

Report

PTEN and p53 are required for hypoxia induced expression of maspin in glioblastoma cells

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Abbreviations: Mdm2, murine double minute 2; PTEN, phosphatase and tensin homolog; wt, wild-type; mt, mutant; IP, immunoprecipitate; IB, immunoblot

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In response to genotoxic stress, p53 induces the tumor suppressors maspin and PTEN. Here we demonstrate that in response to limited oxygen conditions PTEN and p53 work in tandem to induce maspin in glioblastoma cells. In response to hypoxia a portion of PTEN migrates to the nucleus and complexes with p53, while cytoplasmic PTEN prevents Mdm2 nuclear localization by attenuating Akt signaling. Subcellular distribution of PTEN in the cytoplasm or nucleus protects p53 from inactivation and degradation. The presence of nuclear PTEN and p53 coordinates the induction of maspin and p21 (both p53 gene targets) in response to hypoxia. Altering the expression of PTEN and/or p53 attenuated maspin gene induction under hypoxic conditions. Furthermore, implanting U87 (PTEN null) and PTEN reconstituted U87 cells (U87PTEN) in mice we observed by immunohistochemistry and western blot that Maspin was only detectable in cells with PTEN. The integration of PTEN and p53 into a common pathway for the induction of another tumor suppressor, Maspin, constitutes a tumor suppressor network of PTEN/p53/Maspin that is operational under limited oxygen conditions.

Introduction

The tumor suppressor maspin is a 42-kDa member of the serpin family, which does not have protease inhibitory activity.¹ Previous studies have shown the importance of maspin, as it is downregulated in breast and prostate cancer. This may relate to the functions of maspin, which include the inhibition of invasion, metastasis and angiogenesis in breast and prostate cancers.² In addition, it can play a role in sensitizing cells to chemotherapy.³

In response to genotoxic stress the maspin gene is a transcriptional target of the tumor suppressor protein p53.⁴ Additional components

that coordinate to activate p53 to induce maspin have not been determined. Furthermore, it has not been determined if other physiological stimuli, such as limited oxygen, can cause a p53-dependent induction of maspin.

Another transcriptional target of p53 is the tumor suppressor, PTEN, a dual specificity phosphatase.^{5,6} Many glioblastomas and prostate cancers are PTEN-deficient or harbor point mutations in the PTEN gene.⁷ One aspect of the PTEN tumor suppressor axis is achieved through its stabilization of the p53. Considered the guardian of the genome, p53 is responsible for cell cycle arrest and apoptosis in response to cellular stress and genotoxic events.⁸ PTEN dephosphorylates phosphatidylinositol(3,4,5)-trisphosphate (PIP₃),⁹ a crucial second messenger required for the activation of the Akt pathway.¹⁰ Akt mediates Mdm2 nuclear translocation by phosphorylating it on key serines.¹¹ In the nucleus, Mdm2 negatively regulates p53 by direct binding and signaling for destabilization. Therefore, attenuation of the Akt pathway by PTEN protects p53 from Mdm2-mediated degradation and inactivation.¹²

Although PTEN partitions itself into cytoplasmic and nuclear compartments, its nuclear function is still being investigated.¹³ Recent data suggest that nuclear PTEN maintains chromosomal integrity by acting directly on the centromere and inducing the expression of Rad51.¹⁴ Others have shown that nuclear PTEN decreases Mitogen Activated Protein Kinase (MAPK) phosphorylation and leads to cell cycle arrest.¹⁵ Physical interaction between PTEN and p53 in the nucleus enhances the DNA binding ability of p53.²⁰ Current models propose that PTEN predominantly suppresses growth, tumor migration and angiogenesis by antagonizing Akt.

Our data suggest that both PTEN and p53 are required for the production of maspin, a protein that negates factors that promote tumor progression. We show that under hypoxic conditions PTEN and p53 form a complex in the nucleus and induce robust expression of the tumor suppressor maspin. This was evident in cell culture and xenografts of glioblastoma cells. Thus, loss of PTEN attenuates the induction of Maspin even in the presence of wild type p53. Such disruption in the tightly integrated PTEN/p53/Maspin axis may contribute to tumor progression.

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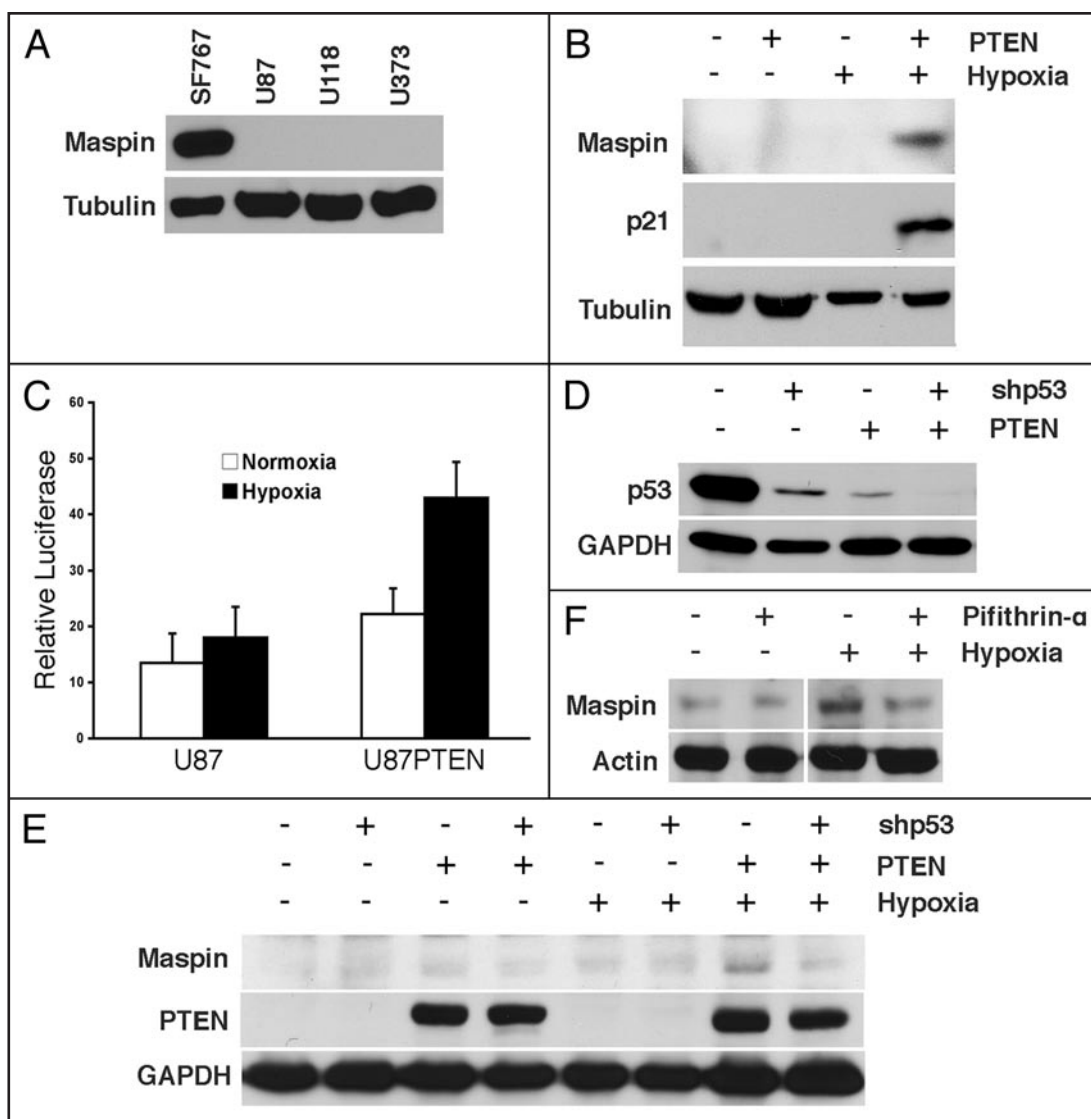


Figure 1. Maspin expression is controlled by p53 and PTEN. (A) A Western blot of Maspin expression in cellular lysates of SF767 (PTENwt/p53wt), U87 (PTEN null/p53wt), U118 (PTENmt/p53mt) and U373 (PTENmt/p53mt) cultured under hypoxia for 24 h. (B) Maspin protein expression in cell lysates from U87 and U87PTEN cells cultured under normal oxygen or limited oxygen for 24 h. (C) U87 and U87PTEN cells were transfected with a maspin reporter construct and placed under respective treatments (hypoxia or normoxia) for 24 h. Cells were lysed and analyzed for Luciferase and RSV- β -galactosidase activity. Bars represent relative luciferase activity (Luciferase Activity/ β -galactosidase Activity) from three independent transfections and standard deviation was calculated from the mean. (D) U87 and U87PTEN cells were transfected with control shRNA or shRNA to p53. Whole cell lysates were prepared to confirm the knockdown of p53 by western blot. (E) U87 and U87PTEN Cells were cultured under hypoxia or normoxia for 24 h and whole cell lysates were analyzed by western blot for maspin, PTEN and GAPDH. (F) U87PTEN cells were pre-treated with Pifithrin- α (20 μ M) or DMSO for 1 h and then cultured under hypoxia or normoxia for 24 h. Cell lysates were analyzed by western blot for maspin and Actin.

Results

Examination of maspin expression in glioblastoma cells. Maspin has been characterized in several prostate and breast cancer cell lines, however, it has not been characterized in glioblastomas. The lack of maspin expression in tumors is an indicator of poor prognosis.¹⁶ We utilized several cell lines to determine the degree of maspin expression in glioblastomas. The cells were incubated under 1% oxygen (hypoxia) for 24 h and cell lysates were prepared for western blot analysis. Of these cell lines, only SF767 cells expressed significant levels of maspin (Fig. 1A). This was the only cell line that was wild-type for p53 and PTEN. According to a previous study limited oxygen can stimulate p53 accumulation and activity.^{17,18}

The possibility of an integrated PTEN, p53 and Maspin tumor suppressor pathway, forced us to further investigate the requirement of PTEN and p53 in the induction of Maspin.

The requirement of PTEN and p53 to induce Maspin in response to hypoxia. The results in Figure 1A indicated that both PTEN and p53 may be required to coordinate the expression of maspin. In order to determine if this was evident we examined maspin levels in U87 (p53wt/PTEN null) cells transfected with a control viral vector (herein referred to simply as U87) and U87 cells with reconstituted PTEN (U87PTEN).¹⁹ Cells were incubated in a hypoxic environment in order to elicit a p53 response. p21 and Maspin, known p53 transcriptional targets, are activated in response to hypoxia, but only in cells that contain PTEN (Fig. 1B). To determine if the induction

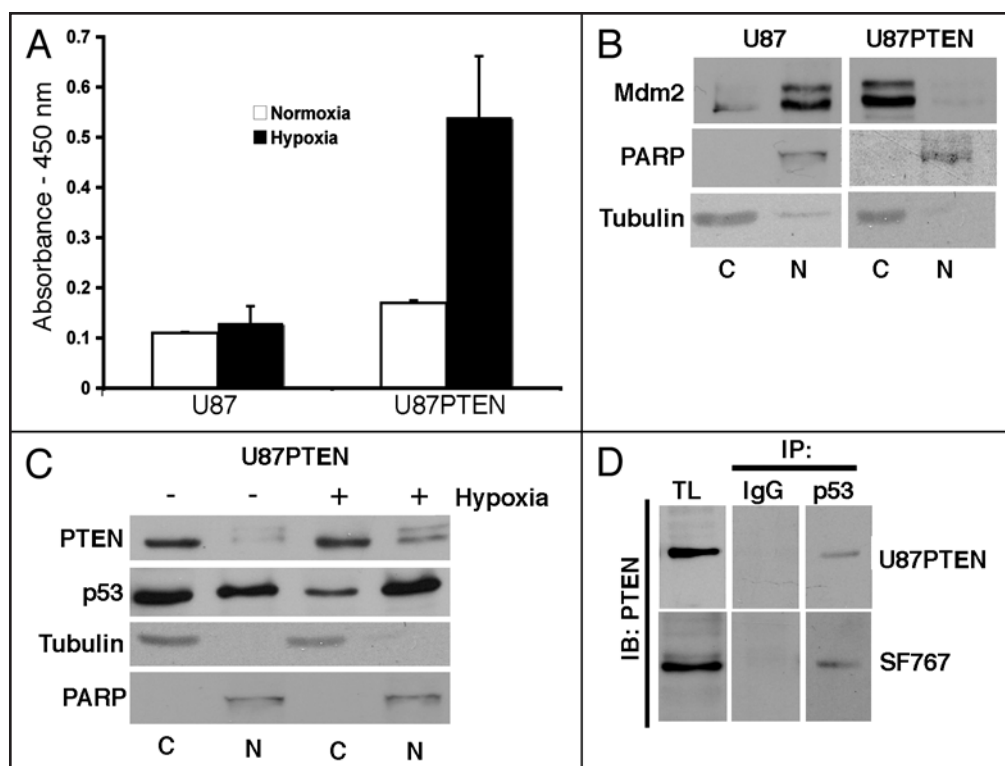


Figure 2. Further characterization of the PTEN/p53/Maspin pathway. (A) U87 and U87PTEN cells were cultured under hypoxia or normoxia for 24 h in serum free DMEM. An ELISA analyzed the media for secreted maspin. (B) Cytoplasmic (C) and nuclear (N) extracts from U87 and U87PTEN cells were analyzed for Mdm2, PARP and Tubulin by western blot. (C) Western blot of PTEN, p53, tubulin and PARP from cytoplasmic and nuclear extracts isolated from U87 and U87PTEN cells. (D) p53 or control IgG was immunoprecipitated from U87PTEN and SF767 nuclear extracts under hypoxic conditions. Western blot detected PTEN. Nuclear extracts (TL) were run to verify the presence of PTEN in the nucleus.

of maspin protein was due to an increase in gene transcription, a maspin reporter construct was used. U87 and U87PTEN cells were transfected with the maspin promoter construct and cultured under normoxia or hypoxia for 24 h. Results indicate that hypoxia promotes greater than 2-fold induction of maspin in U87PTEN cells. When PTEN is absent (U87 cells) maspin induction is limited (Fig. 1C). Co-transfection of a control reporter construct with RSV- β -galactosidase produced relative luciferase values of less than 0.2 under all four conditions (data not shown).

To examine the dependence of p53 and PTEN on the induction of maspin, p53 levels were decreased in U87 and U87PTEN cells by small hairpin RNA (shRNA). One should note that stable reintroduction of PTEN into the U87 cells causes a decline in the amount of p53 protein present, due to a mechanism which remains to be defined (Fig. 1D). However, it should also be noted that although p53 protein level is diminished when PTEN is present, the activity of p53 is increased, as evidenced by strong induction of p21 (Fig. 1B). This is consistent with previous research that shows that the levels of wild-type p53 do not necessarily correlate with transactivation of p53 target genes.²⁰ Furthermore, U87PTEN cells are more sensitive to chemotherapeutic agents as compared to the parental cells.²¹ Reduction of p53 in U87PTEN cells significantly decreased levels of maspin protein as measured by western blot (Fig. 1E). Pre-treatment of U87PTEN cells with Pifithrin- α , a p53 small molecule inhibitor, showed a reduction of maspin protein in response to hypoxia (Fig. 1F). These experiments demonstrate that p53 and PTEN are

necessary to effectively induce maspin under hypoxic conditions.

It is known that maspin is secreted into the extracellular matrix²² and that recombinant maspin in vitro can inhibit endothelial migration.²³ This notes the physiological relevance of maspin secretion. Due to this, we wanted to determine if the maspin being produced by U87PTEN cells was being secreted. Cells were plated and placed under hypoxia in serum free media for 24 h. The media was collected and analyzed by ELISA. Results indicate that a greater than 4-fold increase in maspin protein was secreted into the media by U87PTEN cells in response to hypoxia (Fig. 2A). Thereby supporting the transcriptional data of p53 and PTEN coordinating the induction of the maspin gene.

PTEN sequesters Mdm2 to the cytoplasm. Previous literature has shown that p53 is negatively regulated by Mdm2.²⁴ An E3 Ligase, Mdm2 ubiquitinates p53, which causes its nuclear exportation and subsequent proteosomal degradation. Therefore, if Mdm2 is residing in the nucleus, p53 activity is significantly lower. The nuclear translocation of Mdm2 is regulated in part by Akt. Akt phosphorylates Mdm2 on serines 166 and 186 and these modifications enhance Mdm2's migration to the nucleus.¹¹ PTEN directly antagonizes Akt.⁹ This implies that reintroduction of PTEN should sequester Mdm2 to the cytoplasm. Accordingly, nuclear and cytoplasmic fractions from U87 and U87PTEN cells were prepared to analyze Mdm2 localization. Western blots show that in U87 cells the Mdm2 predominantly resides in the nuclear fraction, while the opposite was evident in the U87PTEN cells having virtually undetectable Mdm2 in nuclear fractions (Fig. 2B).

PTEN and p53 colocalize in the nucleus under hypoxic conditions. Previous work has shown that the transcription of maspin is under the control of p53.⁴ There are two mechanisms by which PTEN stabilizes p53: through a phosphatase-dependent mechanism in the cytoplasm, PTEN attenuates the Akt pathway and sequesters Mdm2 to the cytoplasm;¹¹ through a phosphatase-independent mechanism, PTEN physically interacts with, and stabilizes, p53 in the nucleus.²⁵ To determine if the subcellular localization of PTEN is altered in response to hypoxia, nuclear and cytoplasmic extracts were isolated and western blots were prepared to analyze PTEN localization. Cytoplasmic and nuclear extracts show that under hypoxic conditions the nuclear fraction of PTEN is increased (Fig. 2C). It is important to note that not all of PTEN localized to the nucleus in response to hypoxia over 24 h, supporting the cytoplasmic localization of Mdm2 in Figure 2B. To determine if cytoplasmic and nuclear extracts were pure, PARP and Tubulin were used as controls.

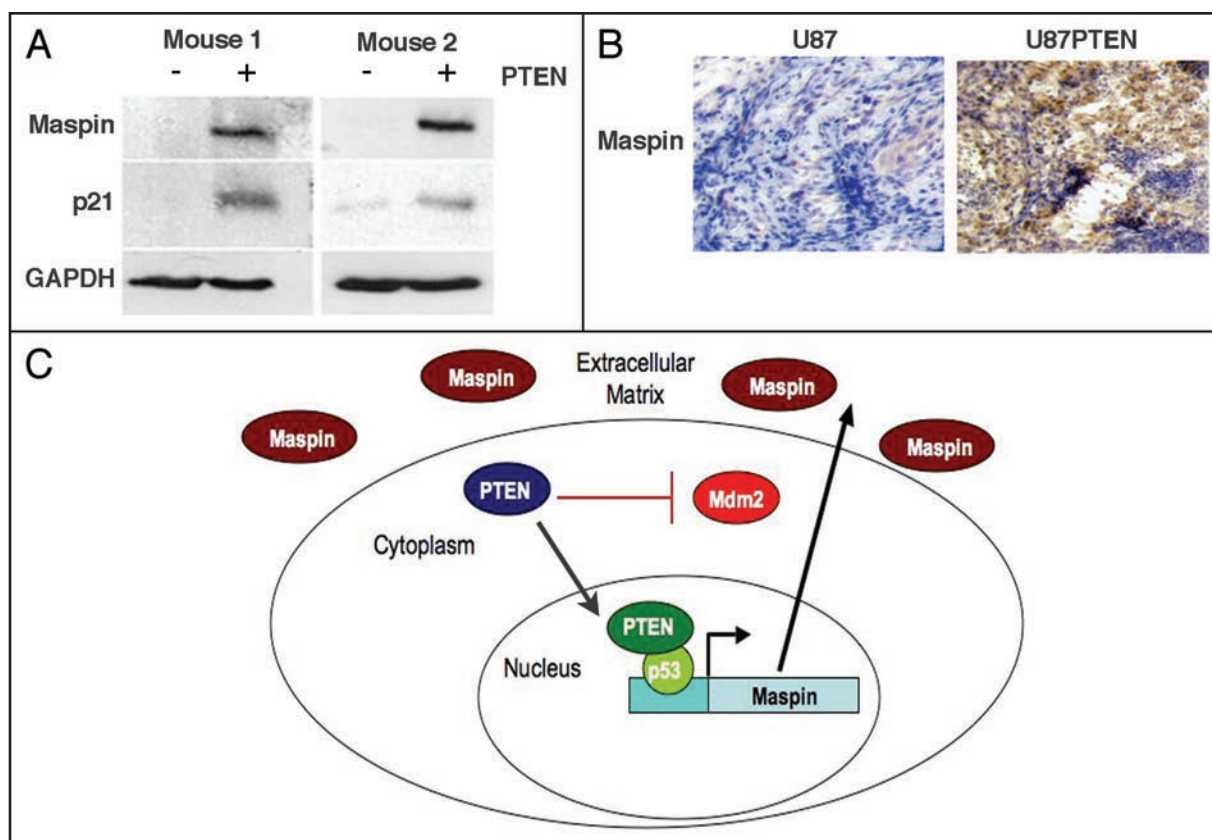


Figure 3. Maspin in mouse xenograft model. (A) U87 and U87 PTEN were injected into the flanks of a mouse and the tumors were isolated two weeks post implantation. Tumors from two mice were harvested and lysates were produced for western blot for detection of p21, maspin and GAPDH. (B) Tumors were stained for maspin using immunohistochemistry. (C) A basic model illustrating the dual role of PTEN in the production of maspin.

We also wanted to determine if PTEN and p53 were physically interacting in the nucleus under hypoxic conditions. It was previously demonstrated that this interaction in the nucleus increases the DNA binding ability of p53 in response to genotoxic stress.²⁵ We immunoprecipitated p53 from nuclear extracts of U87PTEN and SF767 cells treated with hypoxia. Under hypoxic conditions PTEN co-purified with p53 from nuclear extracts (Fig. 2D). Thus, PTEN may be playing a dual role, by both regulating Mdm2 nuclear localization and forming a complex with p53 in the nucleus to augment maspin induction.

Maspin expression in U87 xenografts. To examine the role of PTEN as it relates to maspin induction in vivo, U87 and U87PTEN cells were engrafted into the flanks of nude mice. The tumors were harvested two weeks after implantation and analyzed by immunohistochemistry (IHC) and western blot. Consistent with previous in vitro experiments, U87PTEN cells produced a detectable amount of maspin compared to U87 cells, as determined by western blot (Fig. 3A). Additionally, IHC analysis confirmed the western blot, whereby maspin production was highly elevated in U87PTEN cells compared to U87 cells (Fig. 3B). This is consistent with the fact that as the tumor cells proliferate in the tumor microenvironment they move away from the vessels into a limited oxygen environment. Thus, the xenograft and cell culture models used for investigating the p53/pten/Maspin axis are in agreement.

Discussion

In response to low levels of oxygen PTEN plays a dual role: in the cytoplasm PTEN regulates Mdm2 nuclear localization; in the

nucleus it binds to p53 and induces Maspin (Fig. 3C). The nuclear localization of PTEN is mediated by the Major Vault protein (MVP).²⁶ However, it is unknown if MVP is required for the nuclear localization of PTEN in response to hypoxia. Although the outcome of nuclear PTEN in response to hypoxia is apparent, the mechanism by which this occurs has yet to be defined.

PTEN and/or p53 are lost or mutated in many breast, prostate and brain cancers.²¹ These losses lead to inadequate responses to chemotherapy and other stimuli that typically engage the p53 pathway.¹² Due to this, one could speculate that maspin, an important downstream effector that inhibits angiogenesis, metastasis and proliferation, is not induced. Recent work has shown that haploinsufficiency of the maspin tumor suppressor gene leads to hyperplastic lesions in the prostates of mice.²⁷ This genetic model provides overwhelming evidence for the importance of the maspin tumor suppressor. Altering the expression of Maspin through gene silencing or loss of the PTEN/p53 axis may provide a selective advantage for the tumor. Thus, our work integrating the tumor suppressor network of p53/PTEN/Maspin provides the impetus for investigation of new therapies that reengage aspects of the pathway that may be disengaged. Such therapies, in conjunction with standard treatments, may more effectively target highly proliferative and vascularized tumors.

Materials and Methods

Cell lines and reagents. All cell lines were cultured in DMEM (Invitrogen, GIBCO Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, UT), penicillin/streptomycin and

5% CO₂. For hypoxia treatments cells were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and the air was exchanged with 1% O₂, 5% CO₂, 94% N₂. The cells were then incubated at 37°C for 24 hours. U373 and U118 cells were kind gifts from Lawrence Quilliam and Daniela Matei respectively. Pifithrin- α was purchased from EMD Chemicals (La Jolla, CA).

Plasmids, transfections and viral infection. All transfections were performed using the calcium phosphate method. The production of U87PTEN and U87 (control vector) cells was previously described.¹⁹ Cells stably expressing shp53 or control shRNA were made as follows. Virus was produced by transient transfection of 293T cells with 20 μ g LVTHM-shp53-GFP or a control vector, 15 μ g PMD2G and 6 μ g pCMV dr8.74. Two days following transfection the supernatant was collected with 4 μ g/ml of polybrene and forced through a 0.2 μ filter. Target cells were then repeatedly transduced with the latter described supernatant. GFP expression confirmed the efficacy of the infection to nearly 100%.

Immunoprecipitations and western blots. Cells were grown with the indicated treatments and harvested. Whole cell lysates, and cytoplasmic and nuclear extracts were isolated as previously described.²⁸ Immunoprecipitations from nuclear extracts were conducted as previously described.²⁹ Protein concentrations of lysates were determined using Bradford Reagent (BioRad Laboratories Inc., Hercules, CA). Extracts were then boiled in 6X Laemmli buffer and separated on polyacrylamide gels and transferred to PVDF membrane (GE Healthcare, Little Chalfont, Buckinghamshire UK). The blots were incubated with antibodies against maspin (Chemicon, Temecula, CA), PTEN, p53 (DO-1), p21, α -tubulin, (Santa Cruz Biotechnology, Santa Cruz, CA) Mdm2, (IF2 and 2A10) and GAPDH (Calbiochem, La Jolla, CA) and either goat anti-mouse-HRP or goat anti-rabbit-HRP (BioRad Laboratories, Hercules, CA) for subsequent detection with chemiluminescence (Perkin Elmer, Shelton, CT).

Luciferase assays. The maspin luciferase construct was a generous gift from Dr. Srivastava.⁴ Cells were plated and transfected with the respective luciferase construct and RSV- β -Galactosidase. The following day the cells were treated as indicated. Post-treatment (24 h) the cells were harvested and lysed. The Luciferase Assay System (Promega, Madison, WI) was followed as directed by the manufacturer. The values obtained were normalized against RSV- β -galactosidase activity, as measured using GalactoStar (Tropix, Bedford, MA), to obtain a value for relative luciferase. The data represent independent transfections in triplicates and standard deviation was calculated from the mean.

Enzyme linked immunoabsorbant assay (ELISA). Cells were grown in 6-well plates and then placed under hypoxia or normoxia. The supernatant was collected, treated with protease inhibitor cocktail (Calbiochem, La Jolla, CA), and centrifuged to remove any cellular debris. A 96-well plate was then coated with 50 μ l of anti-maspin mAb (Chemicon, Temecula, CA) diluted in 0.1 M bicarbonate, pH 9.2 for 2 h at room temperature. The wells were cleared and 100 μ l of blocking buffer containing 100 mM phosphate buffer, pH 7.2, 1% BSA, and 0.5% Tween-20 was added and the plate was incubated for 1 h at room temperature. Following blocking, 50 μ l of previously collected supernatant was added and the plate incubated at room temperature for 1 h. The plate was then washed with PBST. Following this, 50 μ l of rabbit polyclonal anti-maspin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 0.1

M bicarbonate, pH 9.2 was added to each well and incubated for 45 minutes. The wells were then washed with PBST. 50 μ l of goat anti-rabbit-HRP (BioRad, Hercules, CA) was added to each well and incubated for 45 minutes. Following washes TMB One Solution (Promega, Madison, WI) was added to each well. The absorbance of each well was read at 450 nm.

Immunohistochemistry. After paraffin removal and blocking, the maspin antibody (1:250) was incubated for 1 h at room temperature. Primary rabbit polyclonal antibody (Santa Cruz Biotechnology) against maspin was used.

Xenografts. U87 and U87PTEN cells (2.0 x 10⁶) were injected into the flanks of nude mice. Two weeks post-injection the mice were euthanized and tumors were harvested and analyzed by western blot and immunohistochemistry.

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