

Profilin potentiates chemotherapeutic agents mediated cell death via suppression of NF- κ B and upregulation of p53

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Abstract The molecular mechanism by which Profilin acts as a tumor suppressor is still unclear. Several chemotherapeutic agents, used till date either have unfavorable side effects or acquired resistance in tumor cells. Our findings show that Profilin enhances cell death mediated by several chemotherapeutic-agents. The activation of NF- κ B and its dependent genes, mediated by paclitaxel and vinblastine, was completely inhibited in Profilin overexpressing cells. This inhibition was due to the Profilin mediated attenuation of I κ B α degradation, thereby preventing p65 nuclear translocation and low NF- κ B DNA binding activity. Moreover, Profilin increases level of p53 in the presence of known inducers, such as doxorubicin, vinblastine, and benzofuran. This increased p53 level leads to enhanced cell death as indicated by activation of caspases 3, 8, 9, which results in cleavage of PARP. Furthermore, knocking down of p53 in Profilin overexpressing cells leads to decreased cell death. Ectopic expression of Profilin in HCT116 p53 knock out cells showed lesser cell death as compared to the HCT116 p53 wild type cells. For the first time, we provide evidences, which suggest that Profilin synergizes with chemotherapeutic drugs to induce tumor cell death by regulating NF- κ B and p53. Thus,

modulation of Profilin may be a useful strategy for effective combination therapy.

Keywords Profilin · NF- κ B · p53 · Chemotherapeutic drugs · Cell death · Caspases

Abbreviations

ANG1	Angiotensin-1
AraC	Arabinoside C
ATRA	All-trans retinoic acid
DAPI	4',6-Diamidino-2-phenylindole
ICAM	Intercellular cell adhesion molecule
IL	Interleukin
Mdm2	Mouse double minute 2 homolog
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B	Nuclear transcription factor kappa B
PARP	Poly-ADP ribose polymerase
Sp1	Specificity protein 1
VEGF	Vascular endothelial growth factor

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Introduction

Profilin is primarily considered as an actin binding protein [1–3]. However it has been shown to interact with variety of regulatory molecules involved in signaling, migration and metastasis, independent of its cytoskeleton related function [4, 5]. Profilin downregulation has been found to be associated with variety of tumors and aids in tumor progression. It acts as a tumor suppressor by upregulating SIRT3 and destabilizing HIF1 α in pancreatic cancer [6]. Additionally, Profilin interacts with Cathepsin X and

cleaves C-terminal of Profilin, involved in clathrin-mediated endocytosis and thereby, increases cell migration and invasiveness of prostate cancer cells, PC3 [7]. Besides, Profilin overexpression suppresses cell proliferation by G1 phase arrest via upregulating p27 in MDA-MB-231 breast tumor cell lines. [8].

Apoptosis is a series of precisely regulated events that efficiently remove dysfunctional cells and frequently found to be altered in tumor cells. Tumor suppressor genes, such as p53, involved in the promotion or inhibition of apoptosis is closely associated with modulation of cell cycle, disruption of which leads to tumor progression and chemoresistance [9–11]. The cell death mechanism triggered by p53 involves transcriptional activation of ‘pro-apoptotic’ Bcl-2 family proteins, such as Bax, Bid, Noxa and Puma [12–14]. Furthermore, p53 promotes mitochondrial release of cytochrome c and APAF-1, leading to the formation of apoptosome complex along with pro-caspase-9 [15, 16]. This complex facilitates the activation of caspases and cleavage of several proteins essential for cell viability, which leads to DNA fragmentation and nuclear condensation. Besides, an E3 ubiquitin ligase, murine double minute 2 (Mdm2), is a key component of p53-mediated signaling pathway. Its expression is regulated by transcriptional activation of p53 [17] and Sp1 [18]. Mdm2, in turn, regulates the levels of p53 by targeting p53 for ubiquitin-mediated degradation [19, 20].

NF- κ B is another transcription factor whose dependent genes are essential for cell survival. Mdm2 induces expression of p65 NF- κ B (RelA) through activation of Sp1 [21]. This NF- κ B exists predominantly as p65-p50 heterodimer, which remains in complex with an inhibitory subunit, I κ B α in the cytoplasm. Upon activation, IKK complex (IKK α , IKK β and IKK γ) phosphorylates I κ B α , leading to its ubiquitination and degradation. The p65-p50 heterodimer gets activated via post-translation modification in the cytoplasm and translocate to nucleus, where it binds to its promoter site and transcribes genes that are involved in cell proliferation and apoptosis. Tumor cells maintain high basal activity of nuclear NF- κ B, which is important for creating an inflammatory microenvironment during malignant progression [22].

In this study, we have shown that Profilin enhances chemotherapeutic agents mediated cell death via two distinct pathways. Firstly, it suppresses NF- κ B and its dependent genes, involved in cell proliferation. Secondly, it enhances expression of p53 by suppressing Sp1 transcription factor, thereby decreasing Mdm2 expression. For the first time, we provide evidences that suggest Profilin inhibits NF- κ B activation and upregulates p53, thereby negatively regulating cell survival.

Experimental procedures

Materials

DMSO, EDTA, EGTA, MTT, doxorubicin, paclitaxel, vinblastine, oleandrin, 4-MUP (4-methylumbelliferyl phosphate), propidium iodide (PI), and antibodies against tubulin and GAPDH, were obtained from Sigma Aldrich Chemicals (St Louis, MO, USA). Penicillin, streptomycin, neomycin, RPMI 1640, DMEM medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY). Antibodies against p21, phospho-p53, cleaved caspase 3, 8 and 9 were obtained from Cell Signaling Technologies (Danvers, MA, USA), whereas antibodies for I κ B α , PARP, cox2, p53, Mdm2, and ICAM1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gel shift oligonucleotides for NF- κ B, p53 and Sp1 were synthesized. Benzofuran was synthesized as reported earlier [23].

Plasmids

Constructs of Profilin1, p65, wild and dominant negative (IKK β -WT and IKK β -DN) were cloned in pcDNA3 as described previously [23]. For p53 gene knockdown, TP53 mission shRNA were obtained from Sigma Aldrich (St Louis, MO, USA). In a typical experiment, various constructs were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) for 6 h and then cultured in supplemented DMEM medium for 12–24 h, followed by various treatments.

Cell culture and generation of Profilin stable cells

The human breast cancer cell line, MDA-MB-231, used in this study was obtained from American Type culture collection (Manassas, VA, USA). HCT116 cells and p53 knockout HCT116 cells were kindly provided by Prof. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Cells were cultured in DMEM medium containing 10 % FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). For stable cell generation, MB-231 cells were transfected with pcDNA3.1 (+) Profilin1 using Lipofectamine 2000 reagent. G418 selection (800 μ g/ml) started 48 h post transfection and was maintained for 2 weeks before switching to regular culture medium. The vector transfected stable MDA-MB-231 cells will be considered as parental cells, whereas stable cells with Profilin will be designated as *Profilin*-stable cells in further studies.

Gel shift assay of NF- κ B, Sp1, and p53

To determine NF- κ B activation, an electrophoretic mobility shift assay (EMSA) was conducted essentially as

described previously [23]. Briefly, 8–10 µg nuclear extract proteins were incubated with ³²P end-labeled double-stranded NF-κB oligonucleotides for 30 min at 37 °C, and the DNA–protein complexes were separated from free oligonucleotides on 6.6 % native polyacrylamide gels. The double-stranded NF-κB oligonucleotide used in the experiment is 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3'. Similarly, Sp1 (5'-ATTCGATCGGGGCGGGGCGAGC-3') and p53 (5'-CCAAGCAAGTCCAGGCAGG-3') DNA bindings were assayed using double-stranded specific gel shift oligonucleotides.

Reverse transcriptase (RT)-PCR

One µg of total RNA, isolated by TRIzol method (Gibco BRL, Grand Island, NY) was reverse transcribed into cDNA by One step Access RT-PCR kit (Promega, Madison, WI), followed by the amplification of the gene of interest using gene specific primers for ICAM1, Cox2, and GAPDH. PCR was performed and amplified products were separated by agarose gel electrophoresis (2 %) and visualized by ethidium bromide staining [24]. The primer sequence and product size are as follows: Profilin: 117 bp {forward} 5'-ACGCCTACATCGACAACCTC-3', {reverse} 5'-TGATGTTGACGAACGTTTTCC-3'; ICAM1: 406 bp {forward} 5'-AGGCCACCCCAGAGGACAAC-3', {reverse} 5'-CCCATTATGACTGGGGCTGCTA-3'; VEGF: 344 bp {forward} 5'-ATGAACTTCTGCTGTCTTGGGT-3', {reverse} 5'-TGGCCTGGTGAGTTTGATC-3'; Angiopoietin 1: 500 bp {forward} 5'-GCCTACACTTTCATTCTTCCAGA-3', {reverse} 5'-TCTTCCTTGTGTTTTCCTTCCAT-3'; IL-8: 186 bp {forward} 5'-GCAGCTCTGTGTGAAGGTGCA-3', {reverse} 5'-CAGACAGAGCTCTCTTCCAT-3'; GAPDH: 192 bp {forward} 5'-ACCTGCCAAATATGATGAC-3', {reverse} 5'-TCATACCAGGAATGAGCTT-3'.

Immunoblotting

In a typical assay, 20–50 µg total proteins were used for Western blot. Cell lysates were resolved in SDS-PAGE, transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA), and blocked in standard Tris-buffered saline-0.15 % Tween-20 (TBST) with 5 % milk or 5 % BSA (Sigma). Primary antibody was incubated in TBST at 4 °C overnight, followed by HRP-conjugated secondary antibody incubation for 1 h. The membrane was developed using the ECL system (170–5040, Bio-Rad).

Immunofluorescence staining

Cells, grown on coverslips were fixed with 3.7 % paraformaldehyde solution in PBS for 15 min. After

permeabilization with 0.5 % Triton X-100 buffer containing 20 mM HEPES (pH 7.4), 50 mM NaCl and 3 mM MgCl₂ for 10 min, cells were incubated with anti-Profilin for 2 h. After the incubation with fluorescent-conjugated secondary antibody (Alexa Fluor 488 or 594 goat anti rabbit or mouse) for 30 min, nuclei were counterstained with DAPI containing mounting medium (Vectashield, USA). All the steps were performed at room temperature, unless otherwise stated. Images were acquired by fluorescent microscope (Olympus, IX51).

Reporter gene transcription assay

The expression of NF-κB or p53-dependent reporter gene was carried out as described previously [25]. Cells were transiently transfected with Lipofectamine with 0.5 µg of reporter plasmid containing p53 binding site cloned upstream of luciferase (designated as *p53-luciferase*) or NF-κB binding site cloned upstream of secretory alkaline phosphatase (*NF-κB-SEAP*) and 0.5 µg of *GFP* constructs. After 6 h of transfection, cells were washed and cultured for 12 h, followed by treatment with different inducers of apoptosis. GFP positive cells were then counted. For luciferase assay, cell pellet was lysed and extract was incubated with the firefly luciferin substrate (Promega). Light emission was monitored with a Luminometer and values were calculated as fold of activation over vector-transfected value. For SEAP assay, the culture supernatant was analyzed for SEAP activity essentially as per the Clontech protocol (Palo Alto, CA) and reported as fold activation with respect to empty SEAP-transfected cells as described previously [26].

Apoptosis assay

Cytotoxicity assay The cytotoxicity was measured by MTT assay [26]. Briefly, cells (1 × 10⁴ cells/well of 96-well plate) were treated with different agents for indicated concentrations and times. Twenty-five microliters of MTT solution (5 mg/ml in PBS) was then added and incubated for 2 h. Thereafter, 100 µl extraction buffer (20 % SDS in 50 % dimethylformamide) was added. After an overnight incubation at 37 °C, absorbance was read at 570 nm with the extraction buffer as blank. **Determination of nuclear fragmentation** Cells, treated with several apoptotic inducers, were harvested and fixed in 80 % methanol, stained with propidium iodide (PI), and viewed under fluorescence microscope [27]. **Live and Dead assay** The cytotoxicity was determined by the Live/Dead assay [28] (Molecular Probes, Eugene, OR). Briefly, cells, after different treatments were stained with 'Live & Dead' cell assay reagent (5 µM ethidium homodimer, 5 µM calcein-AM). Red (as dead) and blue (as live) cells were analyzed under a fluorescence

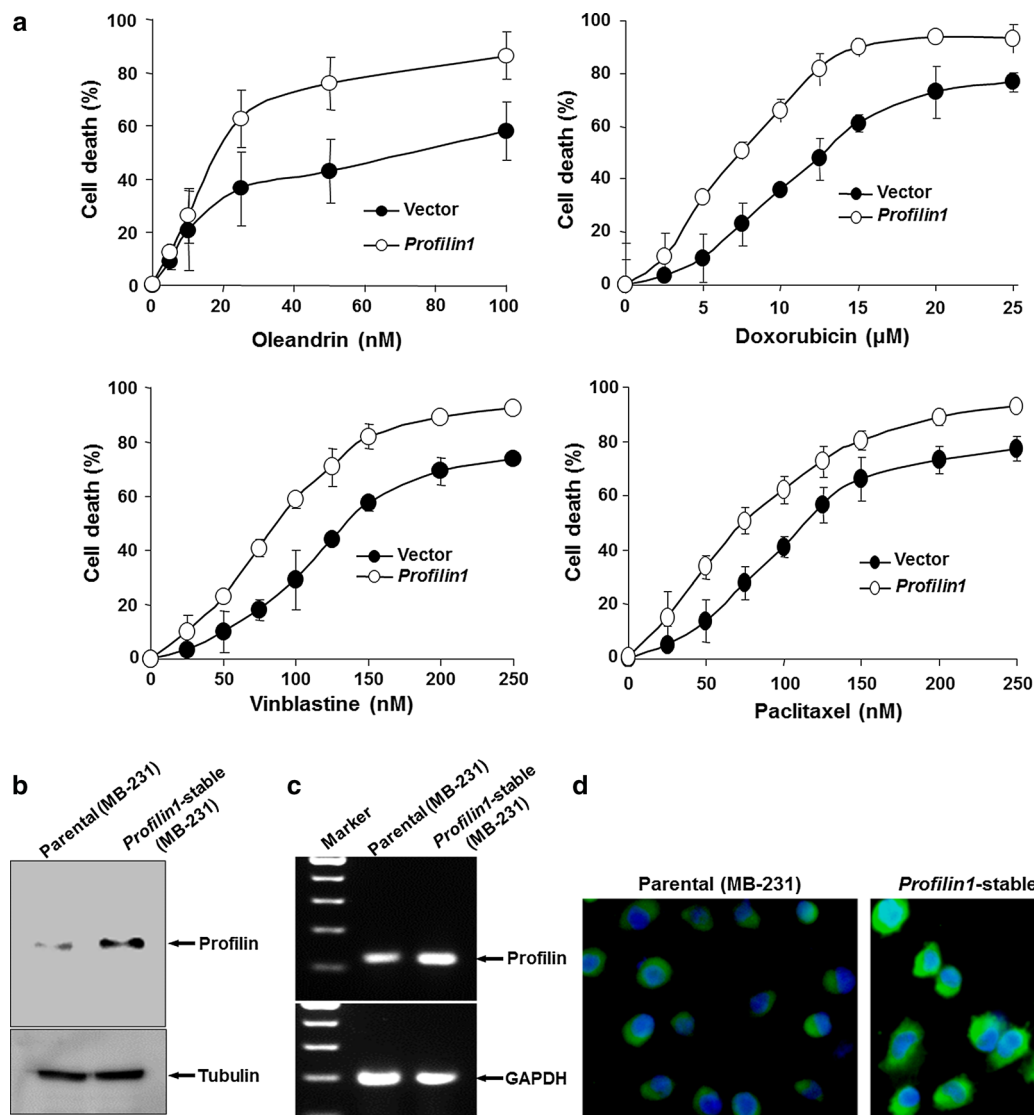


Fig. 1 Effect of Profilin on apoptosis by different apoptosis inducers and generation of *Profilin*-stable cells. **a** MDA-MB-231 cells were transiently transfected with *Profilin 1* for 24 h, followed by treatment with different concentrations of oleandrin, doxorubicin, vinblastine, or paclitaxel for 48 h. The cell viability was assayed by MTT dye and the absorbance was taken at 570 nm and indicated as percentage of cell death. **b, c** MDA-MB-231 cells were prepared for *Profilin 1* stable cells (*Profilin*-stable) as described in the Experimental

microscope. *PARP*, *caspase 3*, *8*, and *9* cleavages Cells, after treatments were pelleted, washed and extracted with whole cell extraction buffer. Caspase 3, caspase 8, caspase 9 and *PARP* (pro and cleaved) bands were detected from 50 to 100 μg extract proteins by Western blot.

Statistical analysis

All data were presented as mean ± standard deviation (S.D.)/standard error of mean (S.E.M.) from three

procedures section. The amount of Profilin was determined by Western blot and RT-PCR, respectively from parental and *Profilin*-stable cells. Tubulin and GAPDH were served as loading control for Western blot and RT-PCR, respectively. **d** Parental and *Profilin*-stable cells were subjected to immunocytochemistry to detect the amount of Profilin using anti-Profilin antibody followed by FITC-conjugated secondary antibody

independent experiments. Statistical analysis was performed using one-way ANOVA followed by a post hoc Tukey test. Results were analyzed and illustrated by SPSS statistical software package (SPSS for Windows, version 16). Comparisons are done within and between the test groups (i.e. Parental cells and *Profilin*-stable cells). Asterisk (*) symbol indicates statistical difference between Parental and *Profilin*-stable cells, whereas Number (#) and Dollar (\$) signs indicate statistical difference within Parental and *Profilin*-stable cells, respectively. Significance of results was determined as $p \leq 0.01$ and $p \leq 0.05$.

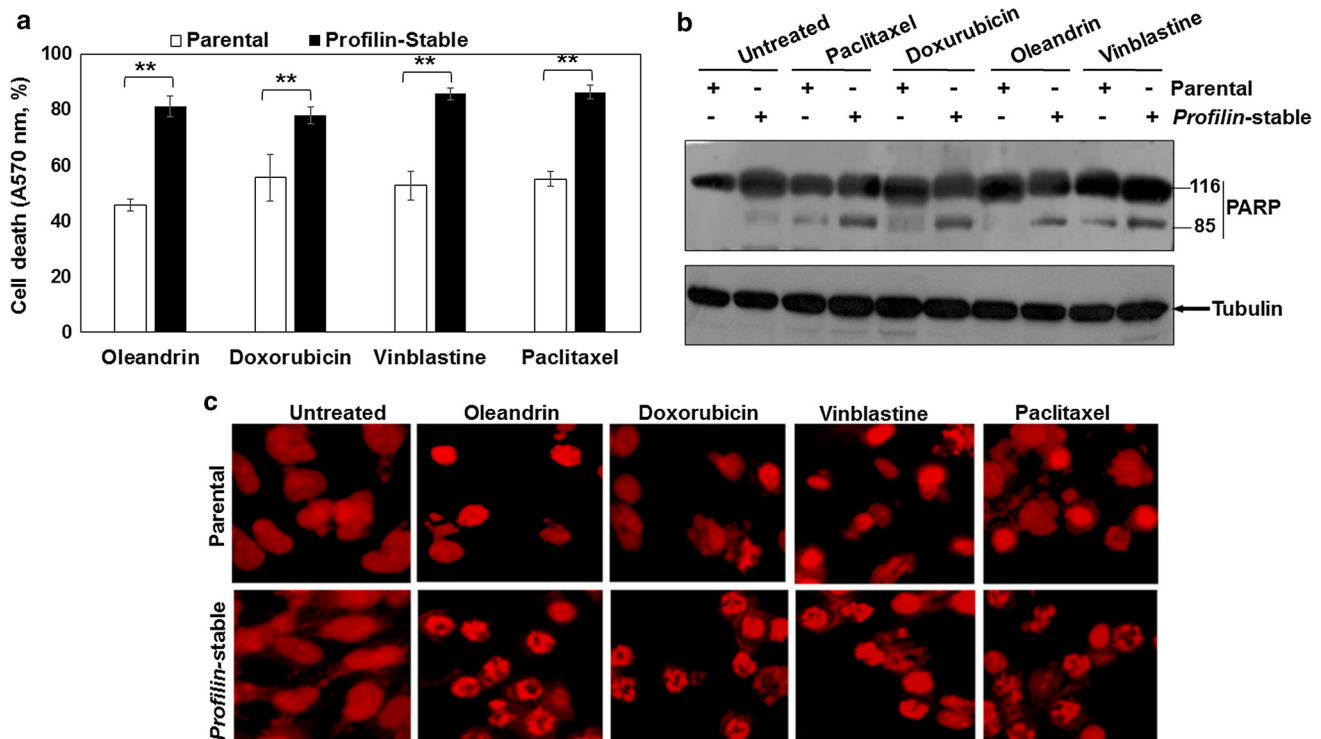


Fig. 2 Effect of Profilin overexpression on cell death. **a** Parental and Profilin-stable MB-231 cells were treated with paclitaxel (0.5 μ M), vinblastine (0.5 μ M), doxorubicin (1 μ M) and oleandrin (100 nM) for 48 h. Cell viability was determined by MTT assay. The experiment was repeated thrice and one of the figures represented here. Error bars represent as S.E.M. Asterisk (*) symbol indicates statistical

difference between Parental and *Profilin*-stable. $**p \leq 0.01$ (one-way ANOVA followed by Tukey test). **b** The amount of PARP was determined from WCE of these cells with similar treatments and detected by Western blot. **c** The cell death was detected by nuclear fragmentation assay from similar treatments and detected by PI stained cells' nuclei

Results

Human Profilin overexpressing breast cancer cell line, MB-231 upon treatment of several agents did not show any cytolysis as determined by lactate dehydrogenase assay.

Profilin transfected cells potentiate cell death upon exposure to inducers of apoptosis

To understand the role of Profilin in cell death, breast tumor cells, MDA-MB-231 were transfected with *Profilin1*. Vector or *Profilin1*-transfected cells were then treated with varying concentration of doxorubicin, paclitaxel, vinblastine, or oleandrin for 24 h and cytotoxicity was assayed by MTT. *Profilin1* transfection alone did not cause any cell death but showed significant cell death in presence of these agents. All the chemotherapeutic-agents induced 20–40 % of cell death in vector-transfected cells whereas, *Profilin*-transfected cells showed 50–80 % of cells death (Fig. 1a). These results suggest that Profilin overexpression sensitizes cell death in MDA-MB-231 cells.

For further study, MB-231 cells were transfected with empty vector or *Profilin1* and stable cells were generated

(to be referred as Parental and *Profilin*-stable cells, respectively). The expression of Profilin was determined by Western blot (Fig. 1b) from whole cell extract proteins, RT-PCR (Fig. 1c) and Immunofluorescence (Fig. 1d) in parental and *Profilin*-stable cells. The amount of Profilin was increased considerably in *Profilin*-stable cells as compared to low basal level in parental MB-231 cells.

Profilin potentiates chemotherapeutic agents mediated cell death

To understand the role of Profilin in apoptosis, MB-231 (parental and *Profilin*-stable) cells were incubated with apoptotic inducers (doxorubicin, oleandrin, paclitaxel or vinblastine) and cytotoxicity was measured by MTT assay. These agents showed almost 40–50 % of cell death in parental cells, whereas, 70–90 % of cell death in *Profilin*-stable cells (Fig. 2a). This cell death was found to be significant in *Profilin*-stable cells as compared to that in parental cells ($p \leq 0.01$). Further, cell death was increased in a concentration-dependent manner in *Profilin*-stable cells in presence of the agents (Supplementary Fig. 1). The PARP (116 kDa protein pro-band) get cleaved to 85 kDa

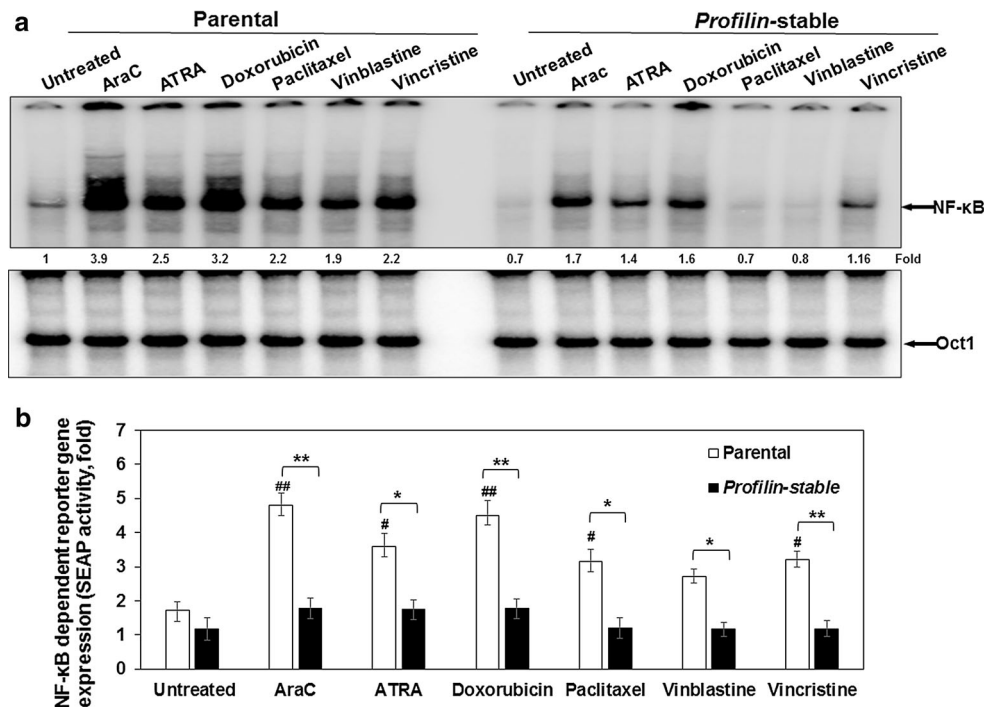


Fig. 3 Effect of Profilin overexpression on NF- κ B activation. **a** Parental and *Profilin*-stable cells were treated with ATRA, AraC, doxorubicin, vinblastine, paclitaxel, or vincristine for 6 h. Cells were washed with PBS and pelleted. Nuclear extracts (NE) were prepared and used to measure NF- κ B and Oct1 DNA binding by gel retardation. **b** Cells, transfected with NF- κ B-SEAP and GFP constructs for 12 h were treated with these agents for 12 h. The

culture supernatant was used to determine the activity of SEAP and indicated in fold change. Error bars represent as S.D. Asterisk (*) symbol indicates statistical difference between Parental and *Profilin*-stable, whereas Number (#) symbol indicates statistical difference within Parental. ** or ## $p \leq 0.01$, * or # $p \leq 0.05$ (one-way ANOVA followed by Tukey test)

band upon treatment of these inducers of apoptosis in parental cells and the intensity of cleaved band increased further in *Profilin*-stable cells (Fig. 2b). Furthermore, fragmented nuclei were observed in parental cells upon treatment of these agents. These nuclei were further increased in *Profilin*-stable cells, after PI staining (Fig. 2c). These results clearly demonstrate that Profilin potentiates cell death.

Profilin suppresses NF- κ B activation

To understand the mechanism of Profilin in potentiation of apoptosis, NF- κ B, the key molecule for cell proliferation, was assayed. To address this, parental cells were stimulated with various inducers of NF- κ B such as AraC, ATRA, doxorubicin, paclitaxel, vinblastine or vincristine and NF- κ B DNA binding was found to increase at different potency, as determined by gel shift assay. In *Profilin*-stable cells, these inducers were either unable to induce or showed mild induction of NF- κ B DNA binding (Fig. 3a, upper panel) whereas, the amount of Oct1 was found to remain unchanged (Fig. 3a, lower panel). Further, the NF- κ B dependent secretory alkaline phosphatase (SEAP)

expression was found to increase in parental cells ($p \leq 0.05$) upon stimulation with these agents. However, it was found to be suppressed significantly in *Profilin*-stable cells upon stimulation with AraC, doxorubicin, and vincristine ($p \leq 0.01$); ATRA, vinblastine and paclitaxel ($p \leq 0.05$), as compared to that in parental cells, as shown by the activity of SEAP in fold (Fig. 3b). These data suggest that Profilin might be involved in suppressing NF- κ B activation upon stimulation of various activators.

Vinblastine or paclitaxel increased the NF- κ B DNA binding in a dose-dependent manner in parental cells, which was completely suppressed in *Profilin*-stable cells (Fig. 4a, b1). Paclitaxel-mediated decrease in the amount of I κ B α was observed in parental cells but not in *Profilin*-stable cells (Fig. 4b2). Further, the amount of ICAM1 was increased by paclitaxel in parental cells, whereas it was completely inhibited in *Profilin*-stable cells, as detected by Western blot (Fig. 4c) and RT-PCR followed by PCR (Fig. 4d). RT-PCR also revealed the decreased expression of NF- κ B-dependent genes involved in angiogenesis such as VEGF, ANG1, and IL-8 in *Profilin*-stable cells (Supplementary Fig. 2). These results confirm the Profilin-mediated inhibition of NF- κ B and NF- κ B-dependent genes.

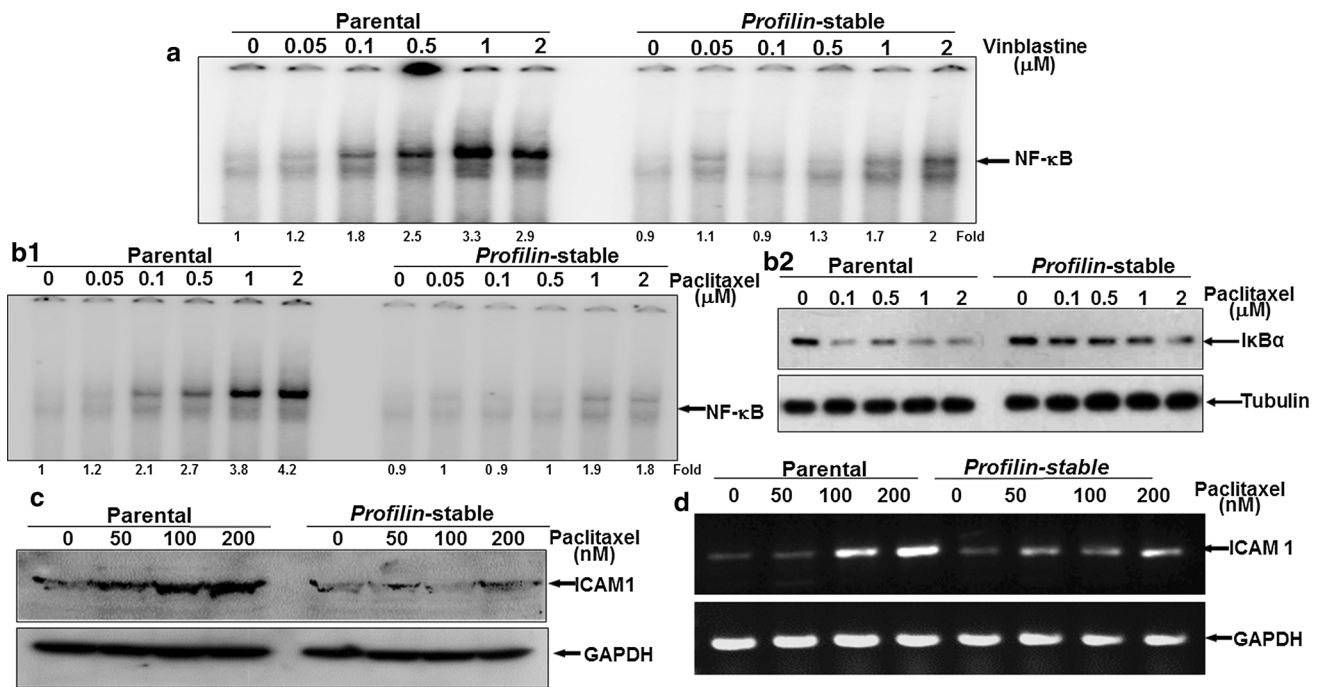


Fig. 4 Effect of paclitaxel and vinblastine on NF- κ B activation in *Profilin*-stable cells. **a**, **b1** Parental and *Profilin*-stable cells were treated with paclitaxel and vinblastine in a dose-dependent manner for 6 h. 8 μ g of NE was used to measure NF- κ B DNA binding by gel shift assay. **b2** Parental and *Profilin*-stable cells were treated with different concentrations of paclitaxel for 6 h. The amount of I κ B α

was determined from WCE by Western blot. **c** ICAM1 was measured from WCE (50 μ g of proteins) by Western blot from different concentrations of paclitaxel stimulated parental and *Profilin*-stable cells for 24 h. **d** Total RNA was also extracted and the amount of ICAM1 was determined by RT-PCR using specific primers, under similar experimental condition

IKK or p65 overexpression partially protects *Profilin*-mediated cell death

To confirm whether suppression of NF- κ B is the possibly involved in *Profilin*-mediated cell death, cells were transfected with *p65*, *IKK β* , or *IKK β -DN* constructs, followed by treatment with Paclitaxel. As expected, cell death was observed in parental cells upon incubation with paclitaxel, which was further potentiated in *Profilin*-stable cells ($p \leq 0.05$). However, in *p65*-transfected cells, the induction of cell death was reduced significantly ($p \leq 0.01$), as compared to empty vector in both parental and *Profilin*-stable cells (Fig. 5a). Moreover, the ectopic expression of *IKK β* significantly rescued the cell death in parental cells ($p \leq 0.01$), but failed to protect from cell death in *Profilin*-stable cells, as compared with *p65*-transfected cells ($p \leq 0.01$). Further, in *IKK β -DN* dominant negative transfected cells (*IKK β -DN*), the cell death was further enhanced significantly upon paclitaxel treatment as compared to that in *p65*-transfection in both types of cells ($p \leq 0.01$). The expression of p65, *IKK β* and *IKK β -DN* was also detected by Western blot (Fig. 5b). These data suggest that the NF- κ B inhibition is important for *Profilin*-mediated cell death, as p65 overexpression is able to rescue paclitaxel-mediated cell death.

Paclitaxel potentiates apoptosis in *Profilin*-expressed cells

Parental and *Profilin*-stable cells were treated with increasing concentrations of paclitaxel. Cell lysate was prepared and the activation of various caspases was detected by Western blot. The results showed that the paclitaxel partially increased the cleaved bands of caspases 3 and 8 in parental cells, whereas it was found to be more significant in *Profilin*-stable cells (Fig. 6a, upper panels). Moreover, the caspase-dependent cleavage of PARP was determined by Western blot. In parental cells, Paclitaxel induced PARP cleavage partially at 24 and 48 h of incubation, whereas in *Profilin*-stable cells, it induced PARP cleavage even at 12 h of incubation (Fig. 6a, lower panel). Moreover, Paclitaxel increased cell death up to 40 % in parental cells, whereas it was almost 80 % in *Profilin*-stable cells, as determined by Live & Dead assay (Fig. 6b, c).

Profilin suppresses Mdm2 expression and thereby upregulates p53

As tumor suppressor p53 plays an important role in mitochondrial pathway of cell death, its association with

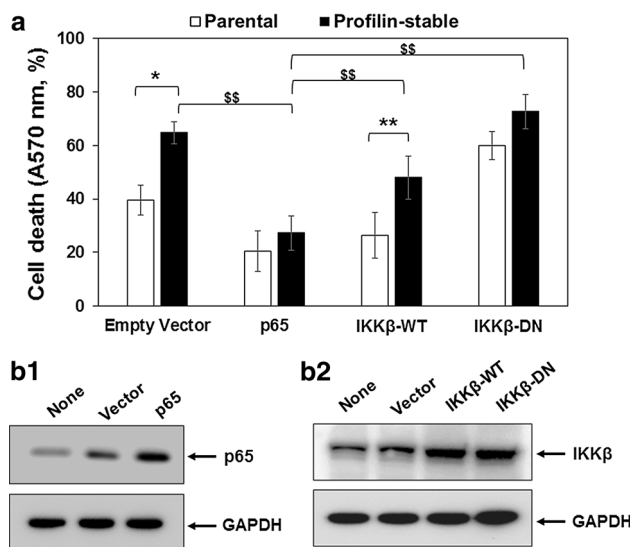


Fig. 5 p65 and IKK rescue paclitaxel-induced cell death in Profilin overexpressed cells. **a** Parental and Profilin-stable MDA-MB-231 cells were transiently transfected with *p65* and *IKKβ* wild and dominant negative constructs. After 12 h, cells were then treated with paclitaxel (1 μ M) for 24 h. Cell death was then measured by MTT assay. Error bars represent as S.E.M. Asterisk (*) symbol indicates statistical difference between Parental and Profilin-stable, whereas Dollar (\$) symbol indicates statistical difference within Profilin-stable. ** or $^{\$}$ $p \leq 0.01$ and * $p \leq 0.05$ (one-way ANOVA followed by Tukey test). **b1**, **b2** The expression of p65 and IKK β was determined from *p65*, *IKKβ-WT* and *IKKβ-DN* transfected cells by Western blot from WCE

Profilin-mediated cell death was also determined. To address this, MDA-231 cells were transiently transfected with Profilin. Profilin-transfected cells showed a high basal activity of p53 than the vector-transfected or non-transfected cells. Further, doxorubicin, paclitaxel or benzofuran increased the levels of p53 in Profilin-expressing cells as determined by gel shift and Western blot (Supplementary Fig. 3). These data suggest that Profilin increases p53 expression.

As Profilin facilitates the availability of p53 in cells, the amount of p53 increased upon treatment of doxorubicin, paclitaxel or benzofuran in Profilin-stable cells than in the parental cells (Fig. 7a1, upper panel). Although, the basal activity of Sp1 was decreased upon benzofuran treatment in parental cells, but the Sp1 DNA binding activity of all these agents was completely suppressed in Profilin-stable cells (Fig. 7a1, lower panel). Moreover, these inducers significantly increased p53-dependent luciferase activity in Profilin-stable cells as compared to parental cells ($p \leq 0.01$) (Fig. 7a2). The levels of Mdm2 were further decreased; whereas the amount of p53 was increased in Profilin-stable cells as compared to that in parental cells. The levels of phospho-p53 and p21, also increased in Profilin-stable cells upon doxorubicin treatment (Fig. 7b). Furthermore, the amount of ‘pro-apoptotic’

Bid and Bax were increased, whereas the level of anti-apoptotic Bcl2 was decreased in Profilin-stable cells upon doxorubicin treatment (Fig. 7c). These results clearly indicate that the Profilin decreases Mdm2 level, thereby increasing p53 level, thus enhances apoptosis.

Knocking out or silencing of p53 decreases Profilin-mediated cell death

To understand the role of p53 on Profilin-induced apoptosis, HCT116 cells (parental and p53 knocked out by homologous recombination) were transfected with Profilin and then treated with doxorubicin. In HCT116 parental cells, Profilin increased the level of cleaved caspases 3, 9 and PARP than that in the *p53*^{-/-} cells (Fig. 8a). Moreover, knockdown of p53 upon transfection with p53 shRNA, doxorubicin-mediated cell death was reduced significantly in Profilin-stable cells (Fig. 8b). These data suggest that p53 has important role in Profilin-mediated cell death.

Discussion

Evasion of apoptosis is one of the key determinants involved in development and progression of cancer. For this, tumor cells acquire increased survival signaling that facilitates to chemo-resistance, making chemotherapeutics ineffective. Moreover, several chemotherapeutic agents used till date, have shown severe side effects in the treatment of cancers, mainly due to their high dose administration. In most of the tumor cells, the microfilament-interacting protein, Profilin is deregulated. Although, several reports suggest its role as a tumor suppressor [6–8, 29] but the molecular mechanism is still unknown. In this report, we provide the evidences that give insight into the mechanism of action of Profilin in tumor suppression and its role in combination therapy. Overexpression of Profilin in MDA-MB-231 breast cancer cells enhances cell death induced by chemotherapeutic drugs, like doxorubicin, vinblastine, and paclitaxel, suggesting the negative role of Profilin in cell survival.

Surprisingly, we have found that therapeutic agents are unable to induce NF- κ B activation in Profilin overexpressing cells. Complete inhibition of NF- κ B and its dependent genes even at higher concentrations of vinblastine or paclitaxel have been observed in Profilin overexpressing cells. Profilin inhibits I κ B α phosphorylation, thereby preventing its rapid degradation and suppresses downstream signaling to activate NF- κ B dependent genes such as ICAM1. Previous reports have shown that the invasiveness of breast cancer cells decreased considerably in Profilin overexpressing cells. Aggressive proliferation of

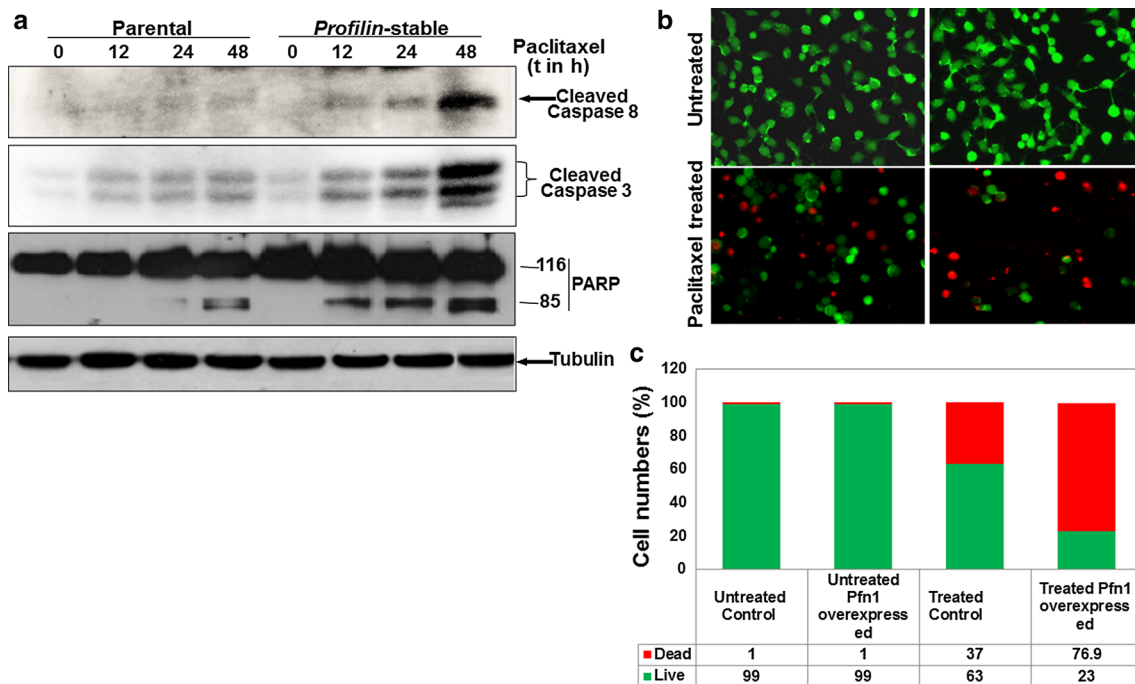


Fig. 6 Effect of Paclitaxel on Profilin overexpression. **a** Parental and *Profilin*-stable cells were treated with 200 nM Paclitaxel in a time dependent manner. Cells were washed and extracted for WCE. 100 μ g WCE protein was then probed for PARP, cleaved caspases 8 and 3 by Western blot. **b** Cell death was detected by Live and Dead

Cytotoxicity assay kit (Invitrogen) from similar treatment and detected by Calcein (for live cells, stained as *green*) and Ethidium homodimer (for dead cells, stained as *red*) stained cells. **c** Live and dead cells were counted at least in triplicates and average is shown in *bar diagram* (Color figure online)

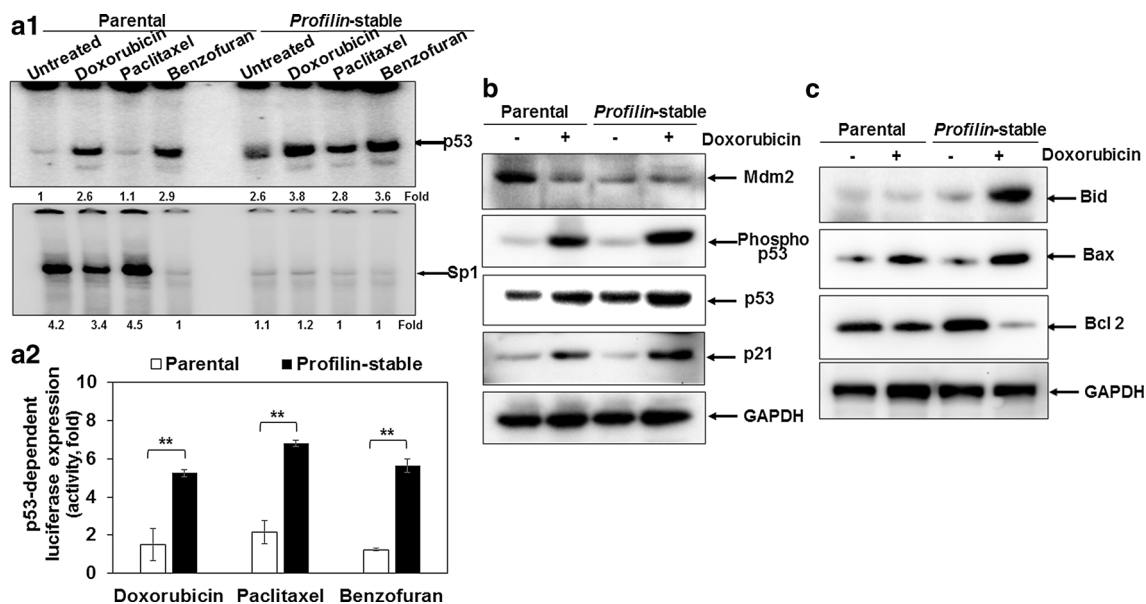


Fig. 7 Profilin overexpression enhances amount of p53. **a1** Parental and *Profilin*-stable cells were treated with doxorubicin, paclitaxel and benzofuran for 6 h. Nuclear extracts (NE) were prepared and 8 μ g of NE were used to measure p53 and Sp1 DNA binding by gel shift assay. **a2** Parental and *Profilin*-stable cells were transfected for 6 h with plasmids for *p53* promoter DNA that had been linked to luciferase reporter (*p53-Luc*) and *GFP*. After washing, cells were cultured for 12 h. The GFP-positive cells were counted, and transfection efficiency was calculated. Cells were treated with

doxorubicin, paclitaxel and benzofuran for 6 h. Cells were lysed and luciferase activity was measured. Results are represented as fold of activation over the non-transfected control. Error bars represent as S.D. Asterisk (*) symbol indicates statistical difference between Parental and *Profilin*-stable. $**p \leq 0.01$ (one-way ANOVA followed by Tukey test). **b** The amounts Mdm2, phospho-p53, p53, and p21 were determined from doxorubicin stimulated parental and *Profilin*-stable cells. **c** The amounts of Bid, Bax, and Bcl2 were determined from doxorubicin stimulated parental and *Profilin*-stable cells

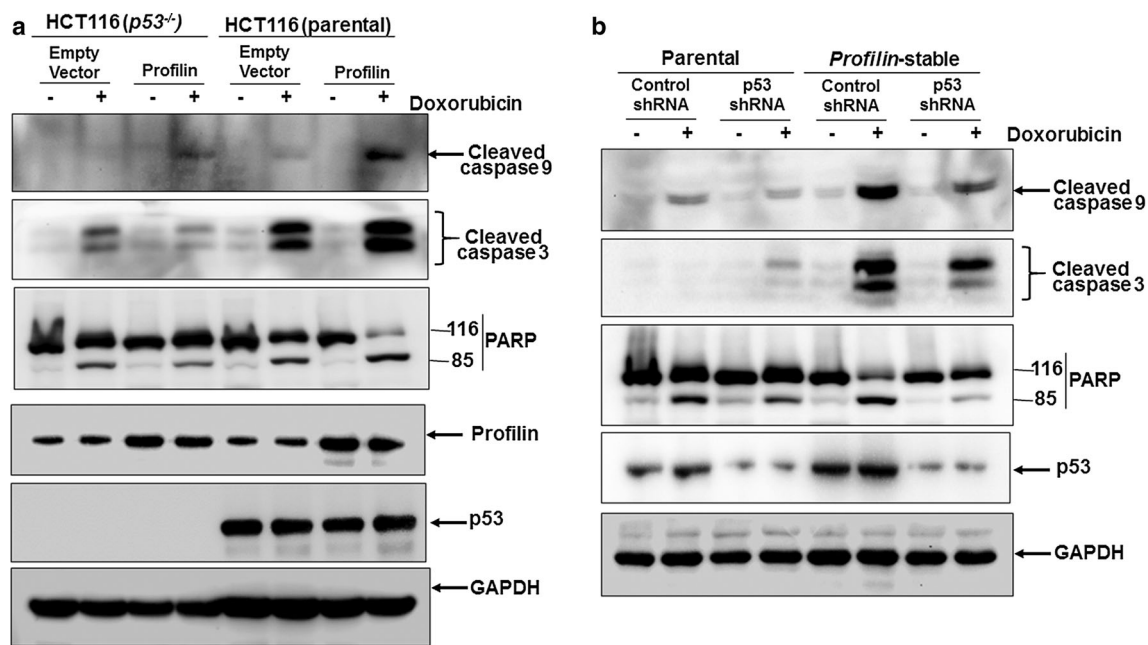


Fig. 8 Effect of Profilin-mediated cell death on p53-null or p53 knockdown cells. **a** HCT116 (parental and p53^{-/-}) cells, transiently transfected with *Profilin* for 24 h were treated with doxorubicin (1 μM) for 24 h. The amounts of cleaved caspase 3 and 9, and PARP

were determined by Western blot. **b** Parental and *Profilin*-stable cells were transfected with p53 shRNA for 24 h. Cells were treated with doxorubicin for 24 h and the amounts of cleaved PARP and caspase 3 and 9 were determined from WCE by Western blot

cells requires several growth factors, like FGF, VEGF, IL-8, etc. [30, 31] and all these factors are dependent on the transcriptional activation of NF-κB [32]. Ectopic expression of p65 in *Profilin* overexpressing cells show significant protection in paclitaxel-induced cell death, whereas overexpression of dominant negative IKKβ further enhances cell death, indicating the possible role of NF-κB signaling in apoptosis. However, IKKβ wild type was partially able to rescue cell death in *Profilin* overexpressing cells, suggesting that *Profilin* might be targeting IKK complex for NF-κB downregulation and requires further investigation.

Since, complete recovery from cell death was not observed, indicating that other signaling pathway might be involved in *Profilin* regulated cell survival. Tumor suppressor p53 is one of the most studied transcription factors, involved in cell survival during stress. Activation of the *p53* gene is known to play a role in arresting cell cycle followed by cell death [11, 12]. We have found that the amount of p53 expression was increased in *Profilin* transfected cells. Also, *Profilin* overexpressing cells showed an increase in p53-dependent luciferase activity in presence of chemotherapeutic agents. Moreover, overexpression of *Profilin* was unable to induce significant cell death in p53-null HCT116 cells as compared to p53 wild-type HCT116 parental cells. On the other hand, knocking down of p53 in *Profilin* overexpressing cells showed considerable decrease in cell death, which establishes the role of p53-mediated cell death in *Profilin* overexpressing cells.

Though p53 is mutated in MDA-MB-231, the studies have shown that mutated p53 was able to induce its dependent genes, p21 upon DNA damage [33]. As the levels of p53 in the cells are tightly regulated by its ubiquitin ligase Mdm2, the amount of Mdm2 expression has shown to decrease in *Profilin* overexpressing cells. Since, expression of Mdm2 depends on the transcriptional activation of NF-κB and Sp1 [18] and *Profilin* has shown to decrease the activity of Sp1. Thus, it is speculated that *Profilin* suppresses the transcriptional activation of Mdm2 and thereby inhibits the proteasome-mediated degradation of p53. As *Profilin* also attenuates the activation of NF-κB, there is a double-edged sword effect of *Profilin* to decrease the amount Mdm2 via inhibition of NF-κB and Sp1. The mechanism by which *Profilin* deregulates the housekeeping transcription factor Sp1 needs to be studied further.

Although, treatment of various chemotherapeutic agents did not show any cytolysis as determined by lactate dehydrogenase assay, a cytosolic marker for necrosis. Moreover, our results clearly show cell death by apoptosis, as indicated by activation of caspases 3, 8 and 9. Activated caspases further cleave PARP, a hallmark of apoptosis, leading to nuclear fragmentation and cell death. Proteolytic cleavage and activation of caspases are often preceded by mitochondrial targeting of p53. *Profilin* increases the levels of p53, which transactivates p21, causing cell cycle arrest. Under severe chemotherapeutic agents stress, elevated p53

levels also triggers the synthesis of ‘pro-apoptotic’ proteins such as Bid, Bax and suppression of ‘anti-apoptotic’ protein such as Bcl2 in Profilin overexpressing cells which results in enhanced cell death.

In conclusion, our observation suggests that Profilin mediates cell death by two distinct yet intervening pathways – i) attenuating NF- κ B activation via inhibition of I κ B α degradation that prevents rapid cell proliferation through NF- κ B-dependent gene activation; ii) upregulating p53 pathway by transcriptionally decreasing the levels of Mdm2 via suppression of Sp1. For the first time, we are providing data that support the role of Profilin in tumor suppression involving NF- κ B deregulation and p53 activation. This study might help us to target Profilin in palliation of tumorigenesis by modulating its expression in cancer. Profilin, in combination of lower doses of existing therapeutic drugs to target tumor cells, can underscore effective therapy with reducing side effects.

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Compliance with ethics standards

Conflict of interest All authors declare no conflict of interest.

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