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*Brief Report*

# YB-1 Oncoprotein Controls PI3K/Akt Pathway by Reducing Pten Protein Level

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**Abstract:** YB-1 is a multifunctional protein overexpressed in many types of cancer. It is a crucial oncoprotein that regulates cancer cell progression and proliferation. Ubiquitously expressed in human cells, YB-1 protein functions are strictly dependent on its subcellular localization. In the cytoplasm, where YB-1 is primarily localized, it regulates mRNA translation and stability. However, in response to stress stimuli and activation of PI3K and RSK signaling, YB-1 moves to the nucleus acting as a prosurvival factor. YB-1 is reported to regulate many cellular signaling pathways in different types of malignancies. Furthermore, several observations also suggest that YB-1 is a sensor of oxidative stress and DNA damage. Here we show that YB-1 reduces PTEN intracellular levels thus leading to PI3K/Akt pathway activation. Remarkably, PTEN reduction mediated by YB-1 overexpression can be observed in human immortalized keratinocytes and HEK293T cells and cannot be reversed by proteasome inhibition. Real-time PCR data indicate that YB-1 silencing up-regulates the PTEN mRNA level. Collectively, these observations indicate that YB-1 negatively controls PTEN at the transcript level and its overexpression could confer survival and proliferative advantage to PTEN proficient cancer cells.

**Keywords:** YB-1; PTEN; cold-shock proteins; proteasome; PI3K/Akt pathway

## 1. Introduction

YB-1 is a DNA- and RNA-binding protein and transcription factor with an evolutionarily ancient and conserved cold shock domain [1]. In healthy tissues, YB-1 is primarily cytoplasmic, where it plays an important role in regulating various aspects of RNA biology [2]. YB-1 is a major component of translationally inactive messenger ribonucleoprotein particles (mRNPs) and is mainly responsible for the storage of mRNAs in a silent state [3].

Since its initial discovery, the Y-box binding protein 1 (YB-1) was linked to oncogenic functions and chemotherapy resistance. YB-1 is upregulated in tumors and its nuclear localization is associated with a more aggressive phenotype indicating a poor prognosis [4–7]. In response to genotoxic stress, YB-1 translocates from the cytoplasm to the nucleus [8], where it acts as a transcriptional regulator to overcome DNA damage-dependent cell cycle arrest and promote cell survival [9].

YB-1 is a direct target of the serine/threonine kinase Akt. Akt is activated by phosphorylation at Ser473. Overstimulated Akt activity in cancer cells [10] induces YB-1 phosphorylation at Ser102 and nuclear accumulation without changing the total amount of the protein. This results in reduced DNA repair in cancer cells after irradiation [3].

The nuclear accumulation of YB-1 in response to DNA damage or transcription inhibition requires a decrease in the cytoplasmic mRNA level [11]. Indeed, like Akt, YB-1 is associated with inactive mRNPs and, activated Akt relieves translational repression of the YB-1-bound mRNAs thereby facilitating translational activation of silenced mRNA species [3,12].

The main negative regulator of the PI3K-Akt pathway remains the PTEN phosphatase. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is recognized as a tu-

mor suppressor due to its negative regulation of the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [13], see Figure 1. The major target of PTEN is phosphatidylinositol (3, 4, 5)-triphosphate (PIP3), which is generated by PI3K and acts as a bridge to recruit 3-phosphoinositide-dependent protein kinase 1 (PDK1) and AKT to the plasma membrane, further activating AKT by phosphorylation at its T308 site [13,14]. PTEN converts PIP3 into PIP2, interrupting the interaction between PDK1 and AKT, and thus negatively mediating the activation of AKT. Apart from its membrane-bound form, nuclear PTEN has multiple functions, including the induction of cell cycle arrest by inhibiting cyclin D1 expression [15], maintenance of chromosomal stability, and DNA double-strand break repair [16].

Loss of PTEN activity has been identified in a wide spectrum of primary and metastatic neoplasms, including breast cancer [17]. This condition, which results in low or null expression of the protein, is believed to be an early oncogenic event in several tumor types [18,19]. Given the ability of Akt to physically interact and activate YB-1 oncogenic functions [3] we hypothesize that YB-1 was, in turn, able to regulate Akt by a positive control that could mirror what happens in vivo when YB-1 overexpression sustains the proliferative and survival potential of cancer cells. Very little information about YB-1 and PTEN functional crosstalk is now available. However, a significant association of YB-1 nuclear accumulation with PTEN deletion in advanced prostate tumor stages was reported by Heumann and collaborators in 2017 thus suggesting a possible reciprocal regulation between the two proteins of the PI3K/Akt pathway [20]. Here we provide compelling evidence that YB-1 can sustain Akt activation by controlling PTEN.
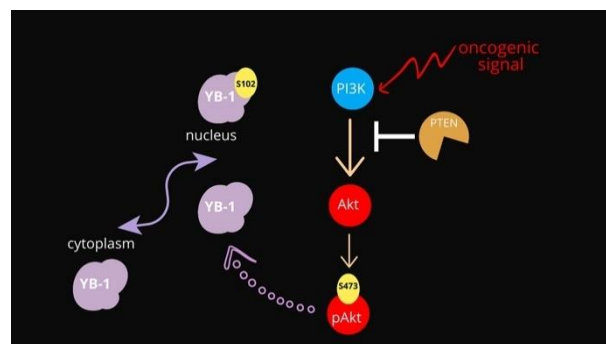


**Figure 1.** Schematic representation of the PI3K/Akt pathway overseeing YB-1 activation.

## 2. Materials and Methods

### 2.1. Plasmids and Reagents

The expression plasmid 3XFlag-YB-1 wt was used for the transfection and provided by Dr. Arezoo Astanehe (Abbotsford, BC, Canada). The pcDNA-GFP plasmid, used as a control, was purchased by Thermo-Fisher Scientific (Waltham, MA, USA). Sodium (meta)arsenite (NaAsO$_2$, S7400, Sigma-Aldrich, St. Louis, MO, USA) and copper (II) sulfate (C1297, Sigma-Aldrich) were used to treat cell culture at 300 μM and 10 μM final concentrations, respectively. MG-132 (M8699, Sigma-Aldrich) was used as a proteasome inhibitor at 10 μM final concentration, for 4h.

### 2.2. Cell Cultures

Human embryonic kidney cells (HEK293T) and HaCaT (human spontaneously immortalized keratinocytes from adult skin) were purchased from Cell Line Service (CLS, Germany) and cultured in a humidified incubator at 37 °C and 5% CO2 in DMEM High glucose (Gibco BRL, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Gibco BRL), 1% L-glutamine (Gibco BRL) and 1% Pen-Strep solution (Gibco BRL). Cells were routinely checked for mycoplasma contamination, using a mycoplasma detection kit (Abcam, Quebec, QC, Canada).

To increase HEK293T adhesion to glass/plastic surfaces, plates were treated with poly-D-lysine (0.1 mg/mL, P7405, Sigma-Aldrich) before seeding cells.

### 2.3. Immunoblotting Analysis

For total protein extraction $2.5 \times 10^5$ cells were seeded in 6-well. After 48 h, cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) with the addition of 1 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Cells were detached with a scraper and left on ice for 30′. Then extracts were clarified by centrifugation at 13,200 rpm for 30′ at 4 °C. The amount of protein in the samples was determined by the Bio-Rad protein assay (Bio-Rad, Milan, Italy).

After the addition of Laemmli buffer (Sigma-Aldrich) samples were boiled at 100 °C for 5 min and resolved by SDS- polyacrylamide gel electrophoresis (SDS-PAGE). About 20 μg of total extracts were separated by SDS-PAGE.

Proteins were then transferred to a polyvinylidene difluoride membrane (PVDF, Millipore) using a Mini trans-blot apparatus (Bio-Rad) according to the manufacturer's instructions. The PVDF membrane was blocked in 5% *w/v* milk buffer (5% *w/v* non-fat dried milk, 50 mM Tris, 200 mM NaCl, 0.2% Tween 20) and incubated overnight at 4 °C with primary antibodies diluted in 5% *w/v* milk or bovine serum albumin (BSA) buffer according to the manufacturer's instructions. Following three washes with TBST (Tris-buffered saline, 0.1% Tween), the blots were incubated for 1 hour at RT with HRP-conjugated secondary antibodies (Sigma-Aldrich). Proteins were visualized by enhanced chemiluminescence (ECL, Bio-Rad) and analyzed by Quantity One W software of ChemiDoc TM XRS system (Bio-Rad).

Band intensities were quantified by ImageJ Software (http://imageJ.nih.gov/ij/, accessed on 21 August 2021, free software, downloaded from the NIH, Bethesda, MD, USA), normalized respect loading control and reported as fold enrichment to the control sample.

### 2.4. Antibodies

The primary antibodies used are: anti-YB-1 raised against the region 1 to 100 of YB-1 protein (12148 Abcam, Cambridge, UK); anti-Actin (8432 Santa Cruz, Dallas, TX, USA); anti-PTEN (Cell Signaling, Danvers, MA, USA, 9559S); anti-Phospho-Akt (Ser473) (193H12) (Cell Signaling, 4058S); anti-Akt (Cell Signaling, 2920S).

### 2.5. Transfections and RNA Interference

Cells were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. Briefly, cells were seeded at 70–80% confluence ($2.5 \times 10^5$) in 6-well and transiently transfected with plasmids at a concentration of 300 ng for 48h. MG-132 was added to the cells at the concentration of 10 μM 4 h before the end of transfection.

YB-1 transient silencing was carried out with IBONI YB-1 small interfering (siRNA) pool (RIBOXX GmbH, Radebeul, Germany) as a pool of 3 different siRNAs and RNAiMAX reagent (Life Technologies), according to the manufacturer's recommendations. Cells were seeded at 70–80% confluence ($2.5 \times 10^5$) in 6-well and transiently silenced with IBONI YB1-siRNA at 150 nM final concentration.

Negative Control siRNA, provided by RIBOXX (Germany) was used as a negative control.

YB-1 guide and passenger sequences:
*h* YBX-1 guide (5′-3′):
UUUAUCUUCUUCAUUGCCGCCCCC
UUAUUCUUCUUUAUGGCAGCCCCC
UAUUUGAUGACCACACCAGCCCCC
*h* YBX-1 passenger (5′-3′):

GGGGGCGGCAAUGAAGAAGAUAAA
GGGGGCUGCCAUAAAGAAGAAUAA
GGGGGCUGGUGUGGUCAUCAAAUA

### 2.6. Co-Immunoprecipitation

For co-immunoprecipitations (Co-IP) $2 \times 10^6$ HEK293T cells were seeded in poly-D-lysine pre-treated 100-mm dishes; the day after Dynabeads Protein A (Invitrogen) were incubated with antibodies against YB-1, 3 μg for 1.5 mg of protein extract for 10′ at room temperature in rotation after protein extract was incubated with the Dynabeads-Ab complex overnight at 4 °C. Immunoglobulin G (IgG) 3 μg for 1.5 mg of protein extract was used as a negative control. Immunocomplexes were resolved with SDS-PAGE; immunoblot was performed with anti-PTEN antibody and anti-YB1 antibody.

### 2.7. Quantitative Real Time-PCR

Total RNA was extracted with Trizol reagent (Gibco) according to the manufacturer's instructions. Reverse transcription was performed using All-In-One 5X RT MasterMix (Abcam). AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) and Brilliant HRM Ultrafast Starter Pack were used. Quantitative relative expression was calculated according to the $2^{-\Delta\Delta CT}$ method (Delta CT method) normalizing to *rpl0* mRNA expression. Sequences of primers used:

PTEN *For:* CTCAGCCGTTACCTGTGTGT
PTEN *Rev*: AGGTTTCCTCTGGTCCTGGT
YB-1 *For*: CGACCAGACTCTCATCCTGC
YB-1 *Rev:* TTTGATGACCACACCAGGCA
RPL0 *For*: GACGGATTACACCTTCCCACTT
RPL0 *Rev:* GGCAGATGGATCAGCCAAGA

### 2.8. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 8.1.2, GraphPad Software Inc., San Diego, CA, USA). Data were presented as the mean ± standard deviation and analyzed for statistical significance using one-way or two-way analysis of variance (ANOVA) and multiple comparisons. For all tests, $p < 0.05$ was considered to indicate a statistically significant difference. To report *p*-values, the *New England Journal of Medicine* (NEJM) decimal format was used; differences were considered statistically significant at * $p < 0.033$, ** $p < 0.002$ and *** $p < 0.001$.

## 3. Results

Firstly, we wondered whether there was a direct functional relationship between YB-1 and PTEN. We hypothesized that YB-1 could control PTEN protein level. To address this point, we decided to analyze the effect of YB-1 downregulation on PTEN protein intracellular level.

To this aim, we used PTEN proficient immortalized HaCaT and HEK293T cells, showing robust expression of endogenous YB-1 resembling actively proliferating pre-malignant cells. YB-1 expression was depleted by RNAi and PTEN levels were checked by immunoblot. Control (siRNA NC) and YB-1 depleted cells (siPool YB-1) were collected 48h after transfection and analysed by immunoblot. As shown in Figure 2A we observed a clear increase of PTEN levels in YB-1 depleted HaCaT cells compared to the control with a concomitant reduction of pAkt$^{S743}$ (Figure 2B) in line with a potential role of YB-1 in enhancing Akt phosphorylation at S743 by directly impinging on PTEN/PI3K pathway. An even more evident increase of PTEN protein levels was observed in HEK293T cells upon YB-1 depletion (Figure 2B). Interestingly, an increase of PTEN protein level was also observed in total extracts from HaCaT and HEK293T cells whose level of YB-1 was reduced by oxidative stress stimuli such as that with NaArs and Cu++ treatments (Figure 2C), as we have previously shown [21].
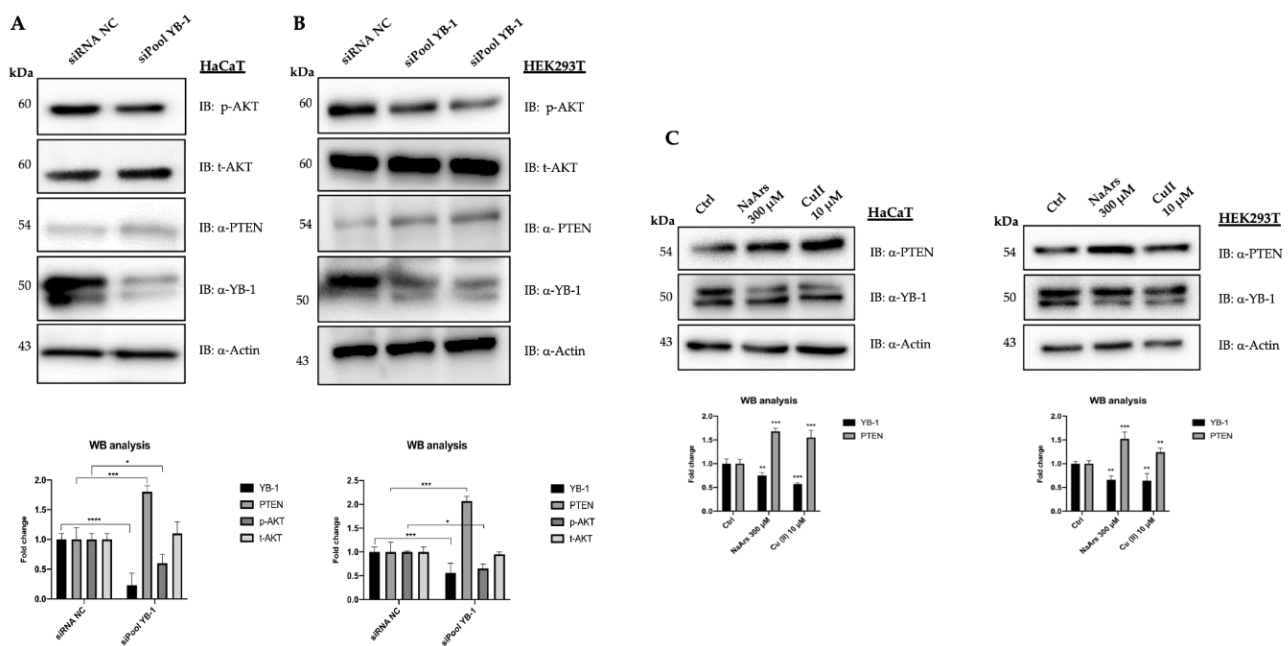
**Figure 2.** YB-1 controls PTEN levels in HaCaT and HEK293T cells. (**A**) HaCaT cells were transfected with 10 nM final siRNA-poolYB-1 or siRNA-Negative Control (NC). The effect on PTEN intracellular levels was evaluated 48 h post-transfection by western blot on whole protein lysates probed with anti-PTEN antibodies. Immunoblots were also probed with antibodies against YB-1, pAkt$^{S743}$, tAKT and actin as a loading control. (**B**) HEK293T cells were transfected with 10 nM final siRNA-NC or siRNA-poolYB-1 (duplicated). Whole protein lysates were collected and analysed 48 h after transfection as described in (**A**); (**C**) HaCaT and HEK293T cells were treated with NaArs and Cu (II) for 2 h to induce oxidative stress. Cell lysates were analyzed by immunoblot with antibodies against PTEN, YB1 and actin used as a loading control. For comparison, siRNA-transfected extracts were used. Statistical analyses were performed using 2-way ANOVA and Sidak's multiple comparison or Dunnett's multiple comparisons test. Levels of significance between points of expression are indicated (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

We then decided to look next at the effect of YB-1 overexpression on PTEN protein levels. To this aim, we transfected YB1 expression vector in HEK293T or HaCat cells treated or not with the proteasome inhibitor MG132. At 48 h after transfection, cells were collected, and the extracts were analysed by immunoblot. As shown in Figure 3A we observed a moderate but significant reduction of PTEN protein level, compared to the control, that was not rescued by MG132 treatment thus suggesting that it does not occur through a proteasome-dependent mechanism. We also tested for a possible interaction between YB-1 and PTEN proteins by co-immunoprecipitation. However, as shown in Figure 3B, YB-1 antibodies were unable to immunoprecipitate endogenous PTEN.

Given the importance of YB-1 function in the control of RNA metabolism, we wished to explore whether YB-1 depletion was able to alter the level of PTEN mRNA. Therefore, we depleted HaCaT and HEK293T cells of YB-1 using the siRNA pool against YB-1 (siPoolYB-1) [21]. Total RNA was extracted and subjected to RT-qPCR. As shown in Figure 4, compared to the control sample the mRNA level of YB-1 was reduced to 0.25 in HaCaT and 0.40 in HEK293T cells while PTEN mRNA was increased to 1.75 in HaCaT and 1.5 in HEK293T cells.
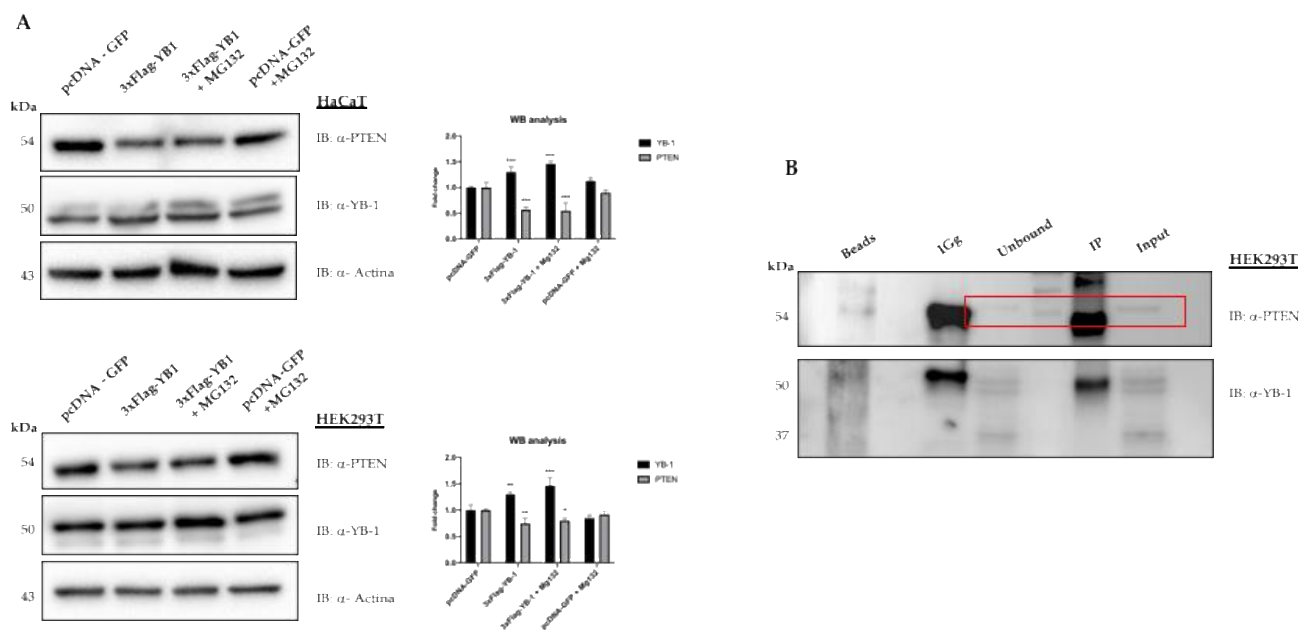
**Figure 3.** YB-1 mediated reduction of PTEN levels requires neither the proteasomal activity nor the physical interaction between the two proteins. (**A**) HaCaT cells (top panel) and HEK293T cells (lower panel) were transfected with 3XFlag-YB-1 plasmid or pcDNA-GFP as a control. Effect on PTEN intracellular levels was evaluated 48 h post-transfection by western blot on whole protein lysates probed with anti-YB-1, anti-PTEN, and anti-actin as a loading control. 10 μM MG132 for 4 h before the end of transfection was used to inhibit proteasome activity. (**B**) HEK293T protein extracts were immunoprecipitated with anti-YB-1 antibodies. Immunocomplexes were subjected to western blot and revealed using antibodies against PTEN. PTEN signal in unbound and input are highlighted in the rectangle box. Statistical analysis was performed using 2-way ANOVA and Sidak's multiple comparison test. Levels of significance between points of expression are indicated (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).
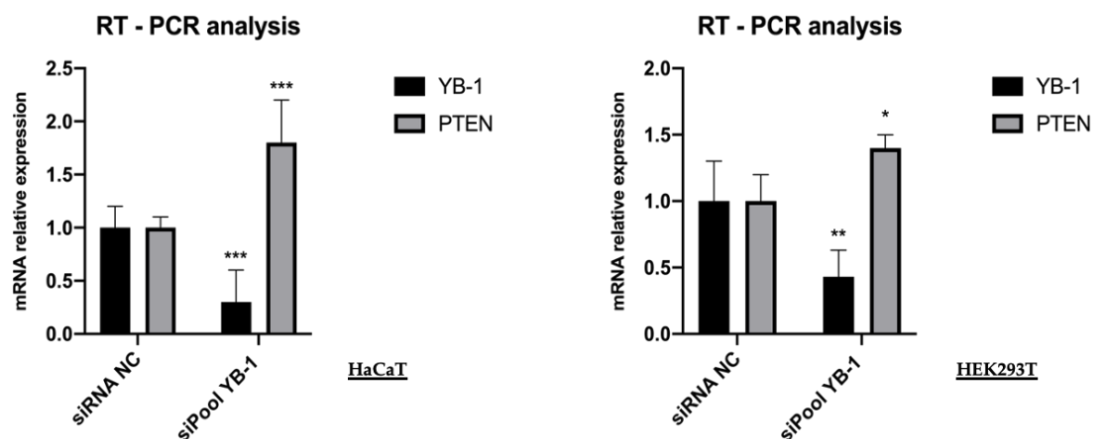


**Figure 4.** YB-1 depletion increases the level of PTEN transcript. HaCaT and HEK293T cells were transfected with 10 nM final siRNA-poolYB-1 or siNC; the effect on PTEN mRNA level was evaluated 48h post-transfection by qRT-PCR analysis. Relative mRNA levels were plotted on the y-axis and siRNA employed are indicated on the x-axis. RPL0 ribosomal protein mRNA was used for normalization. Statistical analyses were performed using 2-way ANOVA and Sidak's multiple comparison test. Levels of significance between points of expression are indicated (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

This result showed that the effect of YB-1 on PTEN was primarily at mRNA level. It is important to remind that PTEN functions in a dosage-dependent manner during tumor development and that moderate PTEN reduction, without complete loss, has been reported to activate the PI3K/Akt pathway and to be associated with chemoresistance and cancer progression [22,23].

## 4. Conclusions

The data presented here show evidence that YB-1 controls PTEN protein levels by acting at the transcript level. Our data are in line with what is already known regarding the function of both proteins, although a direct functional link between PTEN and YB-1 has never been assessed. At the functional level increased expression of YB-1 can restrain PTEN expression thus enforcing Akt activation in premalignant cells (Figure 5). Akt, in turn, increases the pro-proliferative and pro-survival activities of YB-1 by inducing YB1 phosphorylation at S102 thereby promoting its nuclear translocation.

The PTEN level appears to be tightly controlled both transcriptionally and post-transcriptionally [24]. Some oncogenic microRNA and ncRNAs have been found to target PTEN mRNA and regulate malignant progression [25], ncRNAs including lncRNAs and miRNAs act alone or interact with each other to regulate PTEN expression and it has recently been proposed that some of the oncogenic effects of YB-1 in breast cancer may be mediated through its interactions with sncRNAs [26].

Although the precise mechanism through which YB-1 controls PTEN mRNA level remains to be determined, our data suggest the existence of a positive feedback loop between YB-1 and Akt, reinforcing each other, probably occurring at an early step in cancer progression and conferring a selective advantage to premalignant cells. Elucidation of the details about how YB-1 downregulates PTEN expression may provide novel insights into the regulation network of PTEN, which could suggest possible anticancer strategies focusing on targeting both YB-1 and the PI3K/Akt pathway.
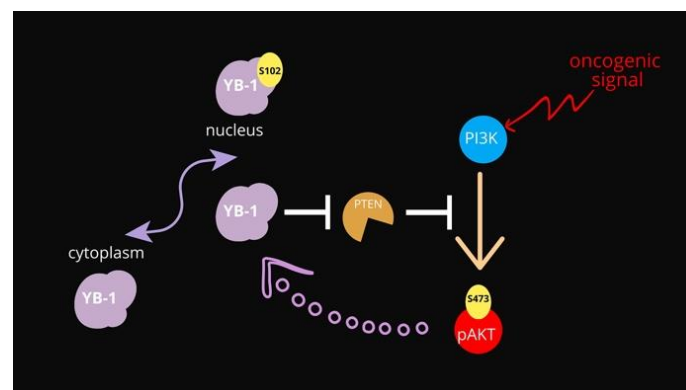


**Figure 5.** Schematic representation of the proposed functional relationships among YB-1, PTEN and Akt. In normal conditions, YB-1 is mainly cytoplasmic. The Akt-dependent phosphorylation of YB-1 at S102 promotes its translocation to the nuclear compartment promoting cell survival.

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