

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Materials and methods

Yeast two-hybrid screen. The screen was done according to the protocol of Matchmaker GAL4 Two-Hybrid System 3 (Clontech, CA, USA). A full length of *APOPTIN* coding region was cloned in frame with the yeast GAL4 DNA-binding domain (BD) into the pGBKT7 vector. It exhibited no toxicity and could not self-activate reporter genes in the yeast strain AH109. A human ovary cDNA library fused to the GAL4 activation domain (AD) in pACT2 vector (Clontech) was screened under the medium-stringency situation (SD/-His/-Leu/-Trp). The candidates were picked up and retransformed into the AH109/BD-Apoptin or AH109/BD-Null strains for high-stringency screening (SD/-His/-Leu/-Trp/-Ade). The positive clones were sequenced and network BLAST searches were conducted, using the NCBI online service.

GST pull-down assay. Full coding regions of *APOPTIN* and *PPIL3* were cloned in frame into the pGEX-4T (for GST fusion, Amersham, UK) or pET28 (for 6× His tag, Novagen, Germany) vectors. The *Escherichia coli* strain BL21 (DE3) transformed with pGEX-4T empty vector, pGEX-4T-APOPTIN, pGEX-4T-PPIL3, pET28-APOPTIN or pET28-PPIL3 were induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h. Cells were then washed and lysed by sonication in ice-cold PBS with proteinase inhibitors. The cleared protein lysates were mixed with 25 μl bed volume of pre-washed glutathione sepharose 4B beads (Amersham). After agitating at 4 °C for 2 h, beads were washed 5 times with PBS-T (PBS plus 0.1% Tween 20) and eluted by Elution Buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0). After boiling for 5 min with equal amount of 2× SDS sample buffer, 20 μl aliquots of the supernatants were analyzed by SDS–PAGE and immunoblotting. The blot was probed with an anti-6× His monoclonal antibody (Novagen) and detected by ECL chemiluminescence kit (Pierce, IL, USA) according to the manufacturer's instructions.

Mammalian cell culture and transfection. Hepatocellular carcinoma cell lines hepG2, hep3B, and huh7 were grown in DMEM medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (PAA, Austria). The cells were grown at 37 °C with 5% CO_2 in a humidified incubator. EGFP and Myc tagged Apoptin/Ppil3 expressing vectors were generated based on pcDNA3.0 or pcDNA3.1-Myc/His vectors (Invitrogen). Full length of *APOPTIN* and *PPIL3* coding sequences were fused in frame to the 3′ of EGFP or 5' of Myc tag. Plasmids were transfected into the preseeded cells by calcium phosphate transfection method. Generally, 150 ng of total plasmids were used for each well of 48-well plate. Two siRNAs specific anti-*PPIL3* (siPPIL3_536 and siPPIL3_676) were designed by an online tool, siDESIGN Center (Dharmacon, IL, USA). siRNAs were transfected by Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's recommendations, at a final concentration of 20 nM. The siRNAs were synthesized as the following sequences:

siNC:

5′-UUCUCCGAACGUGUCACGUTT-3′/5′-ACGUGACACGUUCGGAGAATT-3′

siPPIL3_536:

5′-GGUAAUAGAUGGUCUGGAATT-3′/5′-UUCCAGACCAUCUAUUACCTT-3′

siPPIL3_676:

5′-GGACAAAUAACUUGACAAATT-3′/5′-UUUGUCAAGUUAUUUGUCCAG-3′

sqRT-PCR detection. For expression level detection, total RNAs of the cells were isolated by guanidium thiocyanate–phenol–chloroform extraction and reverse transcribed to first strain cDNAs. Twenty-five to 28 cycles of semi-quantitative RT-PCR were then performed and *HPRT1* served as an internal control. The cDNA specific primers used were list here:

RT-PPIL3:

5′-CACCCAAAACATGTGAGAAT-3′ and 5′-CCAGACCATCTATTACCTTTC-3′

RT-HPRT1:

5′-GCCCTGGCGTCGTGATTAG-3′ and 5′-GTGATGGCCTCCCATCTCCTT-3′

Subcellular localization of Apoptin. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.3% Triton X-100, and then blocked by 5% goat serum. Myc-tag proteins were immunostained with anti-Myc monoclonal antibody (sc-40, Santa Cruz, CA, USA), followed by Alexa-568 conjugated goat anti-mouse secondary antibody (Invitrogen). Finally, 10 μg/ml 4′,6-diamino-2-phenylindole (DAPI) was added to visualize the nuclei of cells. The images were analyzed by fluorescence microscopy (Leica, Germany). Apoptosis was defined as the nuclear condensed cells. The nuclearcytoplasmic distributions of Apoptin were calculated by comparison to nuclei stained by DAPI in non-apoptotic cells.

Results and discussion

Aimed to identify human cellular proteins that interact with Apoptin, we performed a yeast two-hybrid screen, based on Matchmaker GAL4 Two-Hybrid System 3. The positive clones, which specifically interacted with BD-Apoptin, but not BD-Null baits under both medium- and high-stringency situation, were picked up, sequenced, and BLAST searched. This screening resulted in the isolation of several cDNA clones encoding nine Apoptin-associated proteins (data not shown), including several collagen and actin related proteins as observed previously [30]. One of the remaining clones encoded human peptidyl-prolyl isomerase (cyclophilin)-like 3 (Ppil3) protein. This suggested to us that Ppil3 might regulate the localization and proapoptic activity of Apoptin through binding to Apoptin and shifting its conformation just like the functions of other peptidyl-prolyl *cis–trans* isomerase family members.

To confirm the hypothesis, in vitro glutathione-*S*-transferase (GST) pull-down assays were performed first to test the binding of Ppil3 and Apoptin. As expected, the His-tagged Ppil3 was coprecipitated with GST-Apoptin, but not with GST itself. When exchanged the tag, Apoptin-His protein was also only trapped by GST-Ppil3 (Fig. 1). The group consisting of GST-Apoptin and Apoptin-His served as a positive control, because Apoptin could form multimeric complexes by itself [31,32]. These data indicated a specific, direct, and strong interaction between Ppil3 and Apoptin in vitro.

The intrinsic *PPIL3* levels of several cancer cell lines were then detected by semi-quantity RT-PCR. Most cell lines had low *PPIL3* mRNA levels, compared to the internal control, Hypoxanthine phosphoribosyltransferase-1 (*HPRT1*) mRNA (Data not shown). But in huh7 cells, it showed a high level of *PPIL3*, while another HCC cell line hepG2 had a very low level (Fig. 2A). To delineate the characteristic of Apoptin in these cell lines, both EGFP and Myc tagged Apoptin expression vectors were transfected by calcium phosphate transfection method. Forty-eight hours after transfection, as observed previously, in hepG2 cells both EGFP-Apoptin and Apoptin-Myc presented a perfect nuclear localization, only few cells (less than 5%) had weak cytoplasmic Apoptin signals. While in huh7 cells, Apoptin exhibited a partially cytoplasmic rather than completely nuclear localization (Fig. 2B). The two different fusion Apoptin proteins had the similar manners in each cell line, therefore the phenomenon was determined by Apoptin itself but not caused by the tags, although EGFP protein itself did show a little stronger nuclear than cytoplasmic signals. The hep3B cells with a moderate *PPIL3* levels presented a nuclearcytoplasmic Apoptin ratio between hepG2 and huh7 (data not shown). The re-

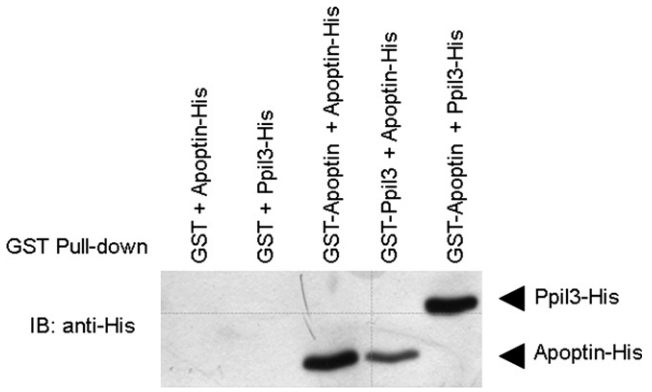

Fig. 1. Direct interaction between Apoptin and Ppil3 shown by GST Pull-down assay. The *E. coli* lysates containing GST, GST-Apoptin, GST-Ppil3, and Apoptin-His, Ppil3-His were coincubated and immobilized on glutathione sepharose beads. The bound proteins were pulled down and separated on SDS–polyacrylamide gel. The anti-His Western blotting showed the tracked His tagged Apoptin and Ppil3.

sults above showed that the cytoplasmic distribution of Apoptin in cells was correlative with *PPIL3* expression levels.

In addition, plasmid based overexpression and siRNA knockdown experiments were designed. EGFP-Apoptin/Ppil3-Myc and EGFP-Ppil3/Apoptin-Myc plasmid pairs were co-transfected into hepG2 cells. Forty-eight hours later cells were fixed and stained. Compared to the cells of low Ppil3 level, hepG2 cells that overexpressed either tagged Ppil3 showed elevated cytoplasmic Apoptin levels (Fig. 3), although they did not aggregate visibly as in normal cells [33] or tumor cells of high intrinsic Ppil3 level (huh7 in Fig. 2B).

On the other hand, GFP-Apoptin was transfected 24 h after the transfection of siRNAs in huh7. Cells were fixed and stained another 48 h later. Both anti-*PPIL3* siRNAs knocked down the intrinsic *PPIL3* level greatly, compared to the negative control siNC (Fig. 2A). By calculating the ratio of cells that were Apoptin cytoplasmic retentive, a statistically significant decrease of the ratio ($p < 0.0001$, revealed by Student's *t*-test) in siPPIL3 groups was found (Fig. 4B). All results above strongly indicated that Ppil3 participated in the nuclearcytoplasmic distribution pathway of Apoptin. But unlike that in normal cells, only part of the cells exhibited absolute cytoplasmic Apoptin [33], many cells had both nuclear and cytoplasmic Apoptin signals.

The C-terminal of Apoptin includes two putative NLS (amino acids 82–88 and 111–121) [8,9] and one NES (amino acids 97–105) [9,10], and it is reported that the phosphorylation of threonine^{108} in cancer cells is important in the regulation of these signals through conformation shifts [7,9,33]. At the same time, Ppil3, as a PPIase, may have the affinity for peptidyl-prolyl bonds to take *cis–trans* isomerization function [18,20]. Given the similar effect to Apoptin of threonine^{108} phosphorylation and *PPIL3* down-regulation, we supposed that the threonine^{108}–proline^{109} peptidyl-prolyl imidic bond of Apoptin might be the substrate of Ppil3, which contributed to the localization of Apoptin in cells (Fig. 4A). Therefore we generated a proline to alanine mutation at 109 (P109A) on Apoptin and fused it to the EGFP tag. Compared to wild type EGFP-Apoptin, EGFP-P109A mutant showed a greater nuclear distribution tendency even in huh7 cells, which was similar to the situation of wild type Apoptin under *PPIL3* knocked down

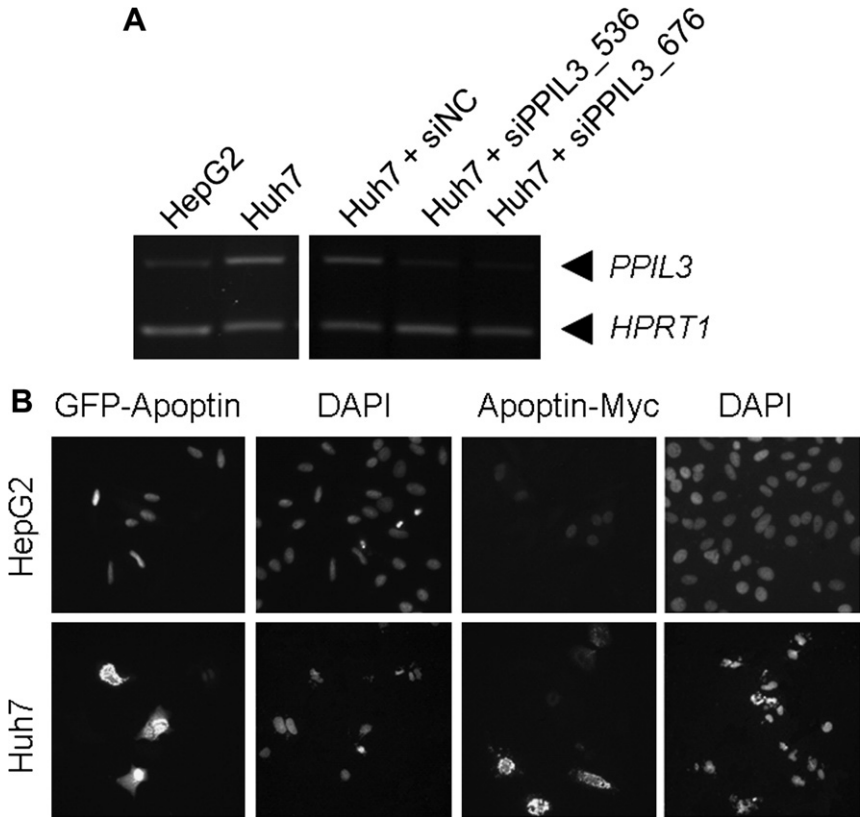

Fig. 2. Association between intrinsic *PPIL3* levels and nuclearcytoplasmic localization of Apoptin. (A) Cellular *PPIL3* mRNA levels detected by semi-quantity RT-PCR. HCC cell lines hepG2 and huh7 showed distinct levels of intrinsic *PPIL3*, compared to the *HPRT1* internal control gene. And after the transfection of siNC control or anti-*PPIL3* siRNAs, *PPIL3* expression levels in huh7 cells were greatly knocked down. (B) Fluorescence microscopy images of the subcellular distribution of Apoptin in hepG2 and huh7 cells. The cells were transfected with the EGFP or Myc tagged Apoptin encoding plasmids. Forty-eight hours later, cells were fixed and immunofluorescence stained. Comparing the fluorescence signals of Apoptin and nuclei shown by DAPI, Apoptin in huh7 cells presented a partially cytoplasmic localization, which was different from that in hepG2 cells.

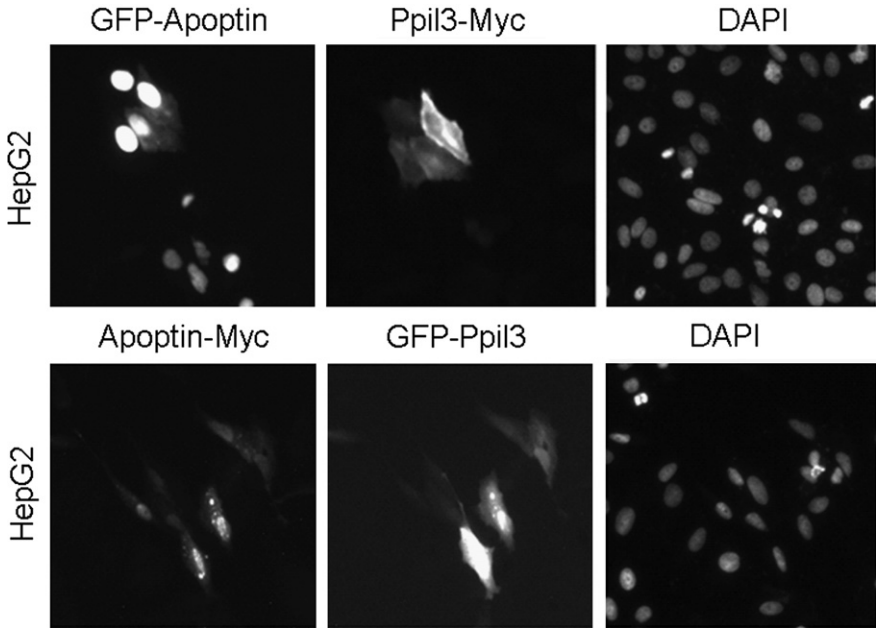

Fig. 3. Extrinsic overexpression of Ppil3 in hepG2 cells increased the cytoplasmic Apoptin. HepG2 cells were co-ransfected with EGFP-Apoptin/Ppil3-Myc and EGFP-Ppil3/Apoptin-Myc encoding plasmid groups. Cells were fixed and immunofluorescence stained 48 h later. Both groups showed elevated cytoplasmic Apoptin levels.

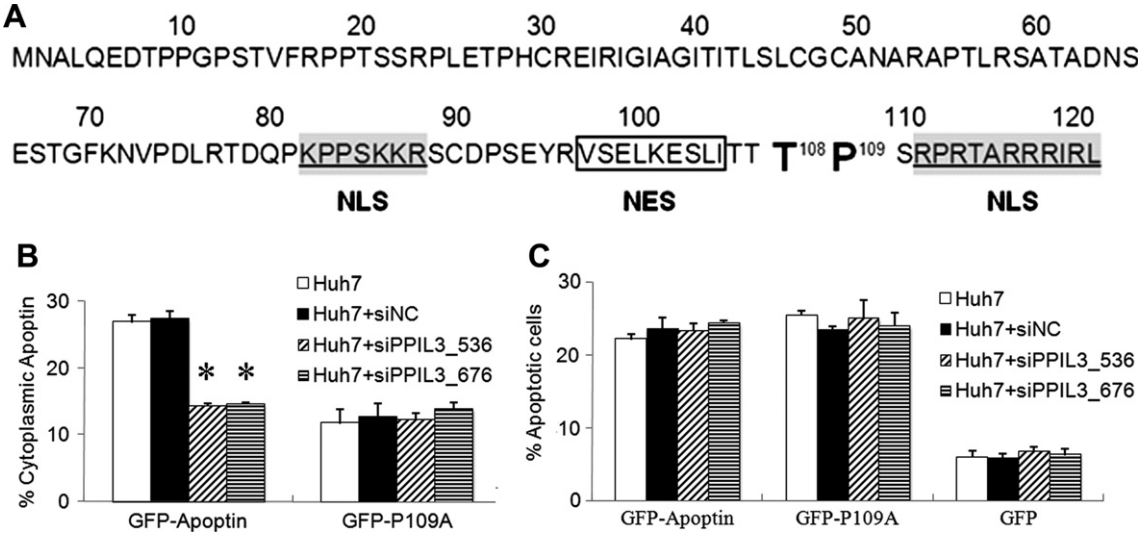

Fig. 4. Apoptin P109A mutant impaired the function of Ppil3. (A) Schematic representation of the Apoptin protein. The bipartite NLS and NES were highlighted, while T108 and P109 were in bold. (B) Huh7 cells were transfected with siRNAs and Apoptin encoding plasmids. Apoptin localization was determined in non-apoptotic cells, by counting 500–1000 positive cells in each experiment. The results show the mean values ± SD, $n = 3$. The data are statistically significant ($^{*}p < 0.0001$), as revealed by Student's t-test. (C) Apoptotic huh7 cells were calculated after siRNA and Apoptin treatment. Apoptosis was defined as the nuclear condensed cells. The results show the mean values ± SD, $n = 3$.

situation (Fig. 4B). This result suggested that the proline[109] of Apoptin was required for the association with Ppil3 and nuclearcytoplasmic shuffling.

Otherwise, the P109A mutant had similar proapoptotic ability to the wild-type Apoptin, with or without Ppil3 (Fig. 4C). In our results, highly expressed Ppil3 just held Apoptin in cytoplasm only in some cells; even in these cells nuclear Apoptin was still observable. The nuclear retention of Apoptin could continue to induce cell death. Therefore, no apparent changes of apoptotic rates before and after siPPIL3 treatments were found (Fig. 4C).

In conclusion, we have shown that Ppil3 bound to the C-terminal of Apoptin, which was crucial for its tumor-specific nuclear localization and proapoptotic activity, and this binding trapped Apoptin in the cytoplasm, even in tumor cells. It's possible that Ppil3 bound to the threonine[108]–proline[109] bond of Apoptin and shifted its conformation, through which Ppil3 modified the activities of the NES and/or NLS nearby. However, Ppil3 alone was not sufficient to completely trap Apoptin in cytoplasm and the interaction between Ppil3 and Apoptin could not affect the proapoptic activity of Apoptin in tumor cells. We think it is reasonable to posit that there are some other factors which take part in the association between Apoptin and Ppil3.

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