

Downregulation of Hsp27 (HSPB1) in MCF-7 human breast cancer cells induces upregulation of PTEN

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Abstract Hsp27 (HSPB1) is usually overexpressed in breast cancers affecting the disease outcome and the sensitivity of tumors to chemotherapy and radiotherapy. Hsp27 interacts with other proteins such as β -catenin, histone deacetylase HDAC6, transcription factor STAT2 and procaspase-3. Phosphatase and tensin homologue (PTEN) is a tumor suppressor gene that is deleted in many human tumors. The PI3K/Akt signaling pathway is negatively regulated by PTEN. Hsp27 is described as a key component of the Akt signaling cascade: Akt, BAD, Forkhead transcription factors, Hsp27, mitogen-activated protein kinase kinase-3 and -6. Here, we have examined whether the downregulation of Hsp27 by siHsp27 affects the PTEN levels in the MCF-7 human breast cancer cell line. PTEN was detected with two different antibodies using

western blots and immunocytochemistry. p-Akt was also evaluated by western blot. In addition, Hsp27 and PTEN were immunoprecipitated to know whether these proteins interact. Intracellular colocalization studies were carried out by confocal microscopy. A significant reduction in the Hsp27 levels was noted in the siHsp27 transfected cells. These Hsp27 downregulated cells showed a significant increased expression of PTEN. The MW 76 and 55 kDa PTEN forms were upregulated as revealed by two different antibodies. The phosphatase activity of PTEN seems to be active because p-Akt levels were reduced. Hsp27 immunoprecipitation was bringing PTEN and vice versa, these two proteins seem to interact at cytoplasmic level by FRET. Downregulation of Hsp27 stabilized PTEN protein levels. Chaperone-assisted E3 ligase C terminus of Hsc70-interacting protein (CHIP) levels were not significantly influenced by Hsp27 downregulation. In conclusion, we report a novel function of Hsp27 modulating the PTEN levels in human breast cancer cells suggesting an interaction between these two molecules.

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Introduction

Hsp27 (HSPB1) is usually over-expressed in breast cancers affecting the disease outcome and the sensitivity of tumors to chemotherapy and radiotherapy (Vargas-Roig et al. 1998; Ciocca and Calderwood 2005; Ciocca et al. 2010). Hsp27 is a molecular chaperone that can interact with other proteins. For example, in human breast cancer tissues and in a rodent breast cancer cell line, Fanelli et al. (2008) reported the interaction of β -catenin with Hsp27 and with HSF1, which may explain some of the molecular pathways that affect the

prognosis of breast cancer patients. A recent study reported that histone deacetylase HDAC6, transcription factor STAT2 and procaspase-3 were degraded in human cancerous cells displaying genetically decreased levels of Hsp27 suggesting that they are client proteins of Hsp27 (Gibert et al. 2012). The downregulation of Hsp27 in cancer cells caused senescence which has been associated with activation of the p53 pathway and induction of p21 (O'Callaghan-Sunol et al.

2007). Nagaraja et al. (2012) have shown that short-term silencing of Hsp25 (rodent counterpart of human Hsp27) enhanced proteasome 28 subunit α mRNA and protein expression, increased proteasome activity as well as CD8+ T-cell-mediated tumor killing and memory responses, inducing the regression of established breast tumors.

Phosphatase and tensin homologue (PTEN) is a tumor suppressor gene involved in the regulation of many

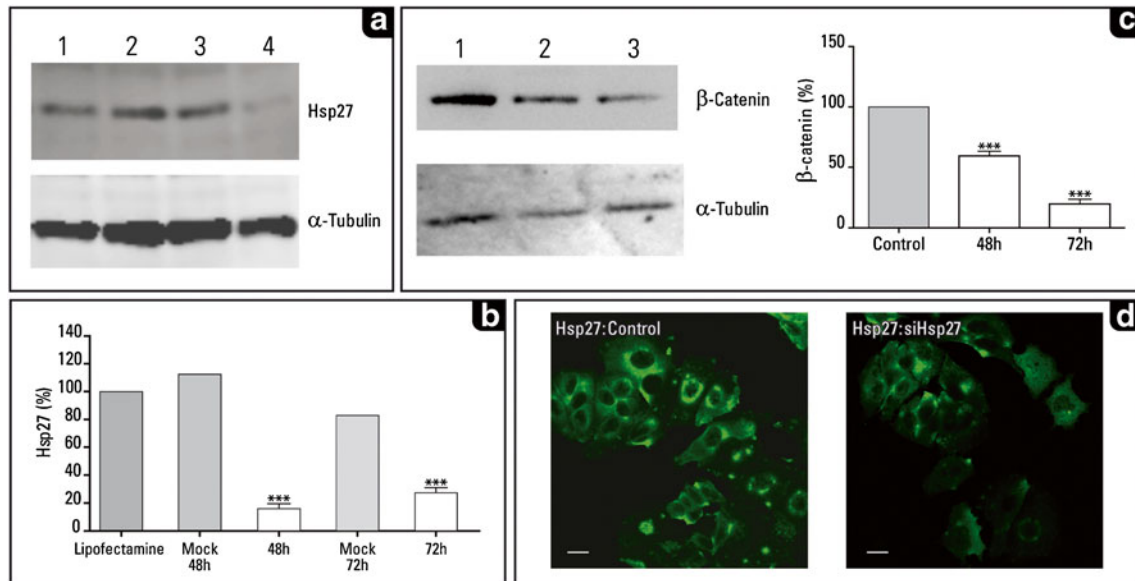


Fig. 1 Downregulation of Hsp27 and its effect on β -catenin expression in MCF-7 cells. **a** Hsp27 levels as revealed in the western blot analysis (performed as described previously; Fanelli et al. 2008). The antibodies used were: **a** mouse monoclonal antibody against Hsp27 (1:1,000; cat. # SPA-800, Stressgen Biotech. Corp., Victoria, Canada), **b** mouse monoclonal antibody against α -tubulin (1:16,000; Sigma Chem. Co., St. Louis, MO, USA), and **c** mouse monoclonal antibody anti- β -catenin (1:500; cat. # 18-0226; Zymed, Carlsbad, CA, USA). **1** Control untreated cells, **2** control cells treated with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), **3** control cells transfected with the empty vector for 72 h, **4** note the decreased expression of Hsp27 in the siHsp27 transfected cells (72 h after transfection). The immunoblots images were capture using LAS-4000 imaging system (Fujifilm Life Sc., USA). **b** Graph showing a significant depletion of Hsp27 at 48 h (immunoblot not shown) and 72 h after siHsp27 transfection. Mock 48 and 72 h were MCF-7 cells transfected with empty vector and analyzed at 48 and 72 h after transfection, respectively. The evaluation of the immunoblots was performed using NIH image V1.62 program (NIH, Bethesda, MD, USA). The data were analyzed with the Prism computer program (Graph Pad Software, San Diego, CA, USA); data shown are means \pm standard errors of the mean of three independent experiments. Statistical significance was assessed by column analyses with one-way ANOVA, the level of significance was set at $p < 0.05$. **c** Immunoblot showing the significant decrease of β -catenin. **1** Control untreated MCF-7 cells, **2** and **3** cells transfected with siHSP27 (48 and 72 h after transfection; $p < 0.05$). **d** Immunofluorescence of MCF-7 cells showing: **a** the basal Hsp27 levels (*left panel*, control cells treated with Lipofectamine™ 2000), and **b** the decreased Hsp27 levels after 72 h of siHsp27 transfection (*right panel*). Bar 10 μ m. The MCF-7 human breast cancer cell line was kindly provided by Dr. MC Abba [Centro de Investigaciones Inmunológicas Básicas y Aplicadas (CINIBA), Universidad Nacional de La Plata, Argentina]. The cells

were routinely cultured in Dulbecco's Modified Eagle Medium (GIBCO, Invitrogen Corp, Argentina) supplemented with 10 % fetal calf serum (GIBCO) and 100 IU/ml penicillin and 100 μ g/ml streptomycin (GIBCO) at 37 °C in an incubator with 5 % CO₂ and 100 % humidity. Subconfluent cells were split twice a week at a ratio of 1:20. For knockdown of Hsp27 expression, transient transfections were done with 2 μ g/ml pSIREN-RetroQ empty vector (Mock-transfection control) and shHsp27-pSIREN-RetroQ vector for 5h using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. The shHsp27-pSIREN-RetroQ vector was gently provided by Dr. MY Sherman (Boston University Medical School, Boston, MA, USA; O'Callaghan-Sunol et al. 2007). The vector contained the sequence of human Hsp27 (accession number NM 001540) as target for RNA interference: shHsp27 (start 701): ATCCGATGAGACTGCCGCAA. The transfection efficiency was evaluated in each experiment using pSIREN-DNR-DsRed-Express (a gift from Dr. MY Sherman). After the start of transfection at 48 and 72 h, cells were washed twice in ice-cold PBS, lysed with cell lysis buffer (triton-x buffer with protease inhibitors) and stored at -80 °C for immunoblotting analysis. Immunofluorescence staining: MCF-7 cells were fixed with 2 % paraformaldehyde in PBS for 10 min at 37 °C, washed with PBS and blocked with 50 mM NH₄Cl in PBS. Then the cells were permeabilized with 0.05 % saponin in PBS containing 0.5 % BSA, and incubated with primary antibody against Hsp27 (1:100). After washing, cells were incubated with secondary antibody conjugated with FITC (1:500; Jackson ImmunoResearch Laboratories Incorporated, West Grove, PA, USA). MCF-7 cells were mounted with Mowiol (Sigma-Aldrich, Argentina) and examined by confocal microscopy using an FV1000 Olympus Confocal Microscope and FV 10-ASW 1.7 software (Olympus, Japan). Images were processed using ImageJ software

cellular processes. Most oncogenes induce tumoral activity by dysregulation of tyrosine kinases, then, the tumor suppressor activity of PTEN may be explained by its protein tyrosine phosphatase activity. Another action of the enzyme encoded by PTEN is the phosphatidylinositol phosphate phosphatase activity. The PI3K/Akt signaling pathway is negatively regulated by PTEN, then, PTEN mutation (as well as deletion or silencing) can lead to increase PI3K activity stimulating downstream Akt signaling promoting: (a) growth factor-independent growth, (b) cell invasion, and (c) metastasis (Hafsi et al. 2012). Activated Akt is a well-established survival factor, exerting anti-apoptotic activity by preventing the release of cytochrome C from mitochondria and inactivating Forkhead transcription factors (FKHR), which are known to induce the expression of genes that are critical for apoptosis. On the other hand, Hsp27 can also regulate transduction pathways upstream of apoptosis by preventing apoptosome formation and the subsequent activation of caspases, interfering the execution phase of apoptosis (Garrido et al. 2006; Paul et al. 2010). Hsp27 is described as a key component of the Akt signaling cascade: Akt, BAD, FKHR, Hsp27, mitogen-activated protein kinase kinase-3 and -6 (Jomary et al. 2006). In the present study, our aim was to examine whether the downregulation of Hsp27 affects the PTEN levels in the MCF-7 human breast cancer cell line.

Results and discussion

A significant reduction in the Hsp27 level was noted in the siHsp27-transfected MCF-7 cells (Fig. 1a). The Hsp27 content was significantly decreased (almost 80 %) at 48 and 72 h after siHsp27 transfection (Fig. 1b). A significant decrease in the expression of β -catenin was also noted after Hsp27 depletion (Fig. 1c). This was not surprising since in a previous study we reported that Hsp27 interacts with β -catenin (Fanelli et al. 2008). This observation suggests that Hsp27 has chaperone activity modulating client proteins like β -catenin. In contrast, downregulation of β -catenin seems not to affect Hsp27 levels, at least in the HepG2 hepatocellular carcinoma cell line (Wang et al. 2011). We already mentioned that other molecules can be client proteins of Hsp27 (Gibert et al. 2012). The roles of the N- and C-terminal sequences of Hsp27 on both, the chaperone activity of Hsp27 and in the self-association of this protein have recently been described (Lelj-Garolla and Mauk 2012). The importance of the chaperone activity of the HSPs in cancer is exemplified by Hsp90. Disruption of the Hsp90 interaction with client oncogenic proteins that are subsequently degraded through the ubiquitin-dependent proteasomal pathway are in ongoing clinical trials (Whitesell and Lin 2012). Regarding Hsp25, the transfection of 4T1 breast cancer cells with siRNA-Hsp25

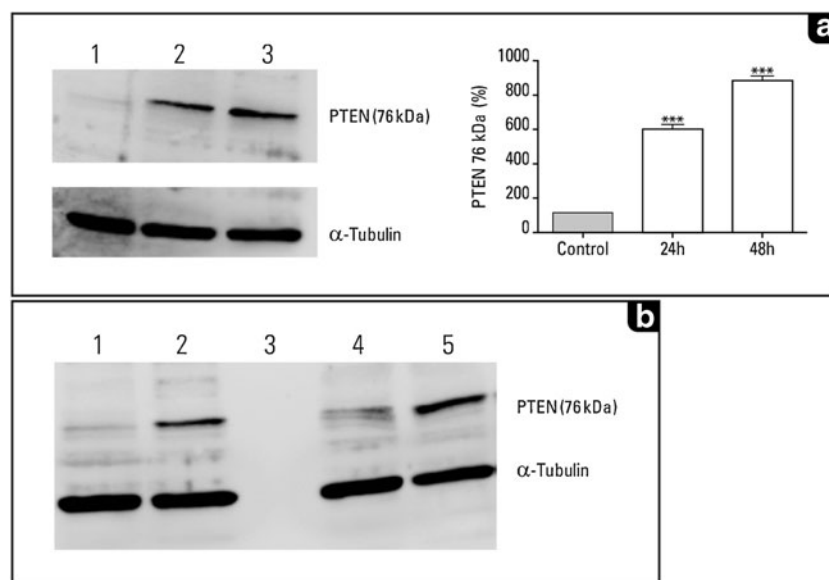


Fig. 2 Depletion of Hsp27 increases PTEN content in MCF-7 cells. **a** Immunoblot showing a significant increase in PTEN at 24 h (2) and 48 h (3) after siHsp27 transfection. 1 PTEN in MCF-7 control cells (Lipofectamine). PTEN was detected using a rabbit polyclonal antibody (1:1,000 dilution) generated against a PTEN synthetic peptide sequence between amino acids 33 to 47 (Perandones et al. 2004). The graph shows the statistical analysis of this blot, data shown are means \pm

standard errors of the mean of three independent experiments (Graph Pad Prism Software, San Diego, CA, USA); $p < 0.05$. **b** Immunoblot showing again the increase in PTEN levels after different times of siHsp27 transfection. 1 Control MCF-7 cells (treated with Lipofectamine), 2 cells after 48 h of continuous transfection with siHsp27, 3 empty lane, 4 control cells (Lipofectamine), 5 cells after 24 h of continuous transfection with siHsp27

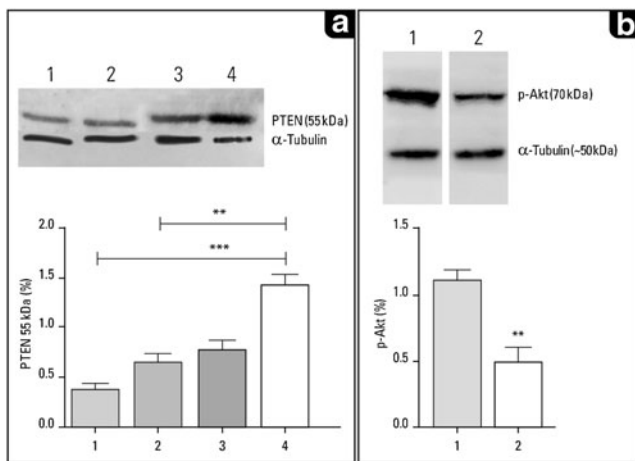


Fig. 3 After downregulation of Hsp27, PTEN is increased and p-Akt is decreased. **a** In this case, PTEN was revealed using an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of PTEN of human origin (1:400; PTEN C-20 sc-6817-R; Santa Cruz Biotech., Santa Cruz, CA, USA). 1 Untreated control MCF-7 cells; 2 control cells treated with Lipofectamine; 3 mock transfection control cells (72 h); 4 siHsp27-treated cells, 72 h after transfection. **b** Basal p-Akt levels (MW 70 kDa) in control MCF-7 cells (1) and in siHsp27-treated MCF-7 cells at 72 h after transfection (2). p-Akt was detected using a rabbit polyclonal antibody (1:1,000 dilution) generated against an epitope corresponding to amino acids 345–480 of Akt1 of human origin. This antibody detects Akt1, Akt2 and Akt3 (H-136, sc-8312, Santa Cruz Biotech., Santa Cruz, CA, USA). p-Akt was analyzed by Mann-Whitney test (graph)

significantly inhibited tumor cell proliferation and migration by a mechanism that is in part due to the repression of matrix metalloproteinase 9 expression, and upregulation of its antagonist tissue inhibitor metalloproteinase 1 (Bausero et al. 2006).

In the present study, the downregulation of Hsp27 after siHsp27 transfection was corroborated by immunofluorescence (Fig. 1d). High Hsp27 perinuclear levels were observed in control MCF-7 cells and the protein significantly decreased at 72 h of siHsp27 transfection.

Using the transfected cells, we found that the downregulation of Hsp27 was accompanied by PTEN upregulation (Fig. 2). A MW 76 kDa PTEN form was significantly upregulated as revealed by an antiserum obtained against the PTEN peptide.

We confirmed the PTEN upregulation in MCF-7 cells depleted in Hsp27 using another antibody obtained commercially (Fig. 3a). Since PTEN phosphatase is a negative regulator of the PI3 kinase/Akt signaling pathway (Cantley and Neel 1999), we were interested in the study of p-Akt. As shown in Fig. 3b, the MCF-7 cells depleted of Hsp27 that had relatively high PTEN levels showed a significant reduction in p-Akt.

In a recent study, Hsp27 has been implicated in the regulation of PEA-15 activity which occurs in an Akt-

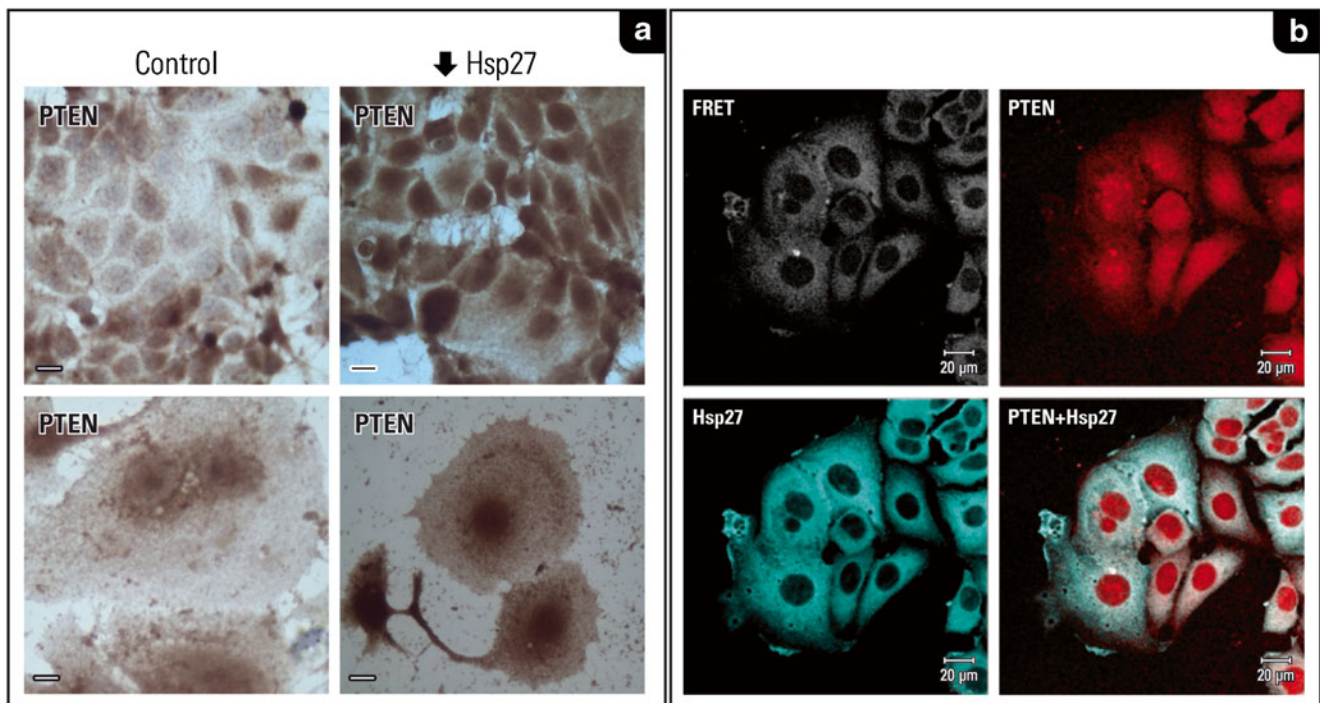


Fig. 4 Immunocytochemistry reveals upregulation of PTEN by siHsp27 and PTEN-Hsp27 interactions in MCF-7 cells. **a** Low-power microphotographs (upper panel; bar 10 μ m) and high-power microphotographs (lower panel; bar 4 μ m) to show the increased PTEN expression in the MCF-7 cells transfected with siHsp27. PTEN can be

seen mainly in the nuclei but also in the cytoplasm of the tumor cells. **b** The confocal microscopy shows that the two proteins colocalize in the cytoplasm. FRET was done according to Kenworthy (2001). PTEN was detected using an aptamer (z7) (Moncalero et al. 2011)

dependent manner (Hayashi et al. 2011). PEA-15 phosphoprotein can sequester ERK in the cytoplasm. MEK1/2 plays a central role in integrating mitogenic signals into the ERK pathway. Significantly, these authors found that Hsp27 silencing in a panel of PTEN wild-type or null cell lines, and in LNCaP cells that express PTEN, resulted in selective growth inhibition of PTEN-deficient cancer cells. They have identified a dual coordinated role of Hsp27 in cell proliferation and Fas-induced apoptosis via Akt and PEA-15. Taken together, these data strongly suggest that Hsp27 should be considered an important regulator of Akt signaling, indicating a

more complex mode of Akt regulation in cancer cells than previously known. This observation is further supported by a recent study where the inhibition of both Hsp27 and p-Akt synergistically decreased glioma cell survival (Schultz et al. 2012). These authors found a complex feedback system between secreted protein acidic and rich in cysteine, Hsp27, and Akt suggesting that this interaction is likely influenced by PTEN activation status.

In the immunocytochemistry study, we found a higher PTEN expression mainly in the nuclei but also in the cytoplasm of the MCF-7 cells transfected with

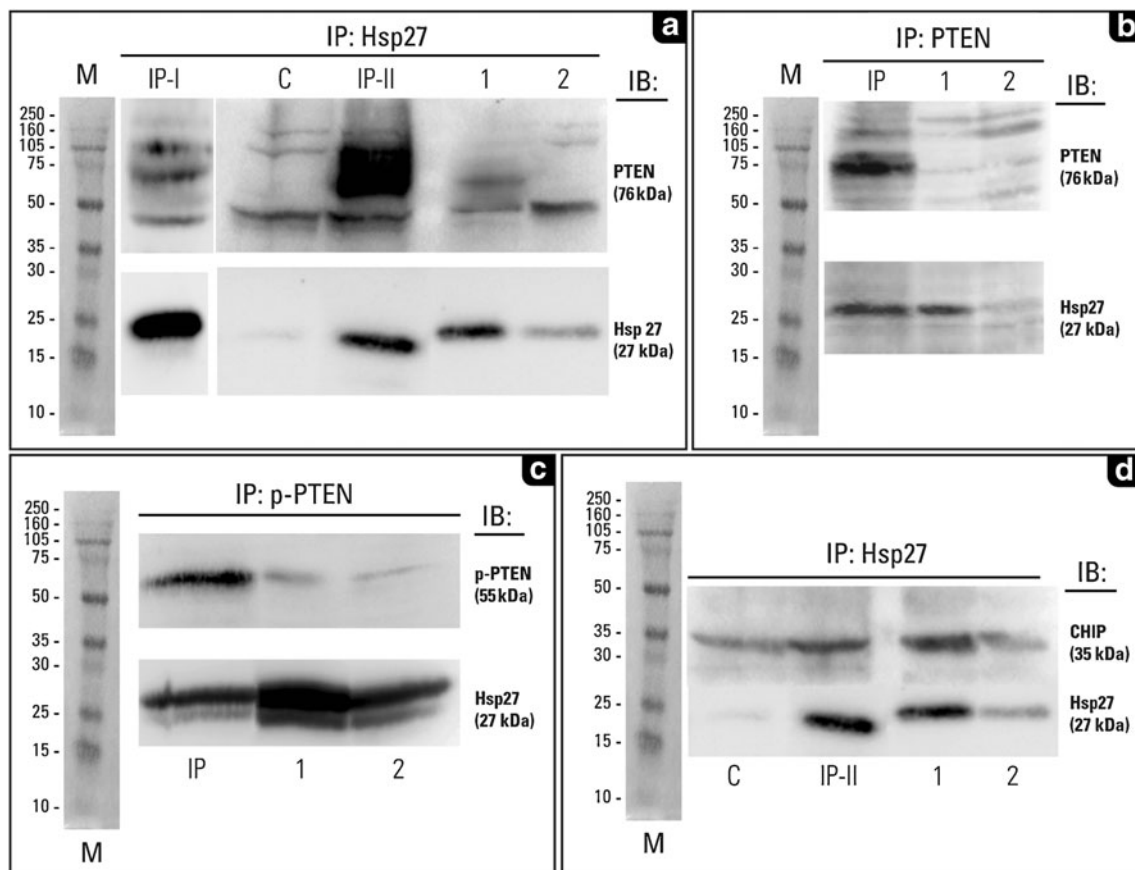


Fig. 5 Immunoprecipitation studies reveal an interaction between Hsp27 and PTEN. **a** Hsp27 immunoprecipitation (using the mouse monoclonal antibody from Stressgen Biotech described before) is presented in two independent experiments (IP-I, IP-II). Note the presence of PTEN in IP-I and IP-II. **M** molecular weight markers (Full Range Rainbow from Amersham, GE Healthcare, UK). **C** control immunoprecipitation using a mouse monoclonal antibody generated against membranes from MCF-7 cells (own antibody). **1** MCF-7 cell supernatant (total lysate) before immunoprecipitation showing both Hsp27 and PTEN proteins. **2** Same as **1** but after immunoprecipitation. **b** After PTEN immunoprecipitation note the presence of Hsp27. The antibody used for PTEN IP was a rabbit polyclonal antibody (Perandonnes et al. 2004). This antibody recognizes PTEN and the ubiquitinated forms of PTEN. **1** MCF-7 cell supernatant (total lysate) before immunoprecipitation showing Hsp27 and a weak PTEN band. **2** Same as **1** but after immunoprecipitation. **c**

Immunoprecipitation was performed with a p-PTEN rabbit polyclonal antibody (ser 370, sc-101787, Santa Cruz Biotech., Santa Cruz, CA, USA), note the presence of Hsp27. **1** MCF-7 cell supernatant (total lysate) before immunoprecipitation showing Hsp27 and a weak p-PTEN band. **2** Same as **1** but after immunoprecipitation. **d** After Hsp27 immunoprecipitation (IP-II) note a weak CHIP band (using a rabbit polyclonal antibody, N-terminal, C9118, Sigma-Aldrich, MO, USA). **C** control immunoprecipitation using a mouse monoclonal antibody generated against membranes from MCF-7 cells (own antibody). **1** MCF-7 cell supernatant (total lysate) before immunoprecipitation showing both Hsp27 and CHIP proteins. **2** Same as **1** but after immunoprecipitation. Immunoprecipitation was carried out with the described antibodies (12 μ g) attached to the Dynabeads M-280 Tosylactivated (cat. # 142.03, from Invitrogen, Argentina), using 500 μ g of total proteins in the lysate. We used the protocol provided by the company

the siHsp27 (Fig. 4a). Figure 4b shows the colocalization of the two proteins in untreated MCF-7 cells. In a previous study, we have shown that Hsp27 protein is located mainly in the cytoplasm of the tumor cells, but that under stress conditions Hsp27 can be translocated to the nucleus (Vargas-Roig et al. 1998). As expected, in non-stressed MCF-7 cells, Hsp27 was mainly found in the cytoplasm while PTEN was located in the cytoplasm and in the nuclei of the tumor cells. In the FRET analysis, both proteins, Hsp27 and PTEN, colocalized/interacted mainly in the cytoplasm (perinuclear region) of the tumor cells.

In a previous study, PTEN has been reported, by immunohistochemistry, both in the cytoplasm and in the nuclei of normal and tumor cells (Naguib et al. 2011). Moncalero et al. (2011) detected the PTEN protein in different subcellular compartments of neurons suggesting the existence of different conformations of phosphatase in the nucleus and in the cytoplasm. In our study, PTEN was localized mainly in the nucleus but part was also located in the cytoplasm. Since Hsp27 is mainly a cytoplasmic protein, we suggest that Hsp27 and PTEN interact mainly while they are in the cytoplasm. Immunoprecipitation studies were done in order to confirm the interactions of the proteins under study (Fig. 5).

Western blot analyses of supernatants (before and after spinning down the immune complex) were performed to measure the percentage of Hsp27 molecules that interact with PTEN. The amount of Hsp27 that remained in the supernatant was 19.5 % (that is, 80.5 % of Hsp27 interacted with PTEN). On the other hand, the amount of PTEN in the original homogenate was very low to be compared with the amount remaining after spinning down the immunocomplex (beads+Hsp27), under these conditions a PTEN band was not observed (in one experiment) or noted with very weak intensity (in the duplicate experiment). To circumvent this problem, we considered the PTEN immunoprecipitation band as 100 % (beads+PTEN), and then we measured the band of PTEN in the reverse immunoprecipitation (beads+Hsp27 revealed to PTEN). We found that 45 % of PTEN was interacting with Hsp27. These numbers support that the significant interaction of Hsp27 with PTEN which occurs mainly in the cytoplasm as seen in the colocalization study. PTEN interacts at a lower level with Hsp27 (PTEN is mainly in the nuclear cell compartment). It is of interest to mention here that even p-PTEN interacted with Hsp27 as shown in Fig. 5c. The amount of Hsp27 that interacted with p-PTEN reached almost 30 %.

Since in a previous study, Ahmed et al. (2012) reported that the chaperone-assisted E3 ligase C terminus

of Hsc70-interacting protein (CHIP), the chaperone associated E3 ligase, induces ubiquitination and regulates the proteasomal turnover of PTEN, we investigated if Hsp27 interacts significantly with CHIP (Fig. 5d). When Hsp27 was immunoprecipitated only a minor CHIP band was observed (after subtracting the control nonspecific band). Moreover, the Hsp27 downregulation by siHsp27 (48 h) did not affect the CHIP levels in the MCF-7 cells (data not shown).

Finally, we investigated PTEN stability after depletion of Hsp27 (Fig. 6). Hsp27 depletion stabilized PTEN. Additional studies are needed to fully understand how Hsp27 may affect PTEN stability, PTEN phosphorylation is essential for the tail dependent regulation of stability (Xu et al. 2010), and here we report that almost 30 % of Hsp27 interacts with p-PTEN. In MCF-7 cells, the PTEN levels are relatively low (as seen in our study) due to accelerated PTEN degradation (Noh et al. 2011).

In conclusion, the present study reveals that the regulation of PTEN, which is critical for the cells, is under significant regulation by Hsp27 (among other chaperones). We suggest that Hsp27 interacts with PTEN decreasing its stability. As many aspects of Hsp27 biology are increasingly well known, Hsp27-targeted therapy should be taken into consideration to achieve an effective anticancer therapy.

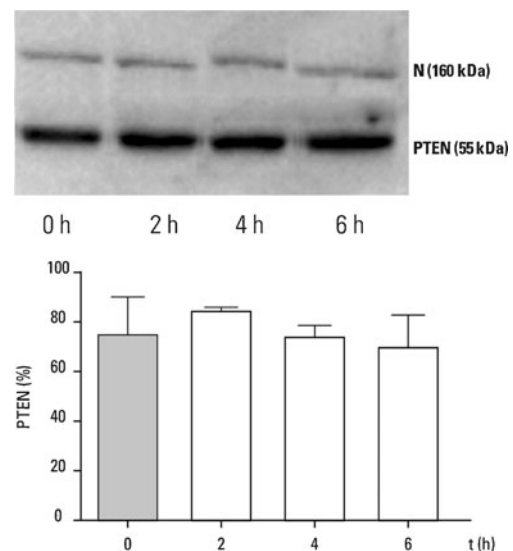


Fig. 6 PTEN stability is maintained after Hsp27 depletion. MCF-7 cells were continuously transfected with siHsp27 for 48 h and then treated with cycloheximide (200 μ g/ml) for the times indicated. Cells were lysed and cell lysates were resolved by SDS-PAGE (10 %), followed by western blot with anti-PTEN (PTEN C-20 sc-6817-R Santa Cruz Biotech., Santa Cruz, CA, USA). N Nonspecific band used as loading control. The bands were quantified with ImageJ software. Data are shown as the mean of two different experiments. Bars standard deviation. Note that the amount of PTEN remained at high levels during the time course of the experiment

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