ATP modulates PTEN subcellular localization in multiple cancer cell lines

Glenn P. Lobo[1,3], Kristin A. Waite[1,3,4], Sarah M. Planchon[1,3], Todd Romigh[1,3], Janet A. Houghton[2,4,6] and Charis Eng[1,3,4,5,6,]*

[1]Genomic Medicine Institute, [2]Department of Cancer Biology, [3]Lerner Research Institute and [4]Taussig Cancer Institute, Cleveland Clinic Foundation, 9500 Euclid Ave, NE-50, Cleveland, OH 44195, USA [5]Department of Genetics and [6]CASE Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH, USA

The tumour suppressor gene *PTEN* plays an important somatic role in both hereditary and sporadic breast carcinogenesis. While the role of PTEN's lipid phosphatase activity, as a negative regulator of the cytoplasmic phosphatidylinositol-3-kinase/Akt pathway is well known, it is now well established that PTEN exists and functions in the nucleus. Multiple mechanisms of regulating PTEN's subcellular localization have been reported. However none are ubiquitous across multiple cancer cell lines and tissue types. We show here that adenosine triphosphate (ATP) regulates PTEN subcellular localization in a variety of different cancer cell lines, including those derived from breast, colon and thyroid carcinomas. Cells deficient in ATP show an increased level of nuclear PTEN protein. This increase in PTEN is reversed when cells are supplemented with ATP, ADP or AMP. In contrast, the addition of the non-hydrolyzable analogue ATPγS, did not reverse nuclear PTEN protein levels in all the cell types tested. To our knowledge, this is the first report that describes a regulation of PTEN subcellular localization that is not specific to one cell line or tissue type, but appears to be common across a variety of cell lineages.

INTRODUCTION

The precise mapping of the Cowden syndrome susceptibility gene to 10q22-q23 in 1996 facilitated the isolation of *p*hosphatase and *ten*sin homologue deleted on chromosome 10 [*PTEN* (MIM 601728)], also known as *MMAC1* or *TEP*1, the following year (1–4). Germline mutations in *PTEN* have been identified in 85% of probands with Cowden syndrome [CS (MIM 158350)], an under-diagnosed autosomal dominant disorder with a high risk of benign and malignant breast, thyroid and endometrial neoplasias (5–7). Subsequently, somatic mutations or deletions of *PTEN* have been identified in variable subsets of a broad range of sporadic tumour types, including those of the breast, thyroid, colorectum, endometrium, melanoma and prostate (8–10).

The role of PTEN's lipid phosphatase activity as a negative regulator of the cytoplasmic phosphatidylinositol-3-kinase (PI3K)/Akt pathway is well known. In recent years, we have begun to elucidate the role of PTEN in regulating cellular pathways in other compartments of the cell. Initially we, and others, had observed PTEN in the nucleus using immunohistochemical analyses in breast, thyroid, endocrine pancreatic tumours and cutaneous melanomas (11–13). Subsequent studies, by us, and others, have shown that PTEN does, indeed, traffic to the nuclear compartment. Further, PTEN immunohistochemistry has revealed nuclear expression in several tissue types, including breast, which appear to be highest in normal cells and diminish, with concomitant increase in expression in cytoplasm, with neoplastic progression, indicating that the subcellular localization of PTEN plays a role in its function (12,13). Indeed, nuclear PTEN has been shown to regulate cell cycle and to regulate MAPK activation (14,15). Nuclear PTEN mediates growth suppression independent of AKT down-regulation and is essential for maintaining chromosomal integrity through physical association with the kinetochore (16).

---

*To whom correspondence should be addressed. Tel: +1 216 444 3440; Fax: +1 216 636 0566; Email: engc@ccf.org

Despite the lack of classical nuclear localization signal (NLS) motifs on PTEN (17–19), recent studies have shown that several mechanisms appear to modulate its localization. These include a cytoplasmic localization signal (CLS) (20), PTEN phosphorylation and involvement of Ran-GTPase activity (21,22), interaction with Major Vault Protein (MVP) via a bipartite NLS (17,23), mono-ubiquitination (18), S6K-mediated export (24) and passive diffusion (25). Despite this diverse range of mechanisms, none of the current modes of regulation appear to be universally applicable across a variety of cell lines or tissue/tumour types.

The modulation of adenosine triphosphate (ATP) levels has been shown to be therapeutically relevant in the cancer field (26). ATP depletion, when used in combination with chemotherapy and/or radiation, has a variety of effects on cancer cells including inducing apoptosis in multi-drug resistant cells and decreasing tumour promotion (26–31). The exact mechanisms of these effects have not been fully elucidated. Interestingly, several have shown that the PI3K/Akt, pathways also modulated by PTEN, may play a role in these effects (32,33).

Active transport, of molecules and proteins, has been demonstrated in a variety of systems (34–37). Interestingly, despite this evidence, there has been no examination of the potential role of ATP in modulating PTEN's subcellular localization. Based on other protein requirements for active transport, we hypothesized that PTEN would require ATP for its nuclear-cytoplasmic shuttling. Therefore, we sought to examine the effects of ATP modulation on intracellular PTEN localization.

RESULTS

Cellular ATP depletion increases nuclear PTEN in breast, colorectal and thyroid carcinoma cells

In order to examine the requirements of ATP for PTEN subcellular localization, we first established a system in which we could deplete cellular ATP levels in a reversible manner. Towards this end, we utilized the conventional method of 2-deoxy-D-glucose (2-DG) and sodium azide (35,37). Treatment using glucose-free media containing 2-DG and sodium azide (ATP depletion media) reduced total cellular ATP levels by ∼90% in 4 h, compared with untreated control cells (Fig. 1). ATP levels could be restored, in a time-dependent manner, by the removal of the ATP depletion media containing the metabolic inhibitors and the addition of fresh glucose-free medium (regeneration media) to each cell line. Within 4 h, cellular ATP levels had been restored to ∼61% of that observed in control cells (Fig. 1). Results comparable to those in Figure 1 were obtained, with each of the other cell lines used in this study, in terms of both the magnitude and time-course of ATP depletion and regeneration (data not shown). In this system, we observed, as expected, decreased phosphorylation of MAPK, Akt and PTEN in the ATP depleted state (data not shown). As expected, these molecules were re-phosphorylated when ATP levels were restored.

Having established a system in which we could reproducibly modulate ATP levels, we next examined the effect of cellular ATP depletion on PTEN subcellular localization. In untreated breast cancer lines, PTEN is present in both the nuclear and cytosolic fractions with MDA-MB-231 breast cancer cells

having a higher basal level of nuclear PTEN levels than MCF7 breast cancer cells (Fig. 2A and B). After ATP depletion, total intracellular ATP levels in all cell lines studied decreased to 8–12% of control non-treated cells. In breast cancer lines, ATP depletion resulted in an increase in nuclear PTEN levels, in a time-dependent manner, peaking at 3–4 h (Fig. 2A and B). This was not simply due to an increase in cytosolic contamination, as α-tubulin protein was not detected in the nuclear fractions. In the lines tested, we did not observe a concomitant decrease in cytoplasmic PTEN. This is not unexpected as the vast majority of PTEN is localized in the cytoplasm with a small, but distinct portion present in the nucleus (Fig. 2A–D). The relatively small cytosolic decrease is difficult to detect by western blot. Nevertheless, our data shows that following ATP depletion, the level of PTEN present in the nucleus increases, resulting in a large relative change (Fig. 2A–D).

Because previous work has suggested that mechanisms of PTEN subcellular localization may be cell line-dependent (19), we examined the effect of cellular ATP depletion in both colon and thyroid cancer cell lines. Similar to what was observed in the breast cancer cell lines, there was a basal level of nuclear PTEN prior to ATP depletion in both colon (Fig. 2C, Supplementary Material, Fig. S1) and thyroid (Fig. 2D) lines. Additionally, as was observed in the breast cancer lines, depletion of cellular ATP in each of the colon and thyroid cancer cell lines resulted in a time-dependent increase of nuclear PTEN protein (Fig. 2C and D, Supplementary Material, Fig. S1). The data presented in Figure 2 demonstrate that the depletion of ATP for 4 h results in a reproducible increase of nuclear PTEN in carcinoma cell lines derived from three different origins.

In order to corroborate the biochemical analyses presented above, we used indirect-immunofluorescence to examine the changes in subcellular localization of endogenous PTEN, following ATP depletion in breast, colon and thyroid carcinoma cells (Fig. 3, Supplementary Material, Fig. S2B and C). In agreement with the biochemical observations above, PTEN is found in both the cytoplasm and nucleus in untreated cells of all cell types (complete medium; Fig. 3A–D). After cellular ATP depletion, a gradual increase in nuclear PTEN levels was observed, which peaked at 4 h in all cell lines (ATP depletion, 2, 3 and 4 h; Fig. 3A–D). Merging of the nuclear (DAPI) and PTEN (FITC) images confirmed that the localization of PTEN became more nuclear following ATP depletion for 3–4 h (Fig. 3A and B, panels i, l, o; Fig. 3C panels f, i, l; Fig. 3D, panels f, i, l). Incubation of cells in glucose-free medium did not alter the localization of PTEN (Fig. 3A, panels b versus e), demonstrating that the localization changes were due to an active depletion of ATP and not simply a withdrawal of glucose from the system.

Regeneration of cellular ATP decreased nuclear PTEN to basal levels

To determine if increased nuclear PTEN localization resulting from cellular ATP depletion was reversible, we depleted ATP in the cells, as previously described. We then removed the ATP depletion media containing the metabolic inhibitors and added fresh glucose-free medium (regeneration media) to each cell line, allowing the regeneration to continue, in a
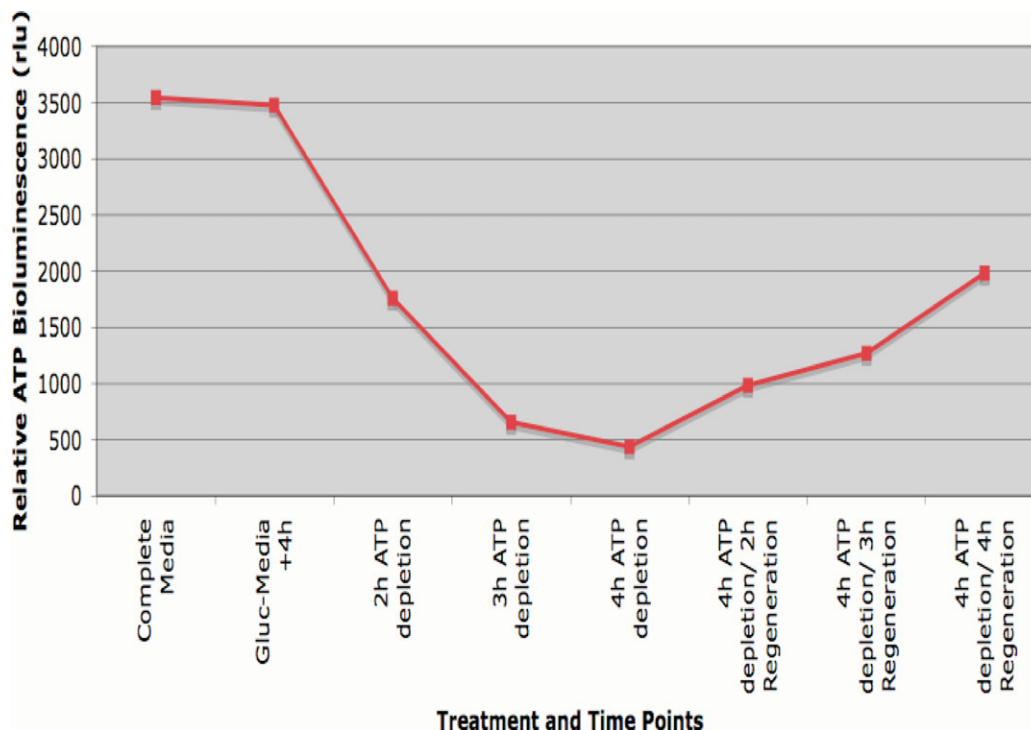
Figure 1. Depletion and regeneration of cellular ATP depletion in cultured cancer cells. For cellular depletion of ATP, cells were incubated in glucose-free media containing 6 mM 2-deoxy-D-glucose and 10 mM sodium azide. ATP levels were measured using the luciferase–luciferin method, as described in the Materials and Methods. Note that ATP depletion effectively reduced total cellular ATP levels by ~90% in 4 h, compared with untreated control cells. ATP levels could be restored by the removal of the ATP depletion media, containing the metabolic inhibitors, and the addition of fresh glucose-free medium (regeneration media). This representative graph shows ATP levels in the MCF-7 breast cell line.

time-dependent manner for 2−4 h. By 4 h, cellular ATP levels had been restored to ~61% of that observed in control cells (Fig. 1). When we examined the levels of nuclear PTEN following ATP regeneration, by both western analysis (Fig. 2A–D) and immunofluorescence (Fig. 3A and B), we found that in each case, nuclear PTEN levels were lower in the cells in which ATP was allowed to regenerate compared with nuclear PTEN levels observed in ATP-depleted cells (Fig. 3). This confirmed that, in all three cell types (regeneration immunofluoroscence data only shown for MCF7 cells), nuclear PTEN levels increased in the absence of ATP, while nuclear PTEN decreased in response to increasing ATP levels.

**Metabolites of ATP, but not ATPγS can modulate PTEN localization**

Based on our results, we chose to examine whether metabolites of ATP are able to reverse the nuclear localization observed in cellular ATP-depleted cells. To this end, following ATP depletion, we supplemented the glucose-free regeneration media with ATP, ADP, AMP, deoxy-ATP (dATP) or the non-hydrolyzable ATP analogue, ATPγS. The addition of each component to the regeneration media affected the regenerated levels of cellular ATP. In adenosine nucleoside- and glucose-free regeneration media, the cellular ATP levels were ~61% of the ATP levels measured in control cells. The addition of ATP to the regeneration media resulted in cellular ATP levels that were ~76% of control cells. The addition of other

components also influenced ATP regeneration, with the addition of ADP resulting in regeneration of ATP to ~71% of control, of AMP ~66% of control, of ATPγS ~12% of control levels and of dATP ~54% of control levels (data not shown).

As shown above, nuclear PTEN increased when cellular ATP was depleted in breast, thyroid and colon cancer lines (Figs 2 and 3, Supplementary Material, Fig. S1 and S2). We found that the addition of ATP, ADP or AMP resulted in a decrease in nuclear PTEN protein across breast, colon and thyroid cancer cells lines (Fig. 4, Supplementary Material, Fig. S3). In contrast, the addition of ATPγS did not reverse the ATP-dependant change in nuclear PTEN levels in any of the cell lines (Fig. 4A–D). Interestingly, supplementation with dATP, after cellular ATP depletion, reversed PTEN nuclear localization only in colon cells. In breast and thyroid cancer cells, this effect was subtle.

DISCUSSION

While there is now increasing evidence of the role of proper intracellular localization in the regulation of PTEN's function, the mechanism of PTEN nuclear transport is not clear (19). PTEN does not possess a traditional NLS. Several mechanisms have been proposed for localization of PTEN to the nucleus; however, to date, a common mechanism has not been elucidated (14,17−25). Interestingly, active transport and the role of ATP has not been previously investigated. Based upon other proteins' requirement for active transport (34−37), we
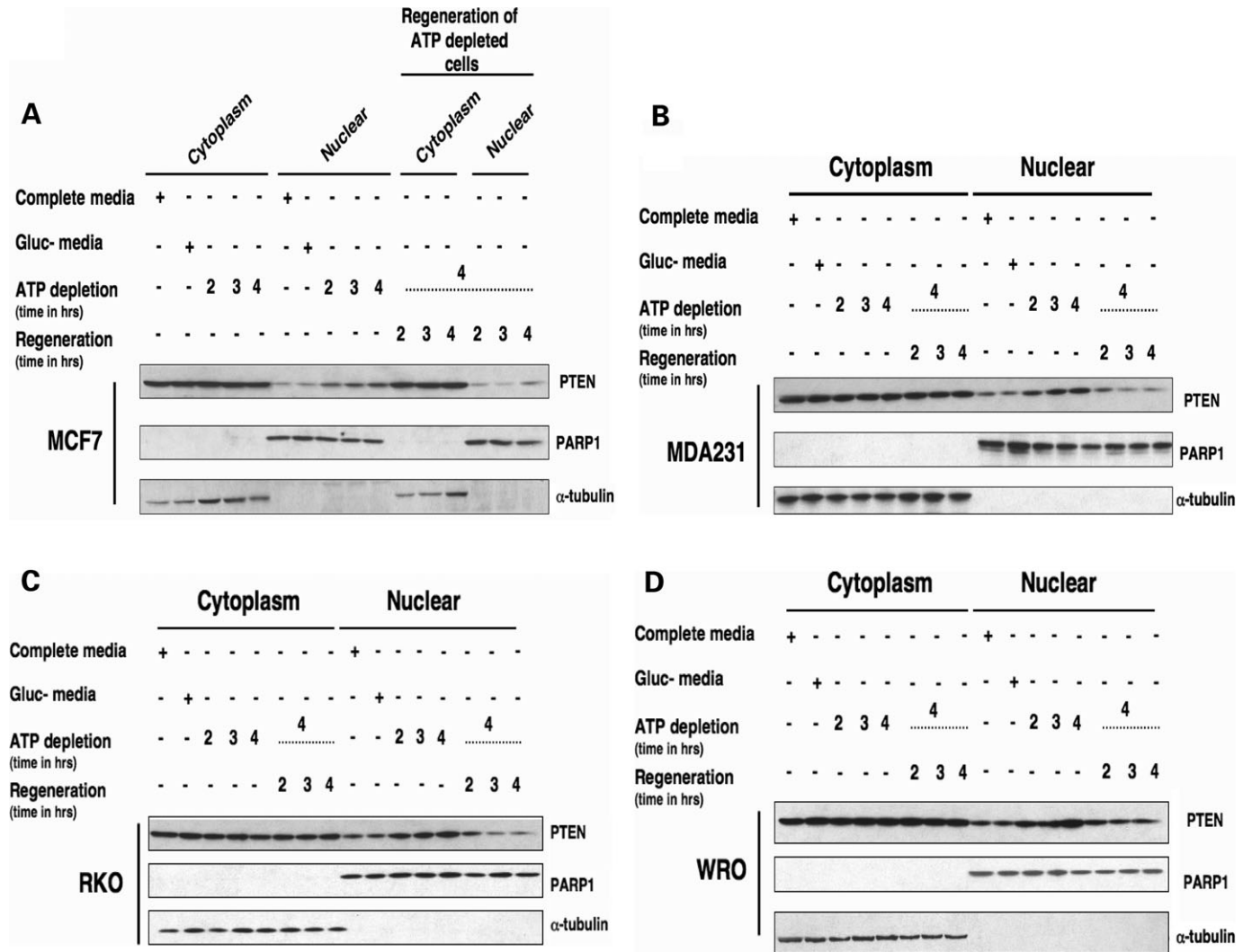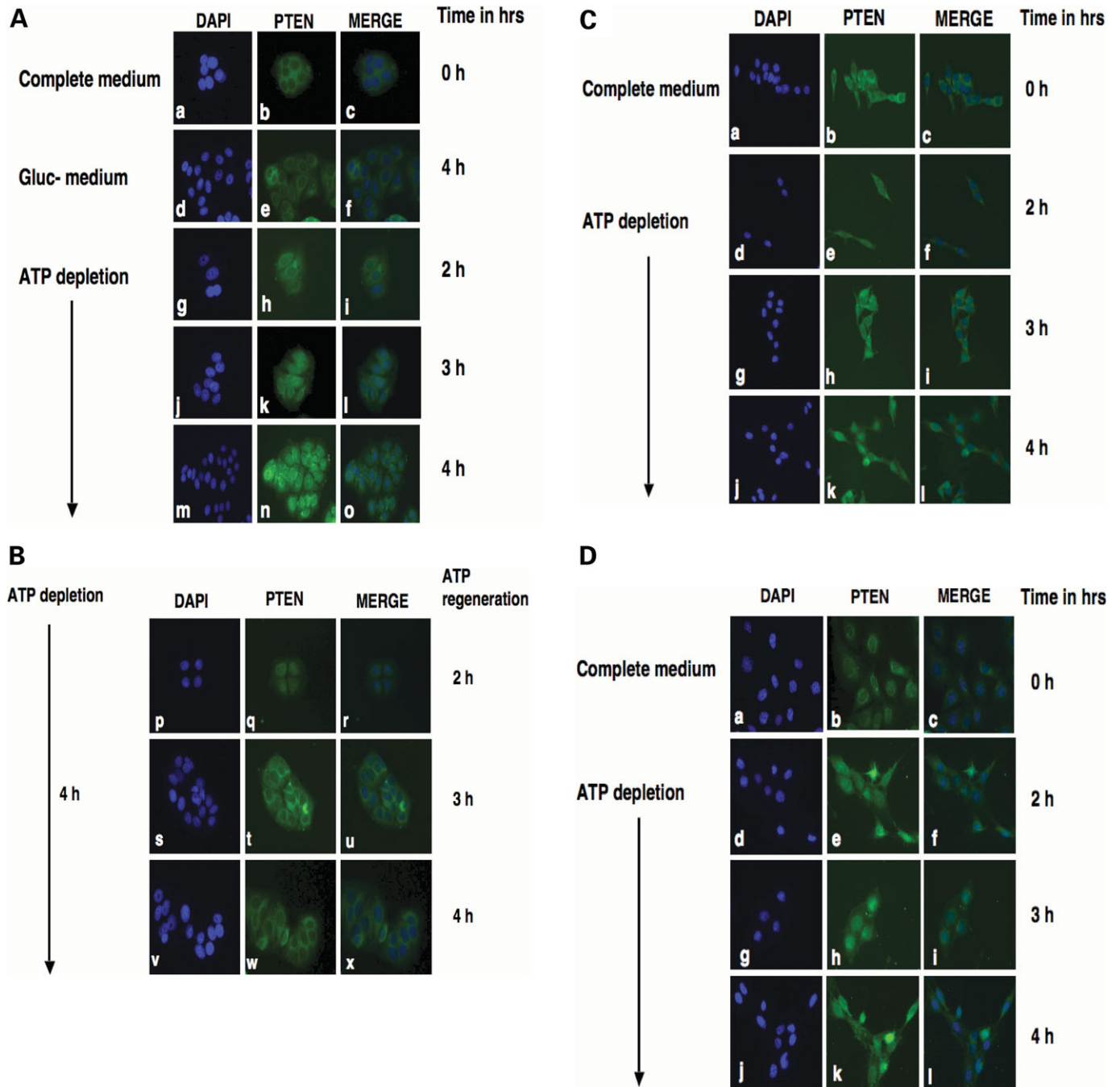
Figure 2. Depletion of cellular ATP increases nuclear PTEN protein levels in a reversible manner. Cells were grown and maintained as described in the Materials and Methods. Control cells were maintained in complete medium or glucose-free medium. Cellular ATP was depleted using ATP-depletion media for up to 4 h. Where indicated, ATP was regenerated for 2–4 h. At the indicated time-points, control, ATP-depleted and regenerated cells were subjected to subcellular fractionation and analyzed by western blot for PTEN, α-tubulin (cytosolic marker) or PARP-1 (nuclear marker). (**A**) MCF7 and (**B**) MDA-MB-231 breast carcinoma cell lines, (**C**) RKO colorectal carcinoma cell line and (**D**) WRO-82-1 follicular thyroid cell line.

hypothesized that PTEN would require ATP for nuclear import. Surprisingly, we found that depletion of cellular ATP resulted in increased nuclear PTEN levels in a variety of cancer cell types.

Our current observations suggest that nuclear PTEN accumulation requires ATP for either active export from the nucleus or that there is a loss of ATP-dependent inhibition of nuclear import. This effect on PTEN localization was reversible as regeneration of ATP, either through replacement of the media or through supplementation, resulted in a decrease in nuclear PTEN levels. Addition of ATP to the regeneration media increased the total intracellular ATP levels as well as reversed the increase in nuclear PTEN observed following ATP depletion. That the ATP-related nucleotides ADP, AMP, and to a much lesser extent dATP, also facilitated these changes suggests that the cells remained capable of producing ATP throughout the depletion process. Supplementation with ATP precursors enhanced the regener-

ation process, and as a result, both increased ATP levels and decreased nuclear PTEN. In contrast, addition of ATPγS to the ATP regeneration media did not result in an enhanced recovery of ATP levels or a decrease in nuclear PTEN. This difference highlights that it is the functional aspects of ATP and/or its related compounds that are important factors in PTEN's localization, not simply the structural aspects of ATP in general.

Our results with ATPγS also suggest that protein phosphorylation may play a role in the subcellular localization of PTEN. Direct phosphorylation has been suggested to play a role in PTEN function. Indeed, phosphorylation of PTEN plays a role in its stability and others have demonstrated that phosphorylated PTEN can modulate its location in specific cell types (21,22). However, we have shown that in MCF7 cells, PTEN nuclear localization is independent of PTEN phosphorylation (17). This system provides further evidence that nuclear import of PTEN is independent of its

Figure 3. Depletion of cellular ATP increases nuclear PTEN protein levels in a reversible manner. Cells were grown and maintained for microscopy studies as described in the Methods. Cells were depleted of cellular ATP using ATP depletion media for 4 h. If indicated, ATP was regenerated for 2–4 h. At the indicated times, cells were fixed using 4% paraformaldehyde and processed for indirect immunofluorescence as described. PTEN localization was determined utilizing a FITC-conjugated anti-mouse secondary antibody and nuclei were stained using DAPI. Representative data are shown for (**A** and **B**) MCF-7 breast carcinoma cells, (**C**) RKO colorectal carcinoma cells and (**D**) WRO-82-1 follicular thyroid carcinoma cells. The other cell lines studied had similar responses and are shown in the supplemental data. All experiments were carried out in duplicate. Approximately 200 cells were counted per individual time point and representative images from three separate experiments are shown. All images were acquired at ×20 magnification.

phosphorylation state, as under decreased ATP conditions (where nuclear PTEN increases) PTEN is not phosphorylated (Supplementary Material, Fig. S4). However, it remains a possibility that the phosphorylation of another key protein is important for either inhibiting ATP import or in facilitating PTEN export. An example of phosphorylation-dependent

export is the DNA-PK-dependent nuclear export of androgen receptor, which has been demonstrated in prostate cancer cells (38). In this system, DNA-PK phosphorylates the androgen receptor or an interacting component, resulting in nuclear export. While we do not postulate that DNA-PK is involved in our system, this system demonstrates the possibility of this
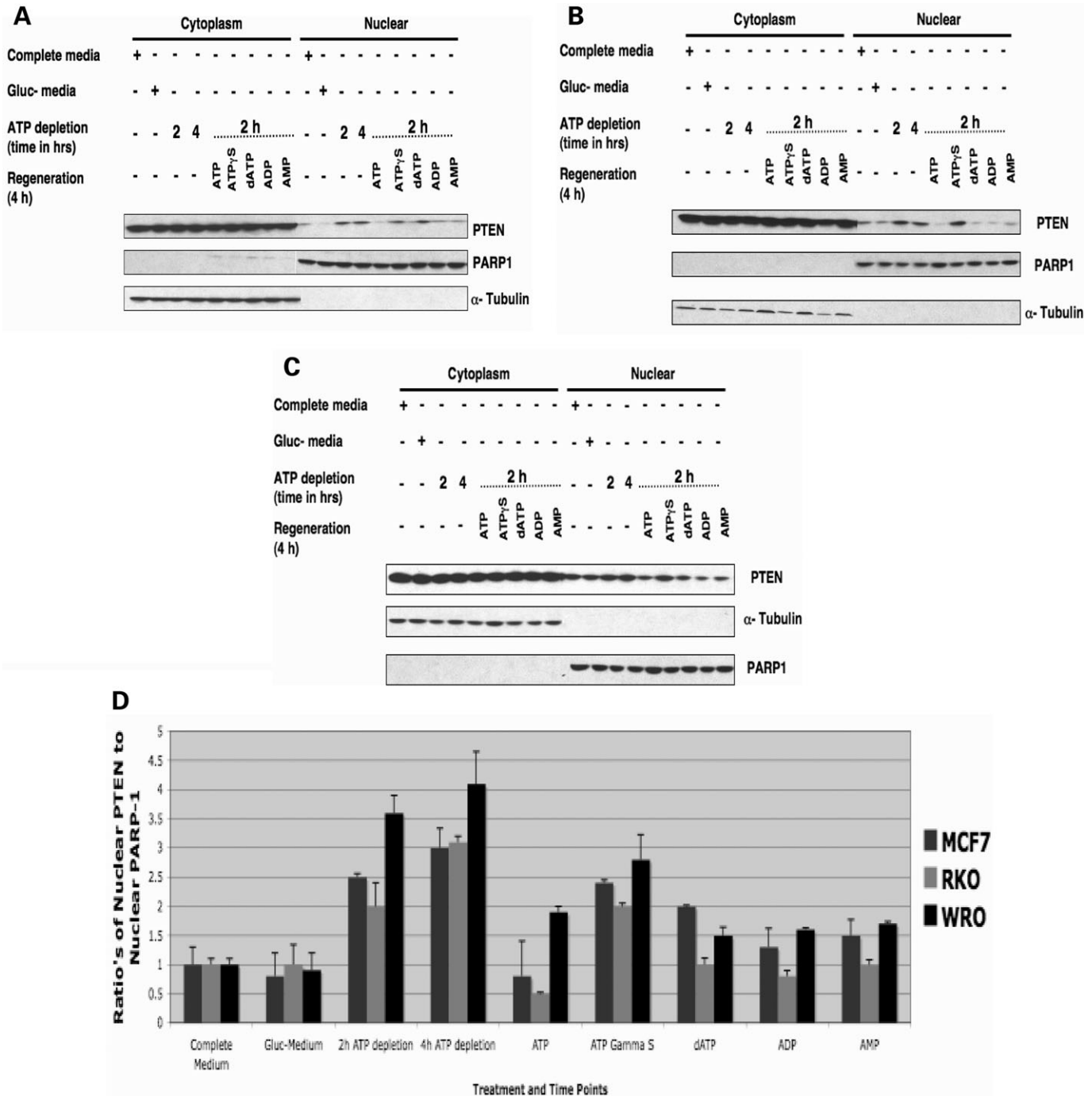
Figure 4. Metabolites of ATP, but not ATPγS, decreased nuclear PTEN levels. Cells were ATP-depleted for 2 h using ATP-depletion media, after which the depletion medium was replaced with glucose-free medium supplemented with 2 μM of either ATP, ADP, dATP, AMP, or ATPγS. Cells were subjected to sub-cellular fractionation and western analysis. Representative autoradiographs are from the analyses of (**A**) MCF7 breast carcinoma cells, (**B**) RKO colorectal carcinoma cells and (**C**) WRO-82-1 thyroid carcinoma cells. The remaining breast and colorectal cell lines behaved similarly and their data can be viewed in the supplemental data file. (**D**) To determine the effects on these tested cell lines, we quantitated the relative amount of nuclear PTEN protein by densitometry to obtain the ratio of nuclear PTEN to the nuclear loading control PARP-1. This ratio of nuclear-PTEN to PARP-1 was normalized to 1.0 in untreated cells (left-most 3 bars). Each bar depicts the mean ± SEM of three individual experiments.

concept. Another example of the role of ATP in nuclear export involves the Ran-GDP/Ran-GTP model. Yoneda and colleagues isolated an ATP-dependent activity that stimulated the release of GDP from Ran in the presence of NTF2 (39). GDP release allowed for an association between Ran and GTP that facilitates exportin-mediated nuclear export. ATP enhanced the GDP release and this enhancement was inhibited by non-hydrolysable ATP analogues. In addition, the inhibition of protein phosphatase activity enhanced this activity, suggesting that it may be regulated by a phosphorylation

event. We do not believe that this exact mechanism is the one that we observe, as the Ran-mediated mechanism of nuclear PTEN import does not appear to be involved in PTEN nuclear import in MCF7 cells (Minaguchi and Eng, unpublished results); however, this does not preclude a similar mechanism from being involved. It is also worthy to note that we have not seen a change in nuclear PTEN protein levels in MCF7 cells treated with the Protein Kinase C inhibitors staurosporin or GF10923X (Minaguchi and Eng, unpublished results). These results may suggest that PKC-dependent protein phosphorylation may not be involved in the common mechanism that we are observing here; however, further studies into this potential mechanism, while beyond the scope of this paper, may be fruitful.

Nuclear PTEN has been shown to be involved in several cellular processes such as cell cycle regulation, down-regulation of the MAPK signalling cascade and increases in apoptosis. Determination of the downstream effects of the ATP depletion-dependent increase in nuclear PTEN is problematic in our model system. The nearly complete depletion of cellular ATP renders examination of protein activation in the MAPK cascade by phosphorylation status uninterpretable, as all proteins become hypo-phosphorylated (including PTEN itself) under these experimental conditions. Additionally, following incubation under ATP-depleting conditions for $>5\,h$, most cells were found to detach from the tissue culture plates, rendering cell cycle analysis difficult. These issues were not surprising considering the importance of ATP in cellular homeostasis. Nonetheless, we found that following $3–4\,h$ under ATP depletion, MDA-MB-231, MCF-7 and RKO cells all demonstrated a slight increase in TUNEL and Annexin V staining (data not shown). The MDA-MB-231 cells demonstrated a 2-fold higher level of TUNEL staining than the MCF-7 and RKO cells. Interestingly, it was the MDA-MB-231 cells that also demonstrated the greatest increase in nuclear PTEN in response to ATP depletion, suggesting that this apoptotic response might indeed be a result of the increase in nuclear PTEN. While these results were preliminary, they highlight the need for future investigations into the role of ATP in modulating nuclear PTEN levels.

That manipulation of ATP levels in the cell results in nuclear localization of PTEN may suggest the involvement of intracellular energy-sensing mechanisms. This is an interesting area that warrants further investigation, particularly in light of potential cancer therapies that could manipulate energy and therefore regulate the localization of PTEN. Recently, work by Liu *et al.* have demonstrated in glioblastoma, that TSC2 and S6Kinase 1/2 activation are involved in the export of PTEN from the nucleus (24). Further elucidation of components of this potential signalling pathway leading to PTEN export, while beyond the scope of this paper, would be recommended.

We present here the first evidence of a common regulation of PTEN localization that is present not only in a few cell lines derived from specific tissues but a variety of cell types, including breast, colon and thyroid lines. Our observations have broad implications, from carcinoma progression, invasion and metastases to therapy. For example, energy levels must vary with the development and progression of carcinomas and is absolutely tied to hypoxia (40). Furthermore, our data may also suggest that the manipulation of cellular energy levels could be an important target for cancer therapy that may have an effect on a variety of cancers. That depletion of ATP results in an increase in nuclear PTEN, in a variety of cell lines from diverse lineages, suggests that altering the subcellular localization of PTEN through modulation of ATP levels may be a beneficial adjunct to traditional therapies in multiple tumour types.

In fact, modulation of ATP levels has been shown to be therapeutically relevant. Maschek and colleagues demonstrated that treatment with 2-DG significantly enhances the effectiveness of paclitaxel and adriamycin in a xenograft model of human osteosarcoma and non-small cell lung carcinoma (27). It may be important to note that clinical treatments that result in ATP depletion, when used in combination with radiation or chemotherapeutic treatment, appears to result in enhanced tumour regression in a variety of preclinical studies (26–31). These preclinical and clinical studies show that ATP depletion may be a well-tolerated adjunctive therapy to traditional chemotherapeutic and radiotherapy regimens. It is intriguing to speculate that the underlying cause of this increase in efficacy might be due to the ATP depletion-mediated increases in nuclear PTEN levels. This both affirms the potential utility of agents that increase nuclear PTEN levels in the development of future combinatorial therapies and demonstrates the need for an increased understanding of the mechanisms of nucleo-cytoplasmic shuttling of PTEN in both sporadic malignancies as well as neoplasias relevant to Cowden syndrome for treatment and prevention.

MATERIALS AND METHODS

Materials

Glucose-free DMEM and RPMI were from GIBCO BRL (Hercules, CA). ATP Bioluminescence Assay Kit CLS II was from Roche Applied Sciences (Indianapolis, IN). Prolong Gold antifade mounting medium was purchased from Invitrogen (Carlsbad, CA). NE-PER kit and BCA protein assay kit were from Pierce Biotechnology Inc. (Rockford, IL). Antibodies used were α-PTEN monoclonal antibody clone 6H2.1 (Cascade Biosciences, Portland, OR), phospho-PTEN, α-tubulin and α-PARP-1 antibody (Cell Signaling Technologies, Danvers, MA). All other chemicals/reagents were obtained from common commercial sources.

Cell lines and culture conditions

MCF7, MDA-MB-231, breast carcinoma lines and WRO-82-1 follicular thyroid carcinoma lines were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin sulphate. HT29, GC3 and RKO colorectal cell lines were maintained in high-glucose RPMI supplemented with 10% FBS, 1% penicillin–streptomycin sulphate. All cell lines were maintained at $37°C$ and 5% Co_2. All cell lines were screened for *PTEN* mutations in the coding, flanking intronic and minimal promoter regions using both DGGE and automated sequencing. All cell lines studied express wild-type PTEN.

Cellular ATP depletion

Cells were seeded at a density of 2×10^6 per 100 mm dish in complete medium and allowed to adhere overnight. Control

cells were maintained in either regular/complete medium or glucose-free medium. For ATP depletion, cells were incubated in glucose-free media (Gluc-medium) containing 10 mM sodium azide and 6 mM 2-DG, supplemented with 10% FBS and 1% penicillin–streptomycin sulphate, at the times indicated (35,37). Intracellular ATP levels were determined using a bioluminescent luciferase–luciferin somatic cell assay kit according to the manufacture's instructions (Roche Applied Sciences, Indianapolis, IN). Briefly, at the time of harvesting, one-third of the cells were collected and processed for recovery of free ATP. Following ATP depletion, the cells undergo morphological changes, similar to those described by others (35,37). While this varies across cell lines, in general, we found that at 2 h post-ATP depletion the cells begin to round up and that by 5 h post-depletion they are beginning to detach from the culture dish. Based on these observations, we chose the 4 h post-depletion time for analysis.

Regeneration of cellular ATP

After cellular ATP depletion, as described above, the media was replaced with fresh glucose-free medium supplemented with 10% FBS and 1% penicillin–streptomycin sulphate. In some experiments, as indicated in figures and figure legends, the ATP-regeneration media was supplemented with 2 µM of either ATP, dATP, ADP, AMP or ATPγS, Regeneration was carried out for a maximum of 4 h as indicated in figures and figure legends. Intracellular ATP levels, post-regeneration, were determined as described above.

Subcellular isolation and western blot analysis

Nuclear and cytoplasmic proteins were isolated as previously described (14,23) using the NE-PER nuclear-cytoplasmic extraction reagents according to the manufacturer's instructions (Pierce, Rockford, IL). Proteasome and phosphatase inhibitors were added to the extractions buffers at the following concentrations: 0.5 mg/ml bezamidine hydrochloride; 2 µg/ml each of leupeptin, aprotinin and pepstatin A; 10 mM β-glycerophosphate; 0.2 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride and 25 mM sodium fluoride. Protein concentrations were determined using the BCA assay, using BSA as a standard. Proteins were separated on 10% SDS–PAGE gels using the Criterion system (Bio-Rad, Hercules, CA) and transferred to nitrocellulose. Equal protein loading was confirmed by staining the nitrocellulose blots with Ponceau S and by western analysis with α-tubulin (cytosolic protein) or α-PARP-1 (nuclear protein). Blots were probed with either, α-PTEN, α-tubulin or α-PARP-1, at 1:1000 dilution, then incubated with the appropriate secondary antibody as previously described (14,23). Proteins were visualized using enhanced chemiluminescence.

Indirect immunofluoroscence

Cells were prepared for indirect immunofluoroscence as previously described (14) with minor modifications. Cells were grown on coverslips in 6-well dishes in tandem with cells for nuclear-cytoplasmic protein extracts. Cells were fixed in freshly prepared 4% paraformaldehyde for 20 min, blocked using blocking solution (2% BSA and 0.2% Triton-X 100 in PBS) for 10 min and immunostained with α-PTEN antibody (1:100) for 1 h at room temperature. Cells were subsequently washed with PBS and incubated with an α-mouse FITC-conjugated secondary antibody (1:100) for 1 h at room temperature. After washing with PBS, coverslips were mounted face down on glass slides (Labtek Inc., Grand Rapids, MI) with a small drop of mounting medium, ProLong Gold antifade containing DAPI. Cells were viewed with a Zeiss Axio fluorescent microscope (Imager A1) (Carl Zeiss Inc., Thornwood, NY) with an Achroplan 20× and 40× objective that had a numerical aperture of 0.50, fitted with filters for FITC and DAPI identification and equipped with a computer-supported imaging system. Images were captured using Zeiss' AxioCam MRc5 high-resolution digital camera in conjunction with the AxioVision 4.5 multi-channel fluorescent acquisition software. Background correction settings with the computer support were standardized for all images. Approximately 200 cells, from 15–20 fields, were counted per slide.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

REFERENCES

1. Nelen, M.R., Padberg, G.W., Peeters, E.A., Lin, A.Y., van den Helm, B., Frants, R.R., Coulon, V., Goldstein, A.M., van Reen, M.M., Easton, D.F. *et al.* (1996) Localization of the gene for Cowdens disease to chromosome 10q22–23. *Nat. Genet.*, **13**, 114–116.
2. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R. *et al.* (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275**, 1943–1947.
3. Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T. *et al.* (1997) Identification of a candidate tumour suppressor gene, MMAC1, at

chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.*, **15**, 356–362.

4. Li, D.M. and Sun, H. (1997) *TEP1*, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.*, **57**, 2124–2129.

5. Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M. *et al.* (1997) Germline mutations of the PTEN gene in Cowden's disease: an inherited breast and thyroid cancer syndrome. *Nat. Genet.*, **16**, 64–67.

6. Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L., Zheng, Z., Liaw, D., Caron, S., Duboue, B., Lin, A.Y. *et al.* Mutation spectrum and genotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. *Hum. Mol. Genet.*, **7**, 507–515.

7. Zhou, X.P., Waite, K.A., Pilarski, R., Hampel, H., Fernandez, M.J., Bos, C., Dasouki, M., Feldman, G.L., Greenberg, L.A., Ivanovich, J. *et al.* Germline PTEN promoter mutations and deletions in Cowdens/ Bannayan-Riley-Ruvalcaba syndrome result in aberrant PTEN protein and dysfunction of the phosphoinositol-3-kinase/Akt pathway. *Am. J. Hum. Genet.*, **73**, 404–411.

8. Eng, C. (2003) PTEN: one gene, many syndromes. *Hum. Mutat.*, **22**, 183–198.

9. Zbuk, K. and Eng, C. (2007) Cancer phenomics: RET and PTEN and illustrative models. *Nat. Rev. Cancer*, **7**, 35–45.

10. Nassif, N.T., Lobo, G.P., Wu, X., Henderson, C.J., Morrison, C.D., Eng, C. and Segalov, E. (2004) PTEN mutations are common in sporadic microsatellite stable colorectal cancer. *Oncogene*, **23**, 617–628.

11. Whang, Y.E., Wu, X., Suzuki, H., Reiter, R.E., Tran, C., Vessella, R.L., Said, J.W., Isaacs, W.B. and Sawyers, C.L. (1998) Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc. Natl. Acad. Sci. USA*, **95**, 5246–5250.

12. Perren, A., Weng, L.P., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Komminoth, P., Lees, J.A., Mulligan, L.M., Mutter, G.L. and Eng, C. (1999) Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. *Am. J. Pathol.*, **155**, 1253–1260.

13. Gimm, O., Perren, A., Weng, L.P., Marsh, D.J., Yeh, J.J., Ziebold, U., Gil, E., Hinze, R., Delbridge, L., Lees, J.A. *et al.* (2000) Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors. *Am. J. Pathol.*, **156**, 1693–1700.

14. Chung, J.H. and Eng, C. (2005) Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. *Cancer Res.*, **65**, 8096–8100.

15. Ginn-Pease, M.E. and Eng, C. (2003) Increased nuclear phosphatase and tensin homologue deleted on chromosome 10 is associated with G0-G1 in MCF-7 cells. *Cancer Res.*, **63**, 282–286.

16. Shen, W.H., Balajee, A.S., Wang, J., Wu, H., Eng, C., Pandolfi, P.P. and Yin, Y. (2007) Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell*, **128**, 157–170.

17. Chung, J.H., Ginn-Pease, M.E. and Eng, C. (2005) Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has nuclear localization signal-like sequences for nuclear import mediated by major vault protein. *Cancer Res.*, **65**, 4108–4116.

18. Trotman, L.C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N.P., Carver, B.S., Cordon-Cardo, C., Erdjumenr-Bromage, H. *et al.* (2007) Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell*, **128**, 141–156.

19. Planchon, S.M., Waite, K.W. and Eng, C. (2007) The nuclear affairs of PTEN. *J. Cell Sci.*, **121**, 249–253.

20. Denning, G., Jean-Joseph, B., Prince, C., Durden, D.L. and Vogt, P.K. (2007) A short N-terminal sequence of PTEN controls cytoplasmic localization and is required for suppression of cell growth. *Oncogene*, **26**, 3930–3940.

21. Liu, J.L., Sheng, X., Hortobagyi, Z.K., Mao, Z., Gallick, G.E. and Yung, W.K. (2005) Nuclear PTEN-mediated growth suppression is independent of Akt down-regulation. *Mol. Cell. Biol.*, **25**, 6211–6224.

22. Gil, A., Andres-Pons, A., Fernandez, E., Valiente, M., Torres, J., Cervera, J. and Pulido, R. (2006) Nuclear localization of PTEN by a Ran-dependent mechanism enhances apoptosis: involvement of an N-terminal nuclear localization domain and multiple nuclear exclusion motifs. *Mol. Biol. Cell*, **17**, 4002–4013.

23. Minaguchi, T., Waite, K.A. and Eng, C. (2006) Nuclear localization of PTEN is regulated by Ca(2+) through a tyrosil phosphorylation-independent conformational modification in major vault protein. *Cancer Res.*, **66**, 11677–11682.

24. Liu, J.L., Mao, Z., LaFortune, T.A., Alonso, M.M., Gallick, G.E., Fueyo, J. and Yung, W.K. (2007) Cell cycle-dependent nuclear export of phosphatase and tensin homologue tumor suppressor is regulated by the phosphoinositide-3-kinase signaling cascade. *Cancer Res.*, **67**, 11054–11063.

25. Liu, F., Wagner, S., Campbell, R.B., Nickerson, J.A., Schiffer, C.A. and Ross, A.H. (2005) PTEN enters the nucleus by diffusion. *J. Cell Biochem.*, **96**, 221–234.

26. Pelicano, H., Martin, D.S., Xu, R.-H. and Huang, H. (2006) Glycolysis inhibition for anticancer treatment. *Oncogene*, **25**, 4633–4646.

27. Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G.F., De Young, L.R. and Lampidis, T.J. (2004) 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res.*, **64**, 31–34.

28. Martin, D.S., Bertino, J.R. and Koutcher, J.A. (2000) ATP depletion+pyrimidine depletion can markedly enhance cancer therapy: fresh insight for a new approach. *Cancer Res.*, **60**, 6776–6783.

29. Zhang, X.D., Deslandes, E., Villedieu, M., Poulain, L., Duval, M., Gauduchon, P., Schwartz, L. and Icard, P. (2006) Effect of 2-deoxy-D-glucose on various malignant cells in vitro. *Anticancer Res.*, **26**, 3561–3566.

30. Zhu, Z., Jiang, W., McGinley, J.N. and Thompson, H.J. (2005) 2-Deoxyglucose as an energy restriction mimetic agent: effects on mammary carcinogenesis and on mammary tumor cell growth in vitro. *Cancer Res.*, **65**, 7023–7030.

31. Aft, R.L., Lewis, J.S., Zhang, F., Kim, J. and Welch, M.J. (2003) Enhancing targeted radiotherapy by copper(II)diacetyl-bis(N4-methylthiosemicarbazone) using 2-deoxy-D-glucose. *Cancer Res.*, **63**, 5496–5504.

32. Elstrom, R.L., Bauer, D.E., Buzzai, M., Karnauskas, R., Harris, M.H., Plas, D.R., Zhuang, H., Cinalli, R.M., Alavi, A., Rudin, C.M. *et al.* (2004) Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res.*, **64**, 3892–3899.

33. Karnauskas, R., Niu, Q., Talapatra, S., Plas, D.R., Grenne, M.E., Crispino, J.D. and Rudin, C. (2003) Bcl-x(L) and Akt cooperate to promote leukomogenesis in vivo. *Oncogene*, **22**, 688–689.

34. Kose, S., Imamoto, N. and Yoneda, Y. (1999) Distinct energy requirement for nuclear import and export of importin β in living cells. *FEBS Lett.*, **463**, 327–330.

35. Schwoebel, E.D., Ho, T.H. and Moore, M.S. (2002) The mechanism of inhibition of Ran-dependent nuclear transport by cellular ATP depletion. *J. Cell Biol.*, **157**, 963–974.

36. Hetzer, M. and Mattaj, I.W. (2000) An ATP-dependent, Ran-independent mechanism for nuclear import of the U1A and U2B" spliceosome proteins. *J. Cell Biol.*, **2**, 293–303.

37. Jeyaraj, S.C., Dakhlallah, D., Hill, S.R. and Lee, B.S. (2006) Expression and distribution of HuR during ATP depletion and recovery in proximal tubule cells. *Am. J. Physiol. Renal Physiol.*, **291**, F1255–F1263.

38. Shank, L.C., Kelly, J.B., Gioeli, D., Yang, C.S., Spencer, A., Allison, L.A. and Paschal, B.M. (2008) Activation of the DNA-dependent protein kinase stimulates nuclear export of the androgen receptor in vitro. *J. Biol. Chem.*, **283**, 10568–10580.

39. Yamada, M., Mattaj, I.W. and Yoneda, Y. (2004) An ATP-dependent activity that releases RanGDP from NTF2. *J. Biol. Chem.*, **279**, 36228–36234.

40. Taylor, C.T. (2008) Mitochondria and cellular oxygen sensing and the HIF pathway. *Biochem. J.*, **409**, 19–26.