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In an effort to identify genetic changes involved in ovarian cancer (OvCa) development, we performed differential display-PCR, cDNA microarray and suppression subtraction hybridization analyses (SHH) to identify early genetic alterations associated with OvCa. These studies resulted in identification of several genes differentially expressed in OvCa, including a novel gene encoding a transcription elongation-like protein with the ability to induce apoptosis and suppress cancer cell growth. We named the protein ProApoptotic Protein on chromosome X (PAPX). Pro-apoptotic protein on X (PAPX) is a novel nuclear protein with sequence homology to transcription elongation factor like 1 (TCEAL1) [1]. PAPX expression is down-regulated in majority of ovarian cancer cell lines and primary tumors [2]. Re-expression of PAPX induces cell death and attenuates cell growth. We therefore proposed to study the functional role of PAPX as a candidate tumor suppressor in ovarian cancer. We proposed to (1) determine effect of PAPX on tumor and cell growth *in vivo* and *in vitro*; (2) analyze genes regulated by PAPX by transcriptional profiling using microarray chips; and (3) identify proteins that interact with PAPX and elucidate the function of PAPX related to tumor suppression.

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ABSTRACT

Pro-apoptotic protein on X (PAPX) is a novel nuclear protein with sequence homology to transcription elongation factor like 1 (TCEAL1) [1]. PAPX expression is down-regulated in majority of ovarian cancer cell lines and primary tumors [2]. Re-expression of PAPX induces cell death and attenuates cell growth. We therefore proposed to study the functional role of PAPX as a candidate tumor suppressor in ovarian cancer. We proposed to (1) determine effect of PAPX on tumor and cell growth *in vivo* and *in vitro*; (2) analyze genes regulated by PAPX by transcriptional profiling using microarray chips; and (3) identify proteins that interact with PAPX and elucidate the function of PAPX related to tumor suppression.

Due to the difficult nature of establishing stable cell lines expressing PAPX (described in detail in the following section) we are generating an inducible system (Flp-In T-Rex) in ovarian cancer cell lines A2780 and SKOV3 **to address the first two specific aims**. As these studies are underway, we initiated studies to elucidate PAPX interacting proteins using an antibody array as described below. Although in the initial proposal, we had proposed to identify PAPX interacting proteins with other techniques such as yeast two hybrid and GST pulldown assays, we also tried the antibody array to achieve the same goal and were successful in identifying PAPX interacting proteins.

To address Aim #3, we utilized antibody array containing antibodies against known proteins involved in apoptosis. Such analysis resulted in the identification of three apoptotic proteins as candidate binding partners for PAPX. We subsequently selected one candidate binding partner, and characterized its interaction to PAPX by immunoprecipitation, including specific domain required for such an interaction. Moreover, we analyzed the effect of PAPX on the transcriptional activity of its binding partner. These studies led to the identification of PAPX as a negative regulator of c-myc. Identification of PAPX as a negative regulator of c-myc is highly significant in light of the fact that c-myc plays an important role as an oncogene in various cancers.

INTRODUCTION

We have recently identified a novel pro-apoptotic nuclear protein with domain homology to $p75^{NTR}$-associated death executor (NADE) [3] and transcription elongation factor A-like 1 (TCEAL1) [1]. Amino acid alignment of PAPX and NADE reveals that PAPX contains a putative nuclear export signal, but no homology with the C terminal region of NADE that is shown to be involved in NGF-dependent regulation of NADE-induced apoptosis [4]. NADE is a nuclear pro-apoptotic protein involved in modulating apoptosis mediated by the low affinity neurotropin receptor [3]. Like NADE, PAPX is a pro-apoptotic nuclear protein, and forced expression of PAPX in ovarian cancer cell lines induces apoptosis. Although NADE is not implicated in carcinogenesis, expression of PAPX is lost or markedly reduced in ovarian cancer. The fact that PAPX is a pro-apoptotic protein and is lost in majority of ovarian tumor is highly significant because loss of expression of a pro-apoptotic protein could confer a survival advantage leading to the development of ovarian epithelial neoplasia.

Therefore, we proposed to (1) determine effect of PAPX on tumor and cell growth *in vivo* and *in vitro*; (2) analyze genes regulated by PAPX by transcriptional profiling using microarray chips; and (3) identify proteins that interact with PAPX and elucidate the function of PAPX related to tumor suppression.

BODY

Aim #1: *Determine the effect of re-expression of PAPX on the colony formation efficiency, soft-agar growth, and tumorigenicity of ovarian cancer cells.*

Since all the ovarian cancer cell lines (five Mayo established cell lines, SKOV3, OVCAR5, A2780, CP70, and C200) do not express PAPX, we were not able to perform RNAi-mediated knock-down to study the effect of PAPX down-regulation on tumorigenicity. Therefore, we elected to re-express PAPX in OV202, SKOV3, and A2780 to perform tumorigenic assays following re-expression. However, due to its pro-apoptotic property, we were not able to establish stable cell lines in these cells. Therefore, we generated tetracycline-inducible SKOV3 cell line (as suggested by one of the reviewers). Although the established tetracycline-inducible SKOV3 cell line proved to be tightly controlled by tetracycline compared to commercially available tetracycline-inducible cell lines, we were again unsuccessful in establishing a stable cell line due to minimal background level leakiness of expression. We are therefore establishing an alternative inducible system using Flp-In T-Rex from Invitrogen. Using established cell line 293T Flp-In T-Rex, we were able to establish a stable cell line expressing inducible PAPX (Figure 1).

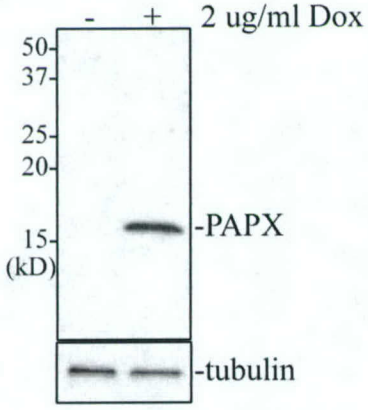


Figure 1. Inducible expression of PAPX in 293T Flp-In T-Rex system. Whole cell lysates were collected from cells treated with doxycycline for 24 h. Western blot analysis using affinity-purified PAPX antibody shows inducible expression of PAPX in doxycycline treated cells.

Therefore, we are now generating Flp-In T-Rex system in SKOV3 and A2780. Based on our initial success in establishing PAPX inducible 293T Flp-In T-Rex cell line, we are confident that we will be able to succeed in establishing stable cell lines in ovarian cancer cells. Once established, we will assess tumor suppressor activity of PAPX in nude mice and in vitro cell culture. **No changes in proposed research design are expected. These studies are to be completed in the third year of grant support.**

Aim #2: *Assess the transcriptional profiles of vector-transfected vs. PAPX-transfected ovarian cancer cells by cDNA microarray analysis.*

Due to the difficult nature of establishing stable cell lines in ovarian cancer as stated above, this project awaits the establishment of tetracycline-inducible ovarian cancer cell lines. Meanwhile, we used transient PAPX expression and performed microarray analysis on these cells. Preliminary profiling analysis indicates upregulation of several apoptotic genes following PAPX transfection (Table 1). Additional microarray analyses are underway using 293T Flp-In T-rex system. Once established, inducible ovarian cell lines will be included in the microarray analysis. Data obtained with 293T and ovarian cell lines will be compared with data obtained from transient transfection of PAPX in ovarian cancer cells, and a list of 10 pro-apoptotic genes upregulated and 10 anti-apoptotic genes down-regulated will be validated by northern blot and quantitative RT-PCR analyses. Once validated, RNAi for apoptotic genes and forced expression for anti-apoptotic genes will be carried out to test whether these measures can counteract the effect of PAPX. No changes in proposed research design are expected. These studies are to be completed in the second year of grant support.

Annotation	Induced		Suppressed
PAPX	51.50	UID=Hs.226124	16.75
Protein phosphatase 2B	13.26	dbEST=377234	15.39
UID=Hs.299544	10.64	UID=Hs.127557	11.91
Interleukin 7 receptor	10.02	UID=Hs.180793	9.57
UID=Hs.130904	9.24	UID=Hs.221513	7.98
UID=Hs.92004	9.05	UID=Hs.298448	7.94
Sema3A	8.54	UID=Hs.292833	7.93
UID=Hs.181054	8.52	Phosphodiesterase 10A	7.01
Disintegrin reprolysin	8.34	UID=Hs.130470	6.94
UID=Hs.104705	8.13	UID=Hs.285673	6.68
UID=Hs.99546	7.90	UID=Hs.114765	5.90
UID=Hs.4791	7.86	UID=Hs.118015	5.88
UID=Hs.218260	7.75	UID=Hs.55305 ESTs	5.88
Prostaglandin E2 receptor	7.29	MEK2	5.65
UID=Hs.20161	7.19	AK000305 unnamed protein product	5.61
Mu-crystallin homolog	7.12	UID=Hs.65828	5.54
UID=Hs.138499	6.96	Retinal fascin	5.41
UID=Hs.107331	6.77	UID=Hs.301584	5.34
UID=Hs.163044	6.76	Diphosphomevalonate decarboxylase	5.32
beta-site amyloid precursor protein cleaving enzy	6.60	UID=Hs.97661	5.25
dermatan/chondroitin sulfate 2-sulfotransferase	6.58	UID=Hs.288837	5.18
Carboxypeptidase N precursor	6.40	UID=Hs.269208 ESTs397620	5.12
Mitochondrial 3-oxoacyl-CoA thiolase	6.38	Troponin I	5.07
UID=Hs.292941	6.31	DKFZp434D1812	4.93
UID=Hs.117955	6.24	UID=Hs.129990	4.78

Table 1. Comparison of gene expression between vector- and PAPX-transfected cells. Left panel shows genes induced by PAPX, while right panel shows genes suppressed by PAPX. Genes highlighted are reported to be involved in apoptosis and/or cell survival in various systems.

Aim #3: *Characterize cellular proteins interacting with PAPX.*

We utilized antibody array (Hypomatrix) containing antibodies against known proteins involved in apoptosis to screen for potential PAPX interacting proteins. This analysis led to identification of three proteins (Bin1, DcR2, and FAST) to be potential interacting proteins (Figure 2).

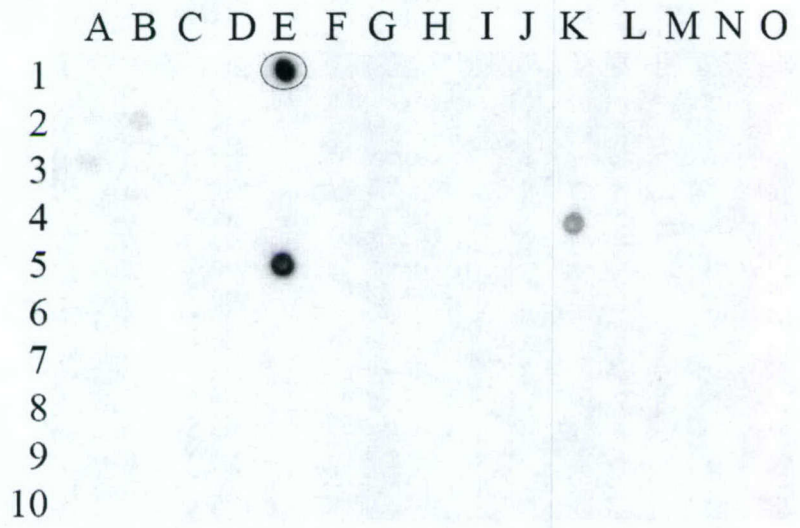


Figure 2. Protein-Protein interaction screening with the Antibody Array from Hypomatrix (CAT # HM4000). Bin1 is indicated by circle. The other two intense dots are DcR2 and FAST.

Bin1 is a c-myc-interacting protein that regulates myc trans-activation [5-7]. Furthermore, Bin1 induces caspase-independent cell death [8]. Since PAPX is also a nuclear protein, it is possible that PAPX may modulate Bin1 interaction with myc. Moreover, PAPX may cooperate with Bin1 in inducing cell death. Therefore, we selected Bin1 for further characterization. Immuno-precipitation results suggest that PAPX interacts with Bin1 via BAR domain of Bin1 (Figure 3).

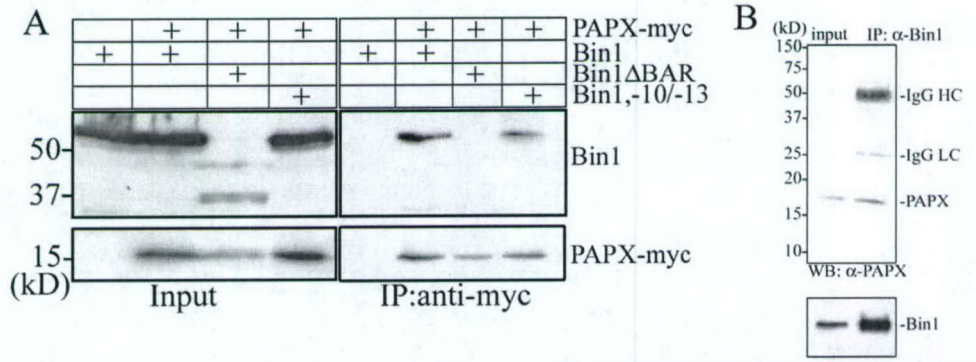


Figure 3. **A**: PAPX co-immunoprecipitates full-length or Myc-binding domain-deleted Bin1 but not BAR domain-deleted Bin1, suggesting that PAPX interacts with Bin1 through BAR domain of Bin1. **B**: Immunoprecipitation of Bin-1 also co-immunoprecipitates PAPX.

To test whether PAPX cooperates with Bin1 in suppressing Myc activity, cells were transfected with PAPX or Bin1 alone or together and myc activity was assessed by luciferase reporter gene containing ODC promoter (a natural promoter containing myc-binding site) or artificial E-box promoter (pEMS) [7]. The result shown in Figure 4, suggest that PAPX suppresses myc activity as determined by pEMS. Moreover, PAPX-induced myc suppression is stronger than Bin1. No potentiation of myc suppression was observed when both Bin1 and PAPX were co-expressed. On the other hand, expression of PAPX and Bin1ΔMBD (myc-binding domain deleted Bin1) showed decreased suppression of PAPX on myc activity suggesting that Bin1ΔMBD antagonizes PAPX by preventing it from interacting with myc. Similar results were obtained with pODC (data not shown). These results establish PAPX as a negative regulator of myc.

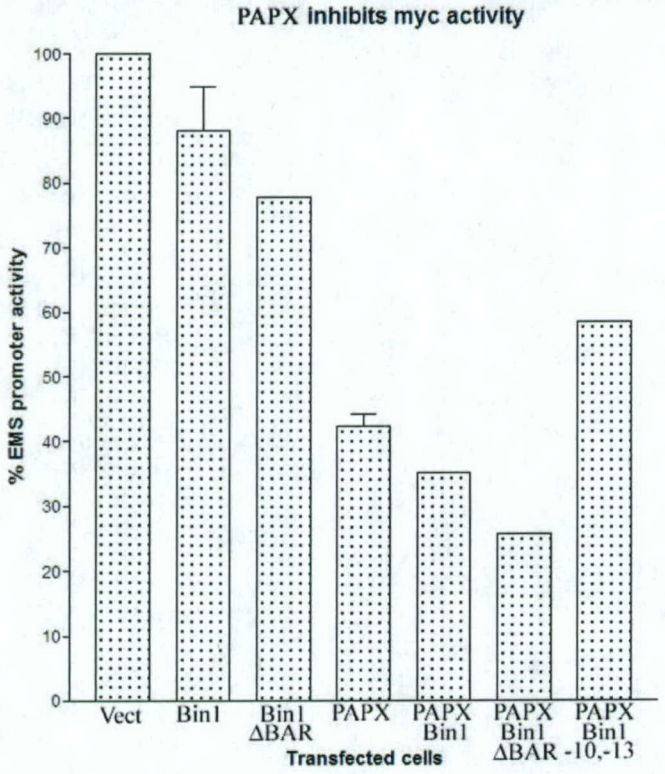
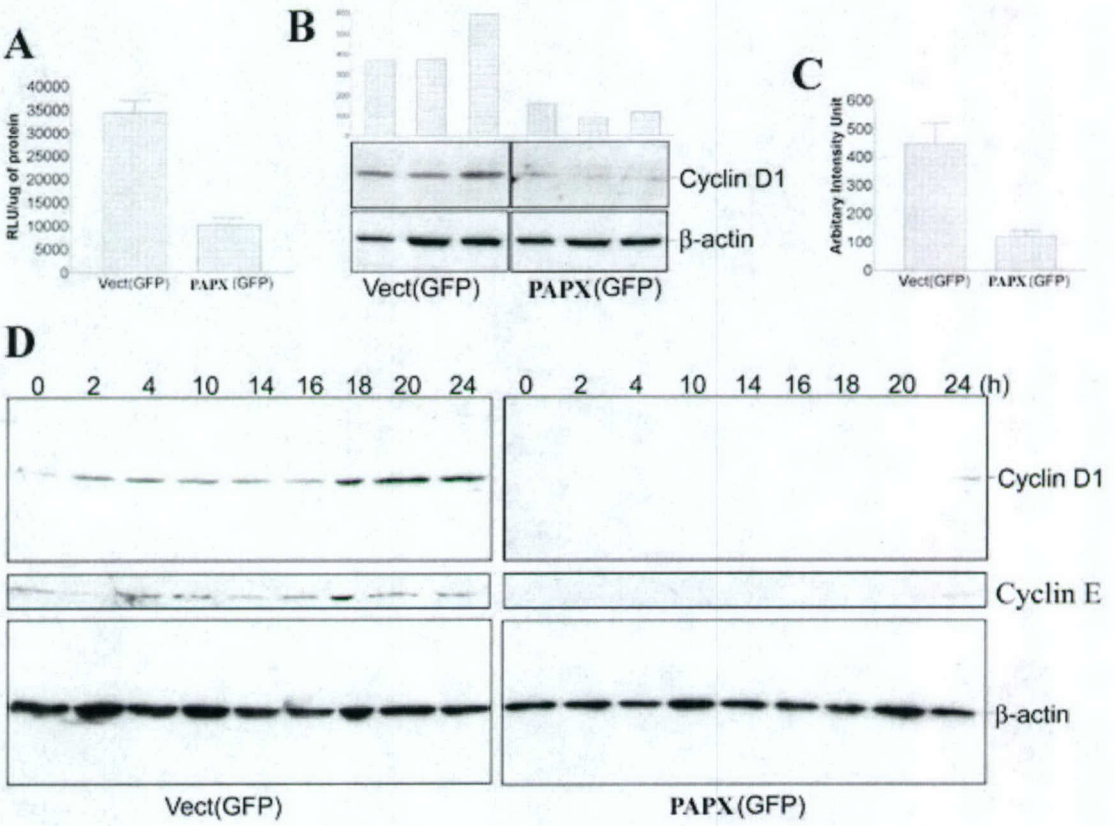


Figure 4. PAPX suppresses myc activity. Bin1 also suppresses myc activity but not as effective as PAPX in suppressing myc activity. Co-expression of Bin1 and PAPX further suppresses myc activity suggesting that they may suppress myc activity independently of each other. However, a naturally occurring exons 10 and 13-deleted isoform of Bin 1 (-10,-13) attenuates PAPX suppression on myc activity, suggesting that Bin1 -10,-13 antagonizes PAPX.

To evaluate the functional significance of such repression of myc, we examined the expression of myc-target gene cyclin D1 following PAPX expression.

As indicated in **Fig 5A**, expression of PAPX decreases cyclin D1 promoter activity. Furthermore, it down-regulates cyclin D1 **expression (Fig 5B**). Densitometric analysis of immunoblot result indicates approximately four-fold decrease in cyclin D1 level following PAPX expression **(Fig 5C).** Analysis of cyclin D1 expression in synchronized HeLa cells suggest that expression of PAPX suppress cyclin D1 and delayed expression of cyclin D1 following nocodazole release of G2/M block **(Fig 5D).** Consistent with these analyses, PAPX localizes to nucleus **(Fig. 5E)** and decreases BrdU labeling **(Fig. 5F).**


A
RLU/ug of protein
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35000
30000
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5000
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Cyclin D1
β-actin
Vect(GFP)
PAPX(GFP)
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Arbitary Intensity Unit
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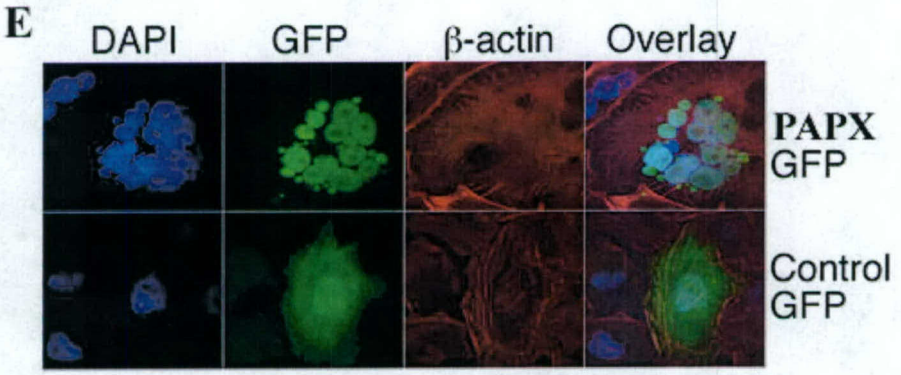
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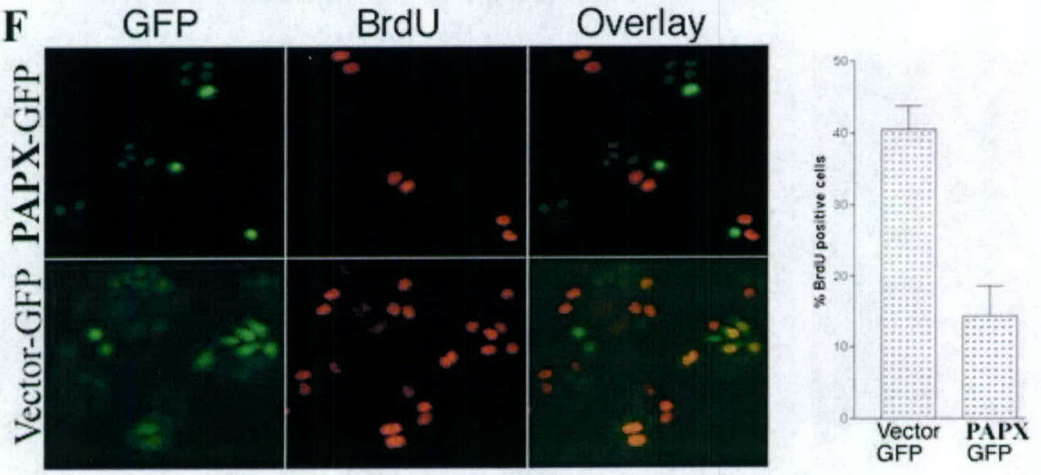

F
GFP
BrdU
Overlay
PAPX-GFP
Vector-GFP
% BrdU positive cells
50
40
30
20
10
0
Vector GFP
PAPX GFP

Consistent with the effect on myc and cyclin D1, expression of PAPX reduced colony forming efficiency of ovarian cancer cells (Fig 6).

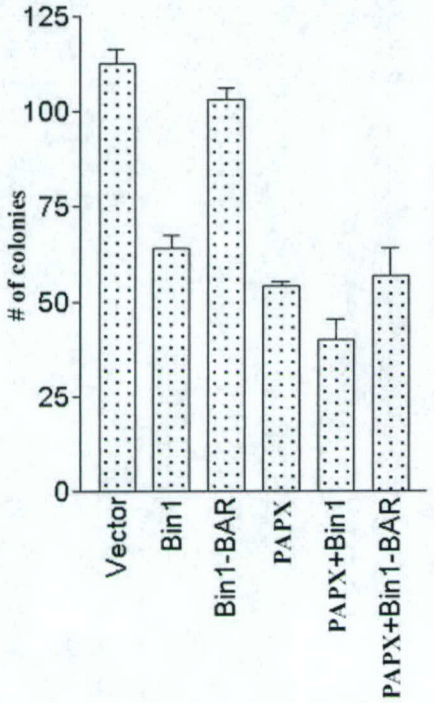


Figure 6. Expression of PAPX reduces colony forming efficiency of ovarian cancer cells. Both Bin1 and PAPX suppresses colony forming efficiency.

Finally, we analyzed the expression of PAPX in various normal tissues and carcinomas. Northern blot analysis of PAPX in multiple tissues indicates varying levels of PAPX expression normal tissues (Fig 7A). Results from multiple tissue expression array also indicate that PAPX in expressed in varying degree in several tissues including ovary (Fig 7B).

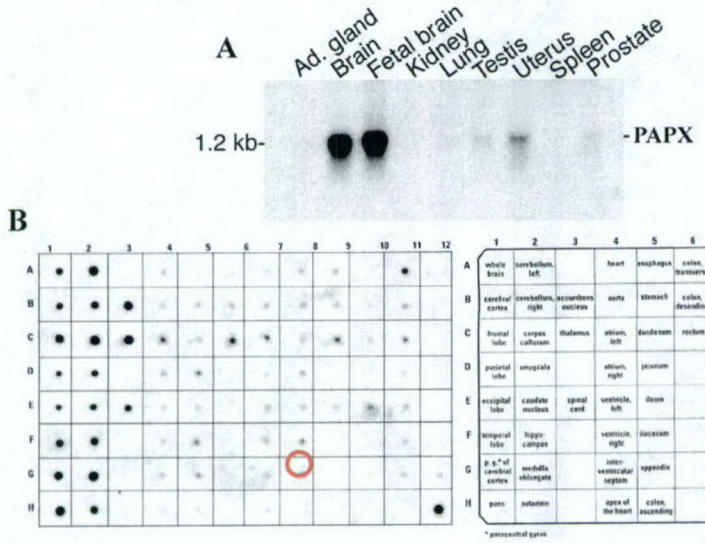
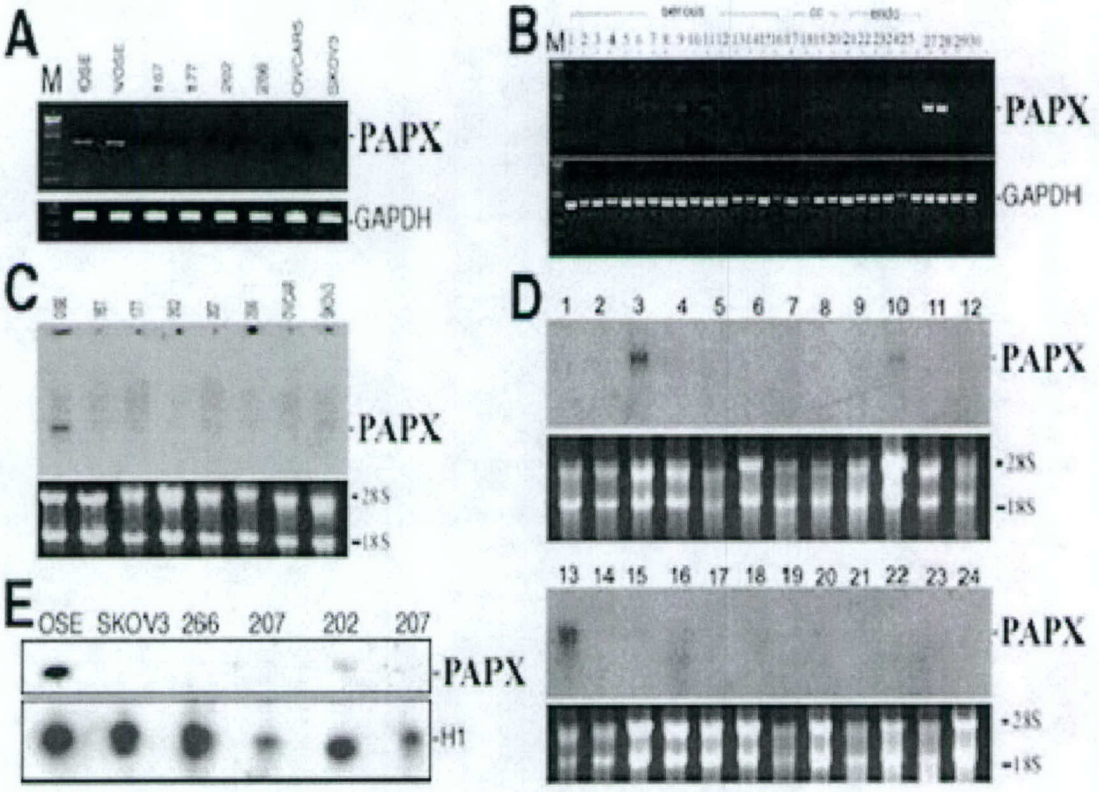
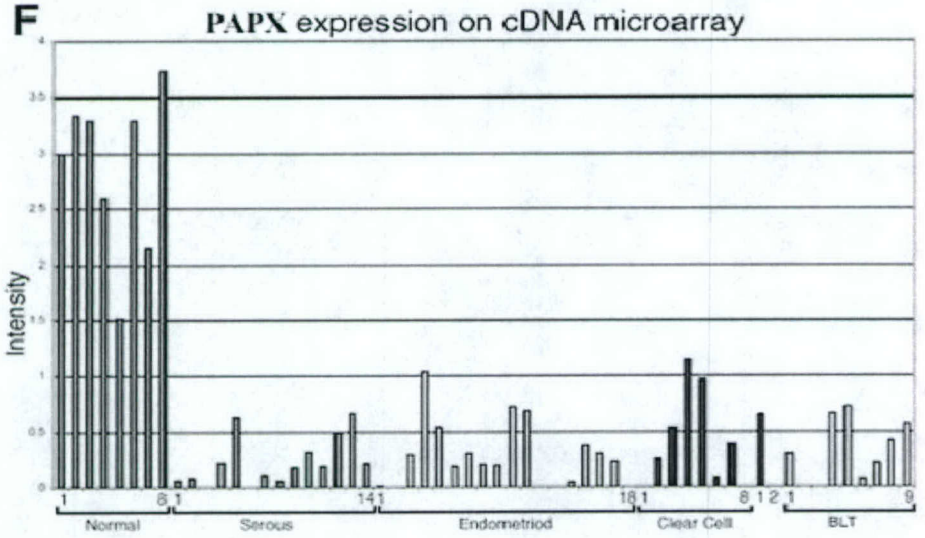


Figure 7: A and B: Multiple tissue northern blot analysis of PAPX expression. PAPX is expressed in various tissues with highest expression in the brain and also in the ovary (red circle in B)

RT-PCR analysis of ovarian cell lines indicates that PAPX is expressed in normal ovarian surface epithelial cells such as short-term culture ovarian surface epithelial cells (OSE) or immortalized OSE (vOSE) but not in cancer cell lines (Fig 8A). RT-PCR analysis of 28 primary ovarian tumors also indicates down-regulation of PAPX (Fig 8B). Northern blot analysis of ovarian cell lines shows that PAPX is expressed in OSE and vOSE but not in cancer cell lines (Fig 8C). Northern blot analysis of 24 primary tumors indicates that only 3 out of 24 tumors expressed PAPX (Fig 8D). Western blot analysis of cell line lysates confirmed that PAPX is down-regulated in cancer cell lines (Fig 8E). Transcriptional profiling of additional 51 primary tumors also indicates down-regulation

of PAPX in these primary ovarian tumors of different histology (Fig 8F). To test the specificity of targeted down-regulation, two genes 20 and 50 kb proximal to PAPX was tested for expression. Expression of these genes were detected in most of the samples tested (Fig 8G).





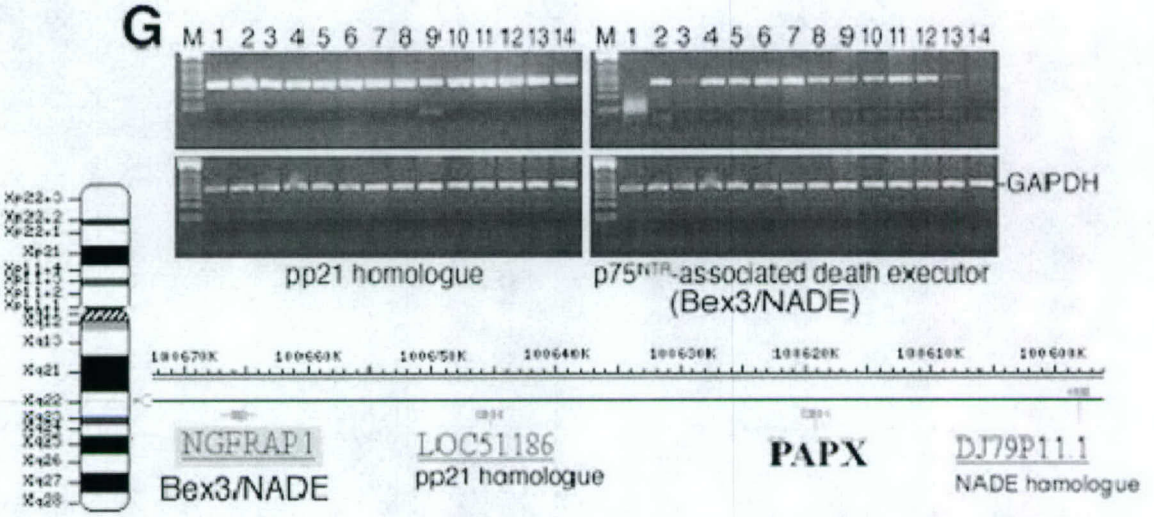
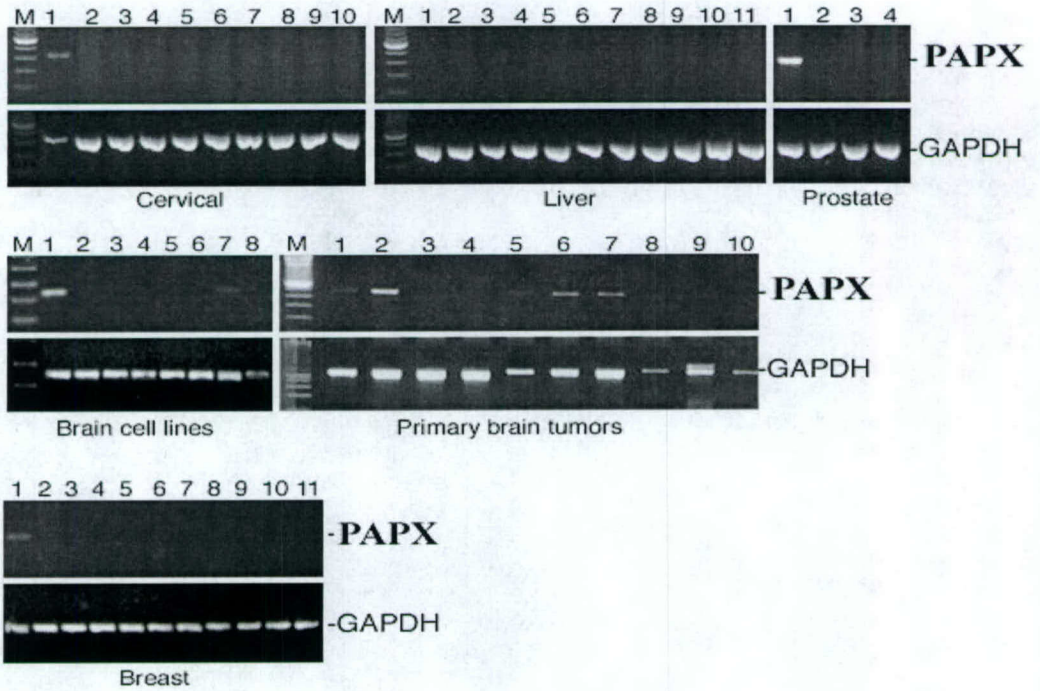


Figure 8. A: RT-PCR analysis of PAPX expression in ovarian cell lines. B: RT-PCR analysis of PAPX expression in primary ovarian tumors. C: Northern blot analysis of PAPX in ovarian cell lines. D: Northern blot analysis of PAPX in primary ovarian tumors. E: Western blot analysis of PAPX in primary tumors. F: Analysis of PAPX expression in 51 primary ovarian tumors and 8 normal ovaries by cDNA microarray. G: Analysis of expression of two genes ditsal to PAPX.

RT-PCR analysis of primary tumors from brain tissues and cell lines from cervical, liver, prostate, brain and breast indicates that PAPX is also down-regulated in these carcinoma also (Fig 9).

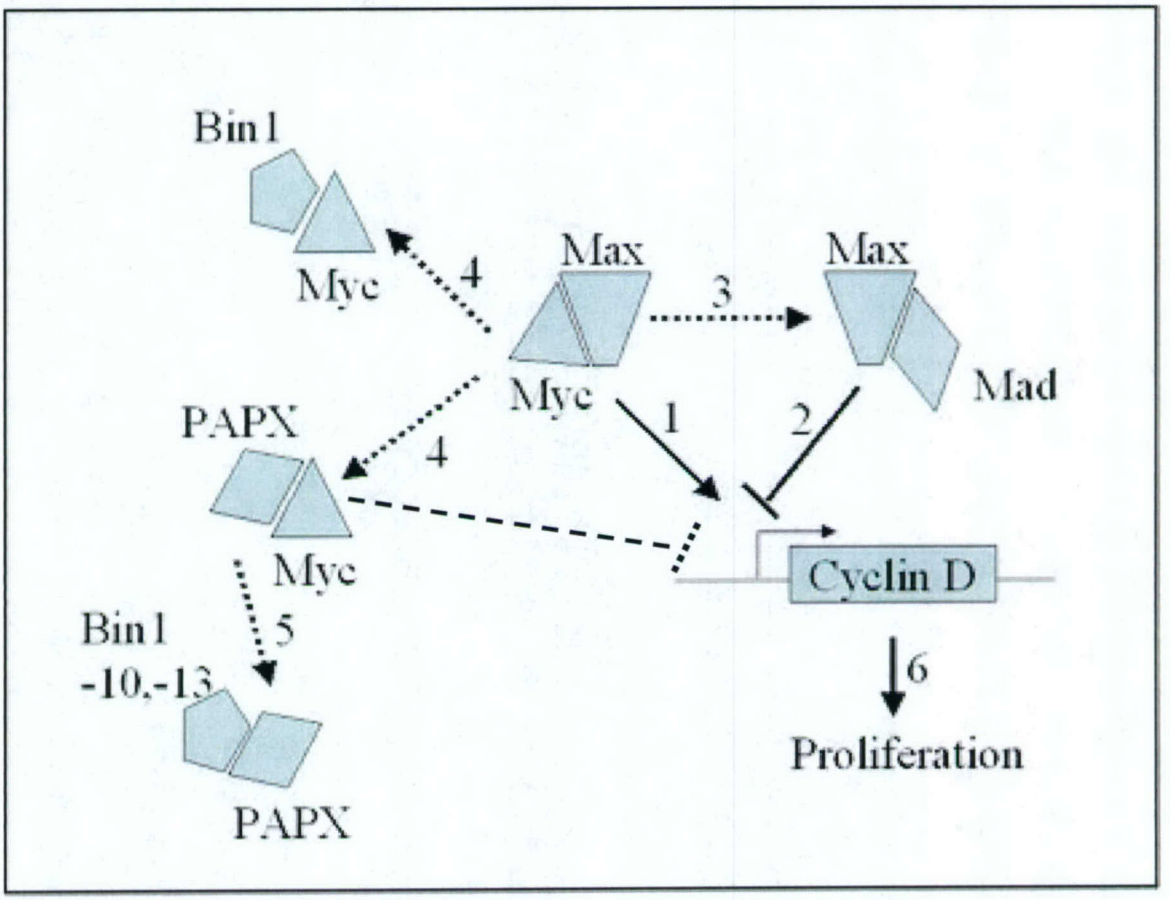
Figure 9. Analysis of PAPX expression in various cancer cell lines and primary brain tumors.



Proposed Model of PAPX in regulating Myc activity

Given that PAPX is a negative regulator of myc activity, down-regulation of PAPX may promote myc activity and may be involved in myc-induced malignant transformation.

Based on these results we propose the following model of interaction: 1) transcriptionally active state induced by Myc:Max interaction [9]. 2) transcriptionally repressive state induced by Max:Mad interaction [10]. 3) Myc interactions with Max allows Max in a transcriptional active state by preventing it from binding to Mad which turns Max off from its transcriptional active state [11]. 4) When Bin1 or PAPX associate with Myc, they recruit Myc away from Max; thus allowing Max to associates with its repressor Mad, leading to repression of Myc target genes. 5) Expression of exons 10 and 13 deleted isoform of Bin1 [7] associates with PAPX but does not associate with myc due to partial deletion of Myc-binding domain. This isoform recruits PAPX away from Myc thus allowing Myc to interact with Max and induce Myc target genes. 6) Therefore, expression of PAPX recruits Myc away from Max, allowing Max to interact with Mad leading to transcriptional repression of cyclin D1, resulting in reduced proliferation as indicated by decrease colony forming efficiency.



KEY RESEARCH ACCOMPLISHMENTS:

- Identification of PAPX as Bin1 binding protein
- Functional characterization of PAPX and Bin1 interaction
- Identification of PAPX as myc regulator
- Validation of PAPX expression in a large set of tumor samples
- Establishment of tetracycline inducible 293T cell line expressing PAPX
- Transcriptional profiling of cells transiently expressing PAPX
- Identification of candidate targets of PAPX

REPORTABLE OUTCOMES:

Abstracts :

Julie Staub, Jeremy Chien, Rajeswari Avula, David I Smith, Scott H Kaufmann and **Viji Shridhar**. Epigenetic silencing of a novel tumor suppressor gene in ovarian cancer. Poster presentation AACR, 95th Annual meeting, Orlando, FL, 2004.

Manuscripts in Preparation

1. Jeremy Chien, Julie Staub, Rajeswari Avula, Wanguo Liu, Lynn Hartmann, David I Smith and Viji Shridhar. Epigenetic silencing of a novel gene, TCEAL7* in ovarian cancer.

2. Jeremy Chien, Pan Yanquin, Jinping Lai, Julie Staub, Lynn Hartmann, George Prendergast, Scott Kaufmann and **Viji Shridhar**. TCEAL7*, a down regulated gene in ovarian cancer interacts with Myc and induces cell cycle arrest.

Please note, PAPX has been renamed to TCEAL7 (transcription elongation factor A (SII)-like 7) in accordance with Genebank nomenclature.

We are planning to submit the first manuscript to the journal of *Gynecologic Oncology* and the second manuscript to *Molecular Cellular Biology*. The support form DOD OCRP will be acknowledged on both the manuscripts.

CONCLUSIONS:

Identification of PAPX as a regulator of myc that is lost in several different carcinomas is highly significant as it may promote myc-induced malignant transformation by relieving repression on myc activity. These findings raise several important questions as well.

1. What is the role of Bin1 –10,-13 isoform?
2. Can it promote myc activity by antagonizing PAPX in PAPX expressing cells?
3. Is Bin1 –10,-13 upregulated in cancer? How is PAPX inactivated in cancer?
4. Could PAPX be targeted for therapy to counteract the effect of myc-induced transformation?

The answers to some of these questions may emerge when studies proposed in Aims #1 and #2 is completed.

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APPENDIX 1

EPIGENETIC SILENCING OF A NOVEL PROAPOPTOTIC TRANSCRIPTIONAL REGULATORY PROTEIN IN OVARIAN CANCER

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Here we report on the cloning of a novel pro-apoptotic protein on chromosome X, first named PAPX due to its ability to induce apoptosis, and describe the functional consequences of its loss in ovarian cancer. PAPX codes for a 1.35 Kb transcript that was at least five-fold down-regulated in all tumors profiled (n=14) by cDNA microarray analysis and was also identified as an under-expressed gene in three of four SSH libraries constructed. Semi-quantitative RT-PCR analysis with primers flanking the open reading frame revealed a complete loss of expression in all seven ovarian cancer cell lines and a complete loss or markedly reduced levels of PAPX expression in 90% of primary ovarian tumors. 5-aza-2'-deoxycytidine treatment of the OV202 cell line induced PAPX expression in a dose-dependant manner implicating methylation in this induction. Methylation specific PCR in normal ovarian epithelial cells and in somatic cell hybrids with either the active or the inactive X as one of its human counterpart revealed that PAPX is subject to X chromosome inactivation. Loss of PAPX expression in 10 primary tumors and 7 cell lines correlated with methylation of the active expressed allele involving either site 3 within exon 1 or site 4 in intron 1. Both PAPX and pp21 homolog have domain homology with the transcription elongation factor A-like 1(TCEAL1). The pp21 homolog and the pro-apoptotic protein $p75^{NTR}$-associated death executor (NADE) are 39% and 37% identical respectively to PAPX. PAPX co-localizes with RNA Pol II in nuclear foci. Forced expression of PAPX in non-expressing ovarian cancer cells induces apoptosis, and reduces colony formation efficiency. These data implicates PAPX as a growth regulatory protein frequently inactivated in a majority of ovarian cancers and suggests it functions as a tumor suppressor. PAPX has been renamed Bex4 due to its homology to a family of brain expressed (bex) genes.

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