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Inducible expression of catalytically active type I serine/threonine protein phosphatase in a human carcinoma cell line

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Abstract

Background : One of the major cellular serine/threonine protein phosphatases is protein phosphatase type I (PPI). Studies employing many eukaryotic systems all point to a crucial role for PPI activity in controlling cell cycle progression. One physiological substrate for PPI appears to be the product of the retinoblastoma susceptibility gene (pRB), a demonstrated tumor suppressor. The growth suppressive activity of pRB is regulated by its phosphorylation state. Of critical importance is the question of the *in vivo* effect of PPI activity on pRB and growth regulation. As a first step towards addressing this question, we developed an inducible PPI expression system to investigate the regulation of PPI activity.

Results : We have established a cell line for inducing protein expression of the type I, alpha-isotype, serine/threonine protein phosphatase (PPI α). A plasmid encoding a fusion protein of the catalytic subunit of PPI α with a 6-histidine peptide (6His) and a peptide from hemagglutinin (HA) was transfected into the UMUC3 transitional cell carcinoma cell line, previously transfected with the reverse tetracycline transactivator plasmid pUHD172-Ineo. A stable cell line designated LLWO2F was established by selection with hygromycin B. 6His-HA-PPI α protein appeared in cell lysates within two hours following addition of doxycycline to the culture medium. This protein localizes to the nucleus as does endogenous PPI α , and was shown to associate with PNUTS, a PPI-nuclear targeting subunit. Like endogenous PPI α , immunocomplexed 6His-HA-PPI α is active toward phosphorylase *a* and the product of the retinoblastoma susceptibility gene, pRB. When forcibly overexpressing 6His-HA-PPI α , there is a concomitant decrease in endogenous PPI α levels.

Conclusions : These data suggest the existence of an autoregulatory mechanism by which PPI α protein levels and activity remain relatively constant. RT-PCR analyses of isolated polysome

fractions support the notion that this putative autoregulatory mechanism is exerted, at least in part, at the translational level. Implications of these findings for the study of PP1 α function *in vivo* are discussed.

Background

One of the major cellular serine/threonine protein phosphatases is protein phosphatase type 1 (PP1) [1]. An abundant enzyme expressed in all cells, complex regulation of PP1 is thought to be essential for proper temporal and spatial regulation of PP1 catalytic activity towards individual substrates [2]. Three different isoforms of the catalytic subunit, designated PP1 α , PP1 δ , and PP1 γ 1, are expressed in many different cell types [3]. Studies employing many eukaryotic systems all point to a crucial role for PP1 activity in controlling cell cycle progression, and an absolute requirement of this activity for mitotic exit [4–7]. There is also evidence to suggest that phosphorylation of PP1 and its associated proteins by the cyclin-dependent kinases may regulate PP1 activity in a cell cycle stage-dependent manner [8–10]. Hence, current efforts are directed towards identification of cell cycle-dependent substrates for PP1, and how regulation of PP1 activity towards these substrates controls the cell division cycle.

One physiological substrate for PP1 appears to be the product of the retinoblastoma susceptibility gene, pRB [11–13], a demonstrated tumor suppressor. The cell growth and tumor suppressive activity of pRB is regulated by its phosphorylation state. pRB phosphorylation varies as a function of cell cycle phase; during G₁ the hypophosphorylated form predominates, while the hyperphosphorylated form accumulates during S, G₂ and M phase [14–16]. It is this hypophosphorylated form of pRB present during early and mid G₁ which functions to modify gene expression. This is achieved by complexing with transcription factors resulting in repression [17] or stimulation [18] of transcription. Under conditions favoring proliferation, the phosphorylation of pRB via cyclin-dependent kinases (CDKs) in mid-to-late G₁ phase results in liberation of E2F and other pRB-bound transcription factors, which then activate the transcription of S-phase genes [19]. In late M phase, pRB returns to its growth suppressive, hypophosphorylated form due to the action of a pRB-directed protein phosphatase belonging to the type 1 class of serine/threonine protein phosphatases [11,20,21].

Of critical importance is the question of the *in vivo* effect of PP1 activity on pRB and growth regulation. To date, several techniques have been employed in this regard to demonstrate PP1 actions in intact cells. Berndt et. al. [22] used electroporation to introduce PP1 protein into tissue culture cells having noted that previous genetic attempts

to accomplish overexpression were not readily accomplished. Using this technique, they showed a G₁ block by PP1 that was dependent upon the presence of pRB. In another report, micro-injection of PP1 decreased extractability of pRB from nuclei, presumably by increasing dephosphorylation and increasing association with nuclear proteins thereby playing a role in G₁/S transition inhibition [13]. Micro-injection of antibodies to the PP1 catalytic subunit has been shown to block cells in metaphase [23]. Since this is the time during mitosis when pRB is normally dephosphorylated [11,24], it is tempting to speculate that prevention of pRB dephosphorylation by inhibiting PP1 activity impedes an orderly progression through mitosis, perhaps contributing towards a transformed phenotype.

Here, using the tetracycline induction system [25], we developed an inducible PP1 expression system to investigate the regulation of PP1 activity. We view this as a first step towards addressing the question of the *in vivo* effect of PP1 activity on pRB and growth regulation. Our results support the notion that induced PP1 α protein functions identically to endogenous PP1 α with respect to non-catalytic subunit binding and activity towards phosphorylase *a* and the tumor suppressor pRB. Localization of this protein to the nucleus also mirrors that of endogenous PP1 α *in vivo*. When using this system to forcibly overexpress 6His-HA-PP1 α , there is a concomitant decrease in endogenous PP1 α levels, suggesting the existence of an autoregulatory mechanism by which PP1 α protein levels remain constant. RT-PCR analyses of isolated polysome fractions supports the notion that this putative autoregulatory mechanism is exerted, at least in part, at the translational level.

Results and Discussion

Induction of 6His-HA-PP1 in LLW02F cells

Figure 1 shows construct of the tetracycline-inducible plasmid used to express 6His-HA-PP1 α . As shown in Figure 2A, 24 hr incubation with doxycycline in the medium has no appreciable effect on endogenous PP1 α protein expression in the untransfected parent cell line (UMUC3) or a cell line transfected with the reverse tetracycline transactivator plasmid pUHD172-1neo only (LLW01). A time course of induction by doxycycline was then carried out in stable clone LLW02F. Harvested at various time intervals, whole-cell lysates were prepared and the proteins were separated by SDS-PAGE for western blotting using antibody to the hemagglutinin tag. As shown in Figure 2B, a

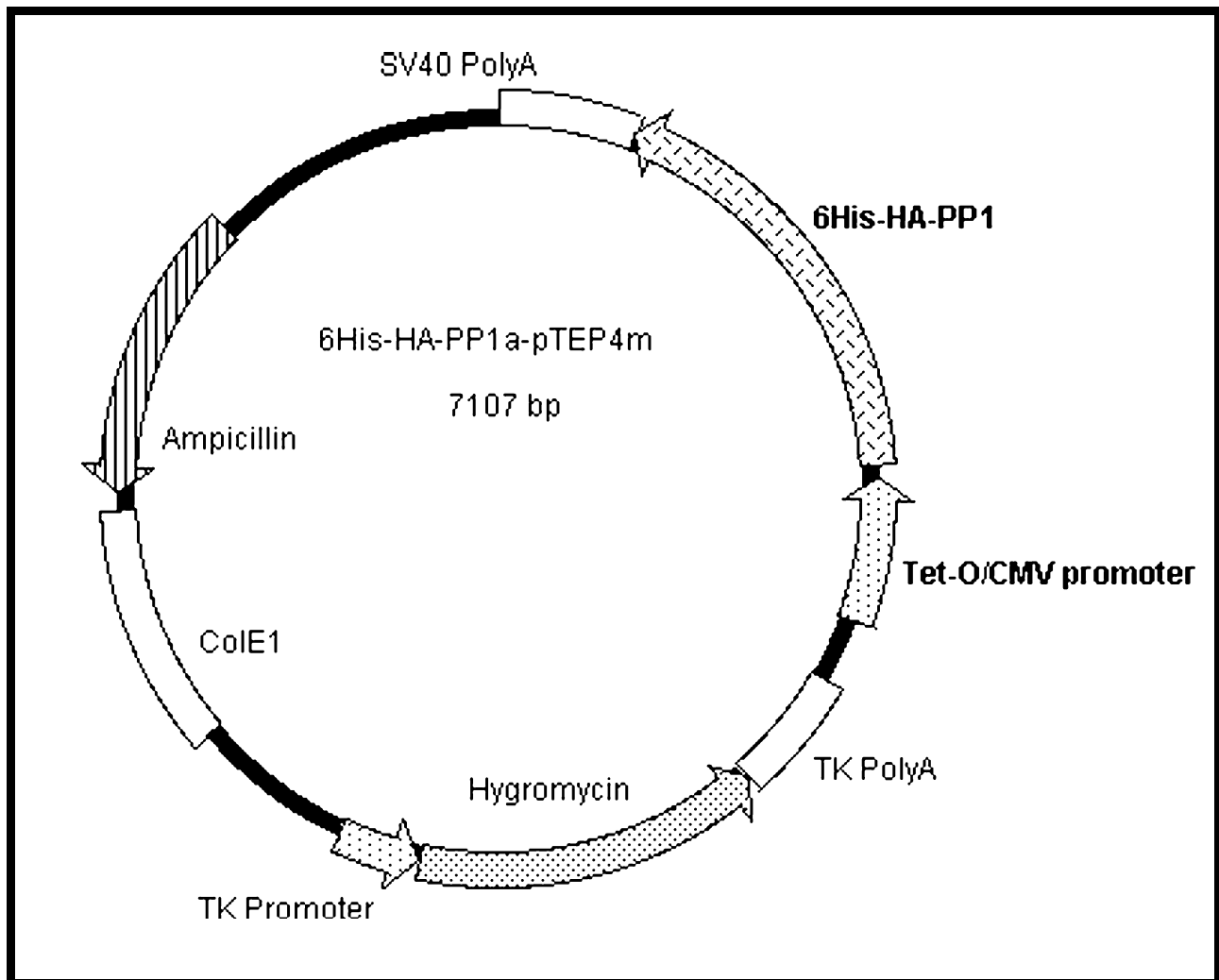


Figure 1

The 6His-HA-PP1 α -pTEP4m plasmid. The plasmid contains the 6His-HA-PP1 α fusion gene under the control of the TetO/CMV promoter. This promoter is active in the presence of the rtTA-nls protein and doxycycline. The plasmid contains an ampicillin resistance gene (Amp^r) for selection in bacteria and the hygromycin resistance gene for selection in eukaryotic cells.

protein with the anticipated molecular weight of 40 kDa was detected. A clear differential of expression of 6His-HA-PP1 α (indicated at the left of the panel) was observed at 1.5 hours after addition of doxycycline, which became more pronounced at later time points. The doxycycline-independent increase in 6His-HA-PP1 α is attributed to background expression when using this system [25]. As predicted for a protein under the control of the doxycycline-inducible promoter, the abundance of 6His-HA-PP1 α decreases in the absence of the inducer over time (Figure 2C). Subsequent time course experiments revealed peak abundance occurring between 16 and 24 hr, and

remaining constant thereafter for at least 72 hr, at which time the experiment was terminated. As such, we have chosen the 24 hr induction time for all subsequent experiments to ensure peak abundance of the induced 6His-HA-PP1 α .

As shown in Figure 2C, a significant reduction in 6His-HA-PP1 α abundance can be observed between 3 and 12 hrs following removal of doxycycline. This reduction continued through the 24 hr time point at which time the experiment ended. Taken together, these data confirm the dependence of 6His-HA-PP1 α protein production on the

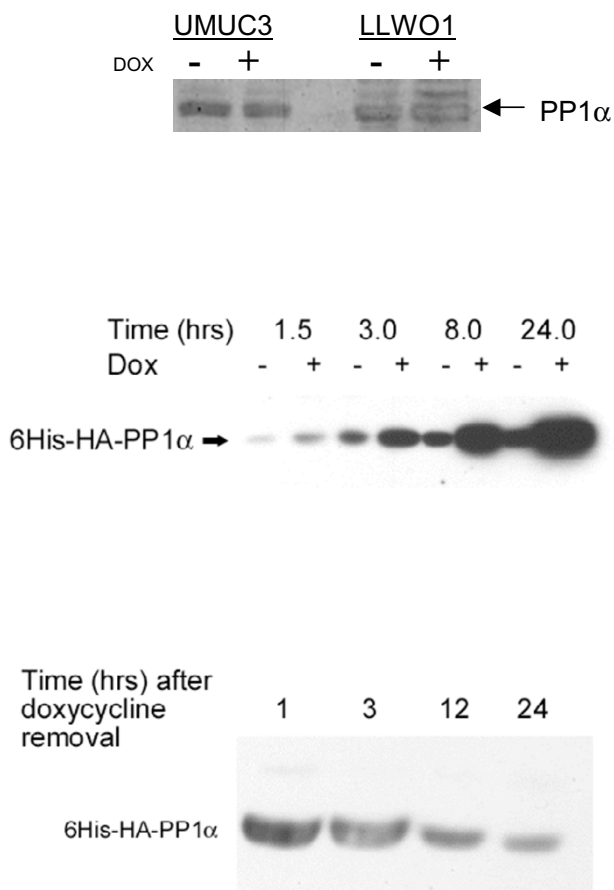


Figure 2
A. Doxycycline has no effect on PP1 α protein expression in control cell lines. Equal quantities (50 μ g) of whole-cell lysates harvested in the absence (indicated above the lanes with a (-) symbol), or presence (at 24 hr following addition, indicated above the lanes with a (+) symbol), of doxycycline were separated by SDS-PAGE and subjected to immunoblotting using antibody specific for PP1 α . UMUC3 – parent cell line from which stable expressor LLWO2F was selected; LLWO1 – cell line generated from UMUC3 which was stably transfected with the reverse tetracycline transactivator plasmid pUHD172-1neo only. **B** – Doxycycline induces a protein in LLWO2F cells that is recognized by antibody to hemagglutinin. Equal quantities (50 μ g) of whole-cell lysates harvested at the various time points in hours after doxycycline addition (indicated above each lane) were separated by SDS-PAGE and subjected to immunoblotting as described in Materials and Methods. **C** – Time-course of recovery from doxycycline induction. LLWO2F cells were induced for 24 hr. At the end of this time, equal quantities (50 μ g) of whole-cell lysates harvested at the various time points in hours following removal of doxycycline (indicated above each lane) were separated by SDS-PAGE and subjected to immunoblotting with antibody to hemagglutinin.

tet-O/CMV promoter, thus demonstrating inducible expression of PP1 α . As expected [25], some low-level expression of 6His-HA-PP1 α in the absence of the inducer doxycycline can also be detected.

Biological and Biochemical Characterization of Inducible 6His-HA-PP1 α

To begin addressing the biological and biochemical characterization of inducible PP1 α , we next localized this protein *in situ* using immunohistochemistry. As shown in Figure 3, there is a predominant nuclear localization of the hemagglutinin epitope (panel A) compared to the low-level cytoplasmic and nuclear background reactivity seen in the uninduced LLWO2F control cells (panel B). Thus, not only can doxycycline-induced expression of PP1 α be detected immunohistochemically, but localization of this protein appears to be identical to that of endogenous PP1 [26].

Targeting of PP1 to the nucleus is due in part to a PP1-associated nuclear targeting subunit (PNUTS; [27]). To further investigate the properties of inducible PP1 α , we performed coprecipitation studies to determine if PNUTS can also be found associated with 6His-HA-PP1 α . Towards this goal, a fusion protein of GST and PNUTS was tested for the ability to capture induced PP1 α . Lysates from induced LLWO2F cells were combined with GST-alone- or GST-PNUTS-loaded glutathione Sepharose beads. Following separation by SDS-PAGE and western blotting, antibody to hemagglutinin was used to detect any associated 6His-HA-PP1 α . Antibody reactivity would indicate a link from the GST epitope to the hemagglutinin epitope via a PNUTS to PP1 α interaction. As shown in Figure 4, the hemagglutinin epitope was captured by the GST-PNUTS fusion protein, but not the GST-alone protein. These data support the idea that induced PP1 α and the nuclear targeting PP1-associated protein PNUTS can form a complex. These results further support the view that induced PP1 α behaves similarly if not identically to endogenous PP1 α , and provides a possible mechanistic explanation for 6His-HA-PP1 α localization to the nucleus.

Two different approaches were taken to address the biochemical activity of inducible PP1 α . First, immunocomplexed 6His-HA-PP1 α was tested for its ability to dephosphorylate phosphorylase *a*. Phosphorylated on a single serine residue, this substrate is often used to monitor the biochemical activity of PP1 *in vitro* [28]. As shown in Figure 5, greater phosphatase activity is detected in immunoprecipitates from induced LLWO2F cell lysates compared to uninduced. This is consistent with the notion that inducible PP1 α is enzymatically active. Our second approach involved testing immunopurified 6His-HA-PP1 α for its ability to dephosphorylate the growth

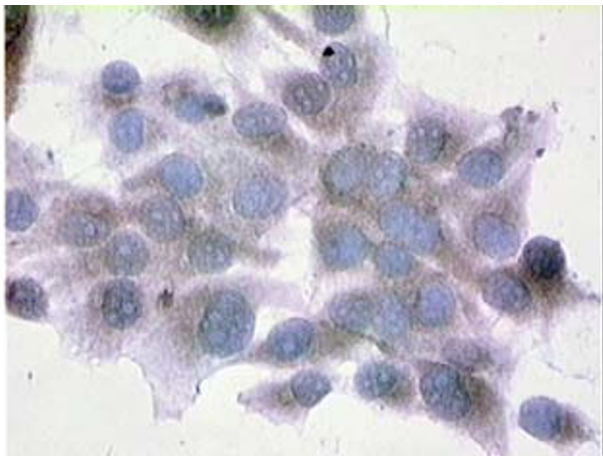
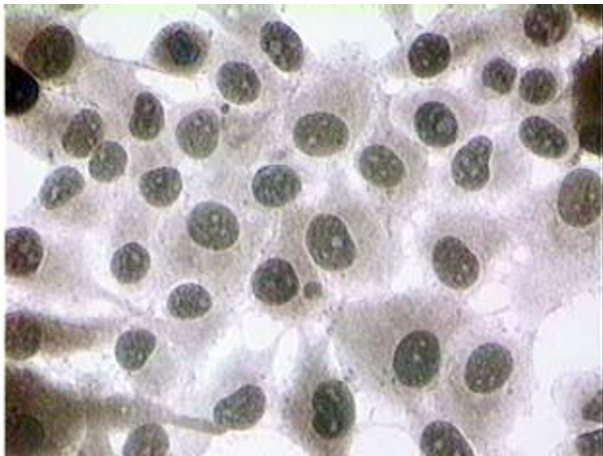


Figure 3
Immunohistochemistry with the antibody to hemagglutinin shows doxycycline-dependent expression of 6His-HA-PP1 α *in vivo*. LLWO2F cells were grown in 12 well tissue culture plates and induced for 24 hours prior to fixation and staining as described in Materials and Methods. Panel **A** – Induced; Panel **B** – Uninduced.

suppressor protein pRB. Having previously reported on the ability of immunocomplexed endogenous PP1 to dephosphorylate pRB [20], we followed the same experimental approach. Figure 6 shows a significant decrease in the ^{32}P radiolabel intensity for pRB following incubation with the anti-hemagglutinin immunoprecipitate compared to the normal mouse IgG control immunoprecipitate. In addition, okadaic acid, a potent inhibitor of PP1 activity [29], significantly inhibits pRB dephosphorylation in this reaction. Taken together, these data indicate that inducible PP1 α is biochemically active.

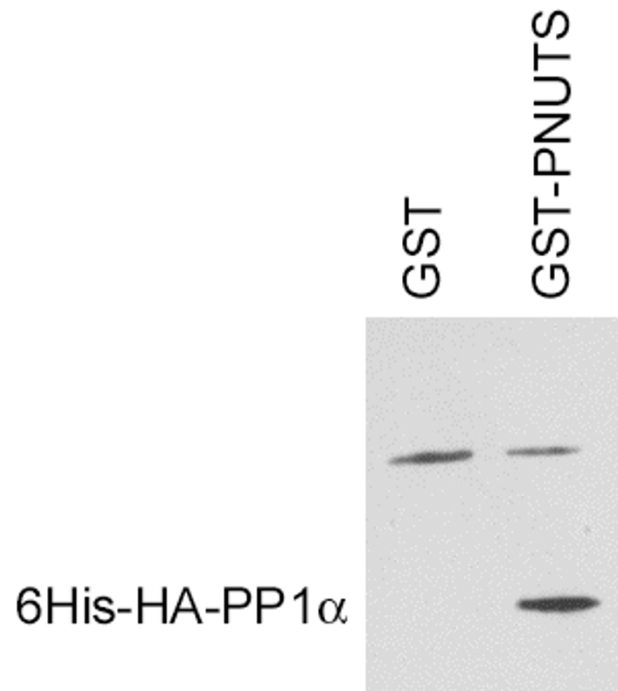


Figure 4
Coprecipitation of 6His-HA-PP1 α with GST-PNUTS. Doxycycline-induced LLWO2F cell lysates were mixed with either GST or GST-PNUTS bound to glutathione-Sepharose beads, washed, and the bound proteins separated by SDS-PAGE and then immunoblotted with antibody to hemagglutinin. Position of 6His-HA-PP1 α is indicated to the left of the panel, and is present only in the GST-PNUTS lane. The band above 6His-HA-PP1 α , which is also present in the GST-alone lane, results from non-specific reactivity with the secondary antibody (horse-radish peroxidase-conjugated anti-IgG) used for chemiluminescent detection

Upregulation of inducible 6His-HA-PP1 α coincides with downregulation of endogenous PP1 α

One prediction would be that by increasing expression of the catalytic subunit of PP1 α , overall PP1-specific activity within the cell would increase. To address this, we tested the phosphatase activity of LLWO2F whole-cell lysate using phosphorylase *a* as the substrate. To inhibit any endogenous PP2A activity, which can also dephosphorylate phosphorylase *a* and thus interfere with PP1 activity measurements, these assays were carried out in the presence of 5 nM okadaic acid, which does not inhibit PP1 activity [30]. Although reproducible, we observed only modest increases in PP1-specific activity found in lysates from induced cells compared to the uninduced controls (Figure 7). One possible explanation for this finding is that the overall level of PP1 α within the cell remains relatively constant regardless of induction. Indeed, a time

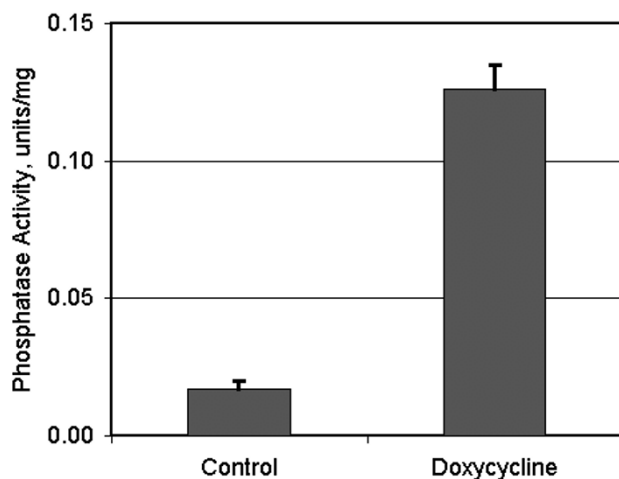


Figure 5

Dephosphorylation of phosphorylase *a* by anti-hemagglutinin immunoprecipitates from uninduced (Control) and induced (Doxycycline) LLW02 cells. Activity was assayed by the release of [32 P] H_3PO_4 from radiolabeled phosphorylase *a* ($1-2 \times 10^5$ cpm/nmol). 1 unit of activity releases 1 nmol of phosphate/min at 30°C.

course of induction followed by western blotting of whole cell lysates using antibody to PP1 α revealed diminution of the endogenous PP1 α signal after 4 hr of induction, while the 6His-HA-PP1 α signal increased (Figure 8). To further test this hypothesis, immunoprecipitation and western blotting experiments were carried out using PP1 isoform-specific antibodies. As shown in Figure 9, top panel, induced (approximate molecular weight of 40 kDa) and endogenous (approximate molecular weight of 37 kDa) PP1 α are clearly resolved by SDS-PAGE. As predicted, both proteins are recognized by antibody specific for PP1 α when performing immunoprecipitation or western blotting. Immunoprecipitation using PP1 α antibody followed by western blotting using the same anti-PP1 α reveals a reciprocal relationship in PP1 α abundance following doxycycline induction; endogenous PP1 α levels decrease in response to increasing levels of 6His-HA-PP1 α (top panel, compare left two lanes). This difference in abundance is more striking in the whole-cell lysate lanes; endogenous PP1 α fails to be detected in lysates prepared from induced cells (compare middle two lanes). Parallel experiments performed using the same lysate and antibody to hemagglutinin for immunoprecipitation and western blotting show the position of 6His-HA-PP1 α and the fact that endogenous PP1 α is not recognized by this antibody (bottom panel). Western blotting for additional isoforms of PP1 reveals that this reduction in PP1 α following induction is isotype-specific; when 6His-HA-PP1 α

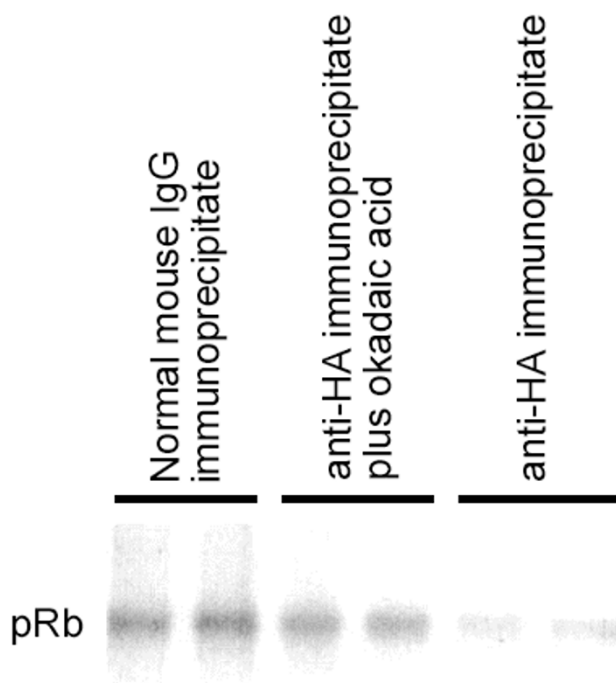


Figure 6

Dephosphorylation of pRB by anti-hemagglutinin immunoprecipitates from induced LLW02 cells. Samples were run in duplicate. Normal mouse IgG immunoprecipitate was used as a negative control. Anti-hemagglutinin immunoprecipitate, reaction run in the presence of okadaic acid (100 nM), a demonstrated PPI inhibitor, was an additional negative control. Anti-hemagglutinin immunoprecipitate, revealing a decrease in ^{32}P -labeled pRB band intensity, indicating removal of phosphate groups by 6His-HA-PP1 α .

levels increase, concomitant with a decrease in PP1 α levels, PP1 δ and PP1 γ 1 levels remain relatively unchanged from those found in uninduced cell lysate (Figure 10). Taken together, these data suggest that an as yet undescribed negative-feedback or autoregulatory mechanism exists for PP1 α which contributes towards maintaining a constant level of protein expression and enzyme activity.

RT-PCR of polysome and total RNA specific for endogenous PP1 α and 6His-HA-PP1 α

To address the possible autoregulatory mechanism(s) of PP1 α expression and activity, we analyzed the RNA levels of both endogenous PP1 α and induced 6His-HA-PP1 α . For uninduced cells, only endogenous PP1 α RNA can be found associated with polysomes (Figure 11, second lane). While some 6His-HA-PP1 α RNA can be detected in the total RNA, the majority PP1 α RNA detected in the total preparation is endogenous (fourth lane). In contrast,

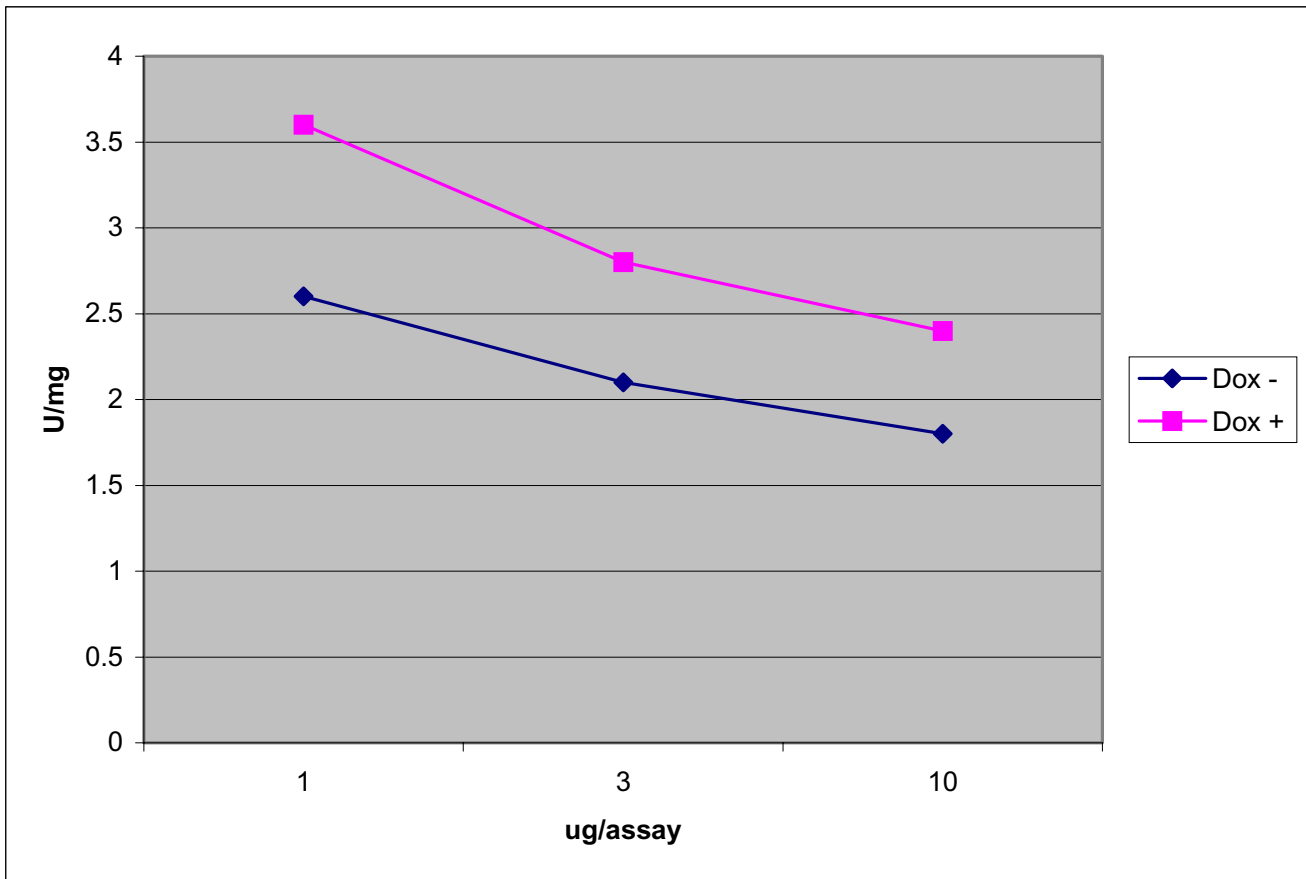


Figure 7

PPI activity in lysates of cells expressing 6His-HA-PP1 α . Cells were cultured for 24 hr either in the absence (closed diamonds) or presence (closed squares) of doxycycline. PPI was assayed using the indicated amounts of lysate/assay. 5 nM okadaic acid was used to inhibit PP2A during the assay. 1 unit of PPI activity releases 1 nmol of Pi/min at 30°C.

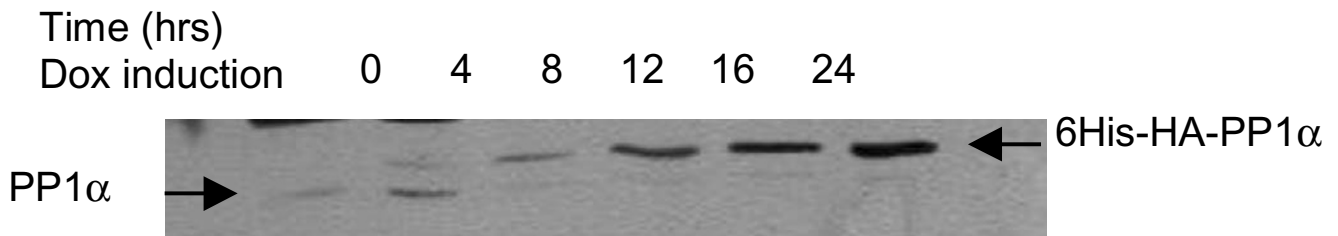


Figure 8

Time course of induction followed by western blotting of whole cell lysates using antibody to PP1 α . Equal quantities (50 ug) of whole-cell lysates harvested at the various time points in hours after doxycycline addition (indicated above each lane) were separated by SDS-PAGE and subjected to immunoblotting using antibody specific for PP1 α . Positions of endogenous PP1 α and 6His-HA-PP1 α are indicated by arrows to the left and right of the figure, respectively.

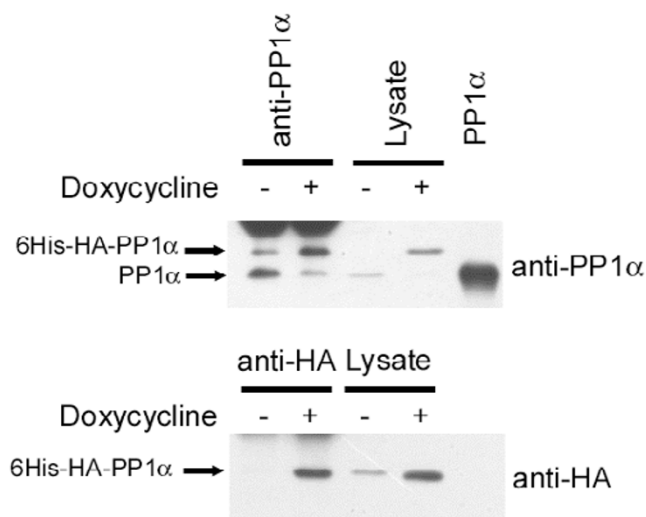


Figure 9
 PP1α isoform-specific antibody immunoprecipitation and detection of endogenous and induced PP1α by western blotting. PP1α isoform-specific antibody was used to immunoprecipitate proteins from equal quantities (50 ug) of uninduced (first lane from the left, top panel) and induced LLWO2F cell lysate (second lane from the left, top panel). The precipitated proteins were then separated by SDS-PAGE and immunoblotted with the same isoform-specific antibody. The positions of 6His-HA-PP1α and PP1α are indicated to the left of the top panel. Whole-cell lysates (10 ug) were separated in the next two lanes. Last lane contains 1 ug of purified recombinant PP1α as a control for endogenous PP1α (37 kDa). Bottom panel shows identical experiment using antibody to hemagglutinin for immunoprecipitation and immunodetection. Although low levels of 6His-HA-PP1α can be detected in cell lysate from uninduced cells following western blotting, this protein is almost undetectable in the same lysate following immunoprecipitation and western blotting. This observation is most likely due to low efficiency of immunoprecipitation.

it appears that for the doxycycline-induced cells (third lane), there is more 6His-HA-PP1α RNA associated with polysomes than endogenous PP1α. However, both messages appear to be present at the same level in the total RNA preparation (fifth lane). This apparent preference for 6His-HA-PP1α RNA association with polysomes suggest that autoregulation takes place, at least in part, at the translational level.

Conclusions

We observed that endogenous PP1α protein levels decrease in response to increasing levels of induced 6His-HA-PP1α protein. This observation suggests that an as yet undescribed negative-feedback or autoregulatory mecha-

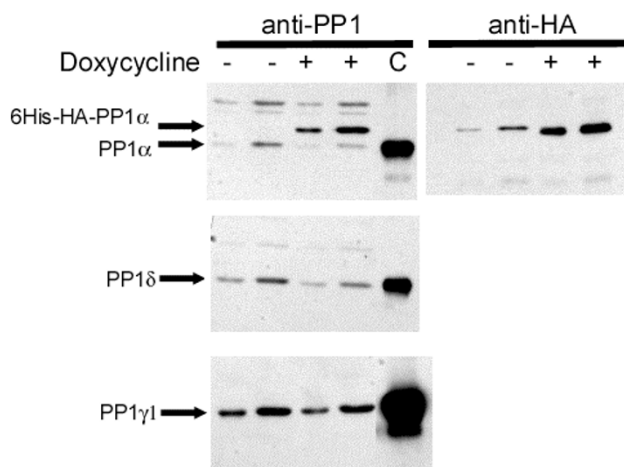


Figure 10
 Isoform-specific antibody detection of PP1 by western blotting induced and uninduced LLWO2F whole-cell lysates. Different amounts (5 ug for the first and third lanes, 10 ug for the second and fourth lanes) of lysate from induced and uninduced cells were separated by SDS-PAGE in parallel and then immunoblotted using PP1α-, PP1δ-, and PP1γ1-specific antibody. Results illustrate downregulation of endogenous PP1α in response to increasing amounts of induced 6His-HA-PP1α, while the levels of PP1δ and PP1γ1 remain relatively constant. Lane C – control lane of isoform-specific antibody immunoprecipitation of respective isoforms from 50 ug of uninduced cell lysate.

nism exists for PP1α which contributes towards maintaining a constant level of this enzyme. Given the difficulty in achieving overexpression of PP1 protein and activity in mammalian cells, this suggestion provides an attractive explanation. This putative mechanism appears to be isotype-specific, since the protein levels of PP1δ and PP1γ1 remained relatively unchanged in response to induced expression of PP1α. Precedent for an autoregulatory mechanism comes from a report by Baharians and Schonthal [31], in which they demonstrate endogenous PP2A protein levels decrease in the presence of increasing amounts of forcibly-expressed exogenous PP2A. Their report also provides evidence that this control is exerted at the translational level and does not involve regulation of transcription or RNA processing. Using a similar approach of analyzing mRNA association with ribosomes as a measure of translation efficiency, a similar level of translational control also appears to exist for PP1α. If mRNA association with ribosomes is truly a measure of translation efficiency, one conclusion from these data is that in doxycycline-induced cells, 6His-HA-PP1α RNA is more efficiently translated than endogenous PP1α. This would help to explain why we observed a decrease in

endogenous PP1 α protein expression in doxycycline-induced cells. Since we did not perform rigorous analyses on PP1 α regulation of transcription or RNA processing, we cannot rule out the possibility that some level of transcriptional control also exists. Indeed, the abundance of endogenous PP1 α RNA found in uninduced cells appears equal to or greater than the combined abundance of endogenous and 6His-HA-PP1 α RNA (Figure 9), supporting the notion that some level of translational control may also exist.

These findings, provided by the development of this inducible PP1 expression system, will afford new research opportunities aimed towards addressing the question of how PP1 activity is regulated. This putative autoregulatory mechanism for the catalytic subunit may help to explain why the overall abundance of PP1 during the various phases of the cell cycle remains relatively constant [32]. Historically, regulation of PP1 catalytic activity has resulted from associated subunits inhibiting or targeting the catalytic subunit towards substrates [33]. While the PP1 catalytic subunit is not found alone in the cell, such unassociated catalytic subunits have been shown to be active, at least *in vitro*. Although speculative, there may be a threshold amount of catalytic subunit *in vivo* above which associated proteins are limiting. Such a situation may compromise the ability of associated proteins to effectively regulate PP1 catalytic activity. To guard against this scenario, control over the amount of PP1 catalytic subunit present via an autoregulatory mechanism would be beneficial.

In summary, we report on the development of an inducible mammalian cell expression system for the catalytic subunit of the α -isotype for PP1. This protein behaves similarly if not identically to endogenous PP1 α with respect to nuclear localization, complex formation to a recently described PP1-associated nuclear targeting protein, and *in vitro* enzymatic activity towards phosphorylase *a* and the cell growth and tumor suppressor protein pRB.

The data presented here validate a useful system with which to manipulate PP1 activity *in vivo* for the purpose of functional studies. Just as is the case for PP2A [31], it seems clear that efficient overall increases in PP1 protein abundance may not be achieved due to a putative negative feed-back or autoregulatory mechanism. However, induced expression of exogenous PP1 which behaves identically to endogenous PP1 while remaining physically distinguishable can facilitate the use of mutants to address functional questions, particularly in the area of cell cycle regulation and tumor cell growth suppression. Indeed, the use of a constitutively active mutant of PP1 has already been shown to result in pRB-dependent G1 arrest in

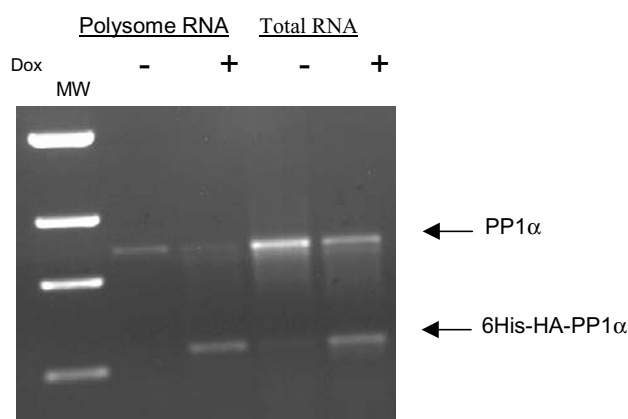


Figure 1

RT-PCR of polysome and total RNA specific for endogenous PP1 α and 6His-HA-PP1 α . PCR products from polysome and total RNA from induced and uninduced cell lysates were prepared and analyzed as described in Materials and Methods. MW – molecular mass standard ladder.

human cancer cells [22]. Placing this mutant into the inducible expression system described here has the potential to down-regulate expression of wild-type endogenous PP1. Doing so in a timed, controlled manner will permit *in vivo* studies addressing PP1 function during discrete phases of the cell cycle, and will be the subject of future endeavors.

Materials and Methods

Tetracycline Induction System

The rabbit PP1 α cDNA in pDR540 [34] was ligated into pcDNA3 (Invitrogen) to create a fusion encoding 6 histidines (6-His) followed by an epitope (YPYDVPDYA) from the Hemophilus influenza hemagglutinin protein (HA). The sequence and reading frame was validated by BigDye DNA sequencing (PE Applied Biosystems). The fusion gene was then cut from the 6His-HA-PP1 α -pcDNA3 plasmid and ligated into pTEP4m, which contains a tetracycline response element for inducible expression [25], and a hygromycin resistance gene for selection in eukaryotic cells (see Figure 1). pUHD172-1neo [25] encodes the reverse tetracycline transactivator protein fused with a nuclear localization signal (rtTA-nls). In addition to the rtTA-nls gene under control of the CMV promoter, the plasmid contains neomycin and ampicillin antibiotic resistance genes.

Cell Culture, Transfection, and Selection

The UMUC3 cell line was obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's minimal essential medium (Life Technologies)

supplemented with 10% fetal bovine serum, 20 units/ml penicillin and 20 ug/ml streptomycin. Cells were incubated at 37°C, in a 5% carbon dioxide containing atmosphere.

Plasmid DNA was introduced into cultured cells using Lipofectamine (Life Technologies). 2 ug of plasmid DNA and 25 ul of Lipofectamine were combined in 200 ul of Opti-mem media (Life Technologies) and allowed to stand at room temperature for 30 minutes. This mixture was diluted to 1 ml with Opti-mem and applied to cells in one well of 6-well tissue culture plates. After two hours, 1 ml of complete medium was added to each well. After twenty-four hours the medium was replaced with complete medium.

Transfection of UMUC3 cells with pUHD172-1neo was followed 48 hr later by selection using geneticin. Isolated colonies were obtained at 800 ug/ml. These cells were passaged and expanded before transfection with pBi-EGFP (Clontech), which contains the enhanced green fluorescent protein coding sequence under control of a bi-directional tetracycline response element. At the end of the 2 hr transfection (as described above) the medium was replaced with complete medium with and without doxycycline at 1 ug/ml final concentration. 24 hr later the cells were examined by fluorescence microscopy. The presence of doxycycline dependent green fluorescence was indicative of stable transfection with pUHD172-1neo encoding the rtTA-nls protein and transient transfection with pBi-EGFP. Parallel cultures were then passaged and transfected with 6His-HA-PP1 α -pTEP4m. Selection with hygromycin was begun 48 hr later. Isolated colonies were obtained at 100 ug/ml hygromycin and were passaged and diluted to extinction. Six cell lines, designated LLW02A through LLW02F, were established. The LLW02F cell line was used for all subsequent experiments. This choice was based on high levels of induced PP1 α expression as assayed by immunoblotting. Doxycycline was used at 1 ug/ml for induction.

SDS-PAGE and Immunoblotting

Cells were lysed for 15 min at 4°C in EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40) containing 10 ug/ml of the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF). The lysates were cleared by centrifugation at 14,000 \times g for 10 minutes. Electrophoresis was performed in SDS-polyacrylamide gels [35] using the indicated amount of total cell protein [36]. After electrophoresis, the proteins were transferred to nitrocellulose paper in buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% v/v methanol, and 0.01% SDS, pH 8.5 [37]. Residual protein binding sites on the nitrocellulose were blocked by incubation for 30 minutes in TBST (25 mM Tris-HCl, pH 8.0, 150 mM NaCl,

0.5% Tween-20) containing 4% non-fat dry milk. Next, the nitrocellulose was incubated in TBST containing 2% non-fat dry milk containing primary antibody directed towards the indicated protein. Following three washes of 10 min each with TBST, the nitrocellulose was probed with horse-radish peroxidase-conjugated anti-IgG (Promega, Madison, WI) and developed using chemiluminescence detection (Pierce, Rockford, IL) according to the manufacturer's instructions.

Immunohistochemistry

Cell monolayers were rinsed 3 times with PBS, fixed for 10 minutes in -20°C methanol, and then air dried for 1 hr. The cells were rehydrated for 20 min in PBS before blocking for 20 min with diluted normal horse serum. The cells were then incubated for 30 min with a 1:200 dilution of monoclonal antibody HA.11 (BAbCO, Richmond, California) which recognizes the influenza hemagglutinin epitope YPYDVPDYA. The cells were then washed 4 times PBS and incubated with biotinylated secondary antibody for 30 min at room temperature. Following 4 rinses with PBS, the cells were then incubated for 30 min at room temperature with avidin-biotin-peroxidase complexes. The cells were rinsed again in PBS 4 times. Diaminobenzidine (DAB substrate kit for peroxidase, Vector Laboratories, Burlingame, CA) was used as the final chromogen. The cells were incubated with this compound for 8 min. Hematoxylin was used as the nuclear counterstain. Following dehydration in ethanol and air drying, the cell monolayers were flooded with glycerol for subsequent observation, photography, and storage.

Phosphorylase Phosphatase Activity of Immunocomplexed 6His-HA-PP1 α

Phosphorylase phosphatase activity was measured as the release of trichloroacetic acid (TCA) soluble counts from ³²P-phosphorylase *a* according to Cohen et. al. [38] Briefly, radiolabeled phosphorylase *a* was synthesized by taking phosphorylase *b* (Sigma), at a concentration of 10 mg/ml, and incubating with 0.2 mg/ml phosphorylase kinase (Sigma) for 1.5 hours at 30°C in kinase reaction buffer (100 mM Tris-HCl; 100 mM sodium glycerol-1-phosphate, pH 8.2; 0.1 mM CaCl₂, 10 mM magnesium acetate, and 0.2 mM [g-³²P]ATP {10⁶ cpm/nmol}). Enzyme samples were immunocomplexed 6His-HA-PP1 α .

Preparation of [³²P]pRB substrate and pRB-directed phosphatase activity assays

To assay pRB-directed phosphatase activity, [³²P]-labeled pRB substrate was prepared by immunoprecipitation with monoclonal antibody to pRB, PMG3-245 (PharMingen), from [³²P]-labeled CV-1P cells as described previously [20]. For the source of enzyme, immunocomplexed 6His-HA-PP1 α from induced LLW02F cells were mixed and

incubated with [³²P]-labeled immunoprecipitated pRB for 30 minutes at 30°C as previously described [20]. Phosphatase reactions were terminated by the addition of SDS-PAGE sample buffer and boiling. Proteins were separated on SDS-polyacrylamide gels. Gels were fixed, dried and subjected to autoradiography.

Type I Phosphatase Activity of Whole-Cell Lysates

PP1 was assayed by the release of [³²P] H₃PO₄ from [³²P]-rabbit muscle phosphorylase *a* (1–2 × 10⁵ cpm/nmol). 5 nM okadaic acid was used to inhibit PP2A during the assay. 1 unit of PP1 activity releases 1 nmol of Pi/min at 30°C.

Immunoprecipitation using PP1 Isoform-specific antibody

LLWO2F cell extracts (50 ug) were immunoprecipitated with approximately 1 ug of isoform-specific rabbit antiserum to PP1 α as previously described [38]. Immunoprecipitation with 1 ug of normal rabbit serum served as a negative control.

GST-PNUTS Affinity Chromatography

Induction of GST-PNUTS fusion protein synthesis and isolation by affinity chromatography using glutathione-Sepharose beads (Pharmacia) was performed as described previously [32]. Induced LLWO2F cell extracts were then incubated for 1 hour at 4°C with recombinant GST or GST-PNUTS fusion protein bound to glutathione-Sepharose. Bound proteins were washed with PBS, separated on 10 % SDS-polyacrylamide gels, and transferred to nitrocellulose. Protein blots were then developed using antibody to hemagglutinin for 6His-HA-PP1 α detection.

Polysome isolation

Uninduced and induced LLWO2F cells (10 × 100 mm dishes) were washed 3-times with ice-cold serum-free DMEM containing 10 ug/ml cycloheximide. After the last wash, cells were scraped into a final volume of 5 ml of this same medium for each 10-dish treatment. The cells were harvested with a brief centrifugation. The cell pellet was resuspended in 0.5 ml lysis buffer (125 mM KCl, 12.5 mM mgCl₂, 10 mM Hepes, pH 6.8, 0.1 mM dithiothreitol, 10 ug/ml cycloheximide, 0.5% Triton X-100, 0.5% deoxycholate) followed by 10 passages through a 22 gauge needle to lyse the cells. The resulting extracts were then layered on the top of a 10–40% sucrose gradient (125 mM KCl, 12.5 mM mgCl₂, 10 mM Hepes, pH 6.8, 0.1 mM dithiothreitol, 10 ug/ml cycloheximide) and centrifuged at 4°C in a SW50.1 rotor at 48,000 rpm for 60 min. After centrifugation, the gradient was fractionated in 200 ul aliquots and analyzed at A₂₆₀ using a Bio-Rad SmartSpec 3000. A single peak of A₂₆₀ absorbance in the bottom portion of the gradient was analyzed as polysome fraction.

RT-PCR analyses of PP1 α mRNA

LLWO2F cells were induced with doxycycline (2 ug/ml) for 24 hr. Negative control LLWO2F cells were not induced with doxycycline. For total RNA experiments, RNA was harvested from monolayered cells using QIAshredder and Qiagen RNeasy mini-kit. Polysome RNA was recovered from polysome fractions by first diluting fractions in 3.5 volumes of lysis buffer from the Qiagen RNeasy mini-kit and then continuing with the manufacturer's instructions. RNA was quantitated by UV spectrometry. 1 ug total RNA (or 200 ng polysome RNA) was used in first strand cDNA synthesis using SUPERScript II reverse transcriptase (Life Technologies) following manufacturer's instructions. cDNA was extracted by one phenol chloroform:isoamyl alcohol extraction followed by one chloroform isoamyl alcohol extraction and then ethanol precipitated. cDNA was resuspended in 20 ul water and used in a 30-cycle PCR reaction with 1 uM of each of the following four primers: {CCACGCTGTTTTGACCTCCAT-AGAAGACAC, CACATAGTCCCCAGAAAAGAGGTAGTT-GCT}, in which product only forms from 6His-HA-PP1 α cDNA, and {GACGCGGGCAAGCAGTCCCTCGAGAC-CATTGCCTGCTG, CTGGAGACCCACGACCTGGCCT-GCCGTTG}, in which product only forms from endogenous PP1 α cDNA. Reaction also contained 200 uM dNTPs, 1 mM Mg⁺⁺, and 5 units Taq DNA polymerase (Life Technologies) with supplied reaction buffer. 2 ul of the PCR product was loaded onto a 1% agarose gel containing ethidium bromide for UV visualization.

Authors' Contributions

JER, JWL, and EMM conceived the study and participated in its design and execution. MPS participated in plasmid constructions. PK performed RT-PCR and polysome analysis. EV-M provided PP1-isoform specific antibodies and participated in PP1 immunoprecipitations and western blots. Authors read and approved the final manuscript.

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