UNIVERSITY OF CALIFORNIA, SAN DIEGO

Phosphorylation of PUMA at Serine Residues -96 and -106 is Involved in Regulation of Autophagy but not Apoptosis

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Biological Sciences

by

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The Thesis of Nicole Gabriele Nalbandian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2014

This Thesis is dedicated to my parents and loved ones

who have supported me through every step of my academic journey.

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ABSTRACT OF THE THESIS

Phosphorylation of PUMA at Serine Residues -96 and -106 is Involved in Regulation of Autophagy but not Apoptosis

by

Nicole Gabriele Nalbandian

Master of Science in Biology

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Professor Yang Xu, Chair

PUMA is a Bcl-2 pro-apoptotic protein known for its ability to mediate cell death by apoptosis and, more recently, autophagocytosis. Previously three serine phosphorylation sites have been identified which are involved in the post-translational regulation of PUMA, S-10 -96 and -106. Thus far, serine-10 is best understood for promoting cell survival when phosphorylated by destabilizing PUMA and decreasing apoptosis, however the regulatory function of S-96 and S-106 phosphorylation is still unknown. To address this question we developed a PUMA S96,106A non-phosphorylation mutant and compared its function with wild-type PUMA. We first used flow cytometry to determine the regulatory function of S-96

and S-106 in apoptosis and found and insignificant difference between wild-type and mutant PUMA. Next, flow cytometry was used to quantify autophagy in cells overexpressing PUMA wt and S96,106A, revealing that simultaneous elimination of S-96 and S-106 phosphorylation significantly decreases cellular autophagy. This result was confirmed by quantifying Light Chain-3-II (LC3-II) by protein immunoblotting and fluorescence microscopy. Lastly, the protein stability of wild-type and mutant constructs was quantified by western blot showing that elimination of S-96 and S-106 phosphorylation slightly decreased PUMA stability, however these results were not significant enough to draw any definite conclusions. We conclude that serine-96 and -106 phosphorylation sites are involved in regulating PUMA's mediated autophagy but not apoptosis or protein stability. The aim of this study is to better understand how PUMA is post-translationally regulated important for the application of PUMA in pathologies including but not limited to cancer, alzheimer's and colitis.

Introduction

In 2001 a protein called PUMA (p53 upregulated modulator of apoptosis) was identified as a key mediator of cellular apoptosis (1). *PUMA* is a gene on chromosome 19q containing four exons which can encode four transcript variants, PUMA α, β, γ and δ (Figure 1a) (2,3). PUMAα and β are the only two variants which express the BH3 and MLS (Mitochondrial-Localizing Sequence) functional domains necessary for initiating cell death by mitochondrial outer membrane permeablization MOMP (3,4). Katsinori Nakano established that PUMAα and β are identical in sequence and function with only slight variation in their N-terminal sequences (4). The PUMAα variant, consisting of exons 1a, 2, 3 and 4 and encodes a 193 amino acid sequence, is the only thoroughly studied transcript. We will use the PUMAα variant, which will hereafter be referred to as PUMA, to explore mechanisms of post-translational regulation of PUMA (Figure 1b) (4).

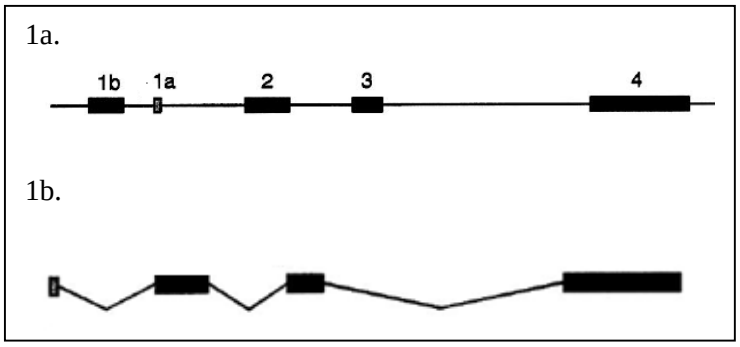


Figure 1 *PUMA* structure: **a**. Complete *PUMA* gene with exons 1a, 1b, 2, 3 and 4 shown **b**. The PUMAα variant of *PUMA* transcribed from exons 1a 2 3 and 4, consisting of 193 amino acids. Figure modified from Katsunori Nakano's paper (4).

p-53 Dependent and Independent Transcriptional Activation of PUMA

PUMA is expressed in all cell types at moderate levels which rapidly increase anywhere from three to ten fold in the presence of cellular stress to prevent dissemination of

damaged cells (5,6,7). The p53 tumor suppressor, known for regulating the cell cycle and cell death pathways, is one of the best understood transcriptional regulators of PUMA in response to DNA damage or UV radiation. P53 initiates apoptosis by interacting with PUMA via the BS2 reporter, located 144bp upstream of PUMA's transcription start site, increasing reporter expression 400-fold within hours of DNA damage (5, 8).

PUMA can also be upregulated independently of p53 in response to genotoxic stress by transcription factors including p73, FOXO3a as well as ER stress, hypoxia and IL-3 cytokine or growth factor deprivation (9). C-Myc, a proto-oncoprotein known to activate p53 expression, is capable of directly upregulating PUMA in the absence of p53 by binding one of several high-affinity sites along *PUMA* (1). The net effect of increasing PUMA expression in the cell is the initiation of cell death pathways be either apoptosis or autophagocytosis (1).

How PUMA Mediates Apoptosis through the BH3 Domain

The most conserved feature of all B-cell lymphoma-2 (Bcl-2) proteins is the binding homology (BH) domain which functions as a gatekeeper in mitochondrial-induced apoptosis and autophagocytosis (10). Four differing forms of BH domains exist, BH1 -2 -3 and -4, with the BH3 domain found most commonly in pro-apoptotic proteins (10). The BH3 domain of PUMA, spanning amino acids 137-150, was proven to be critical to its apoptotic function when PUMA-ΔBH3 expression vectors were over expressed in HeLa cells resulting in a complete loss of PUMA driven apoptosis (Figure 3) (2).

Apoptosis is a means of inducing cell death activated by genotoxic stress (10). The ability of cells to initiate apoptosis is a critical tumor suppressive function which is lost in most cancer cells (11). PUMA is one of the most potent mediators of apoptosis which drives cell death by modifying mitochondrial outer membrane permeablization (10). Overexpression of PUMA induces apoptosis by competitively binding homologous BH3 domains found on anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-X_L (12). This interaction sequesters

apoptotic inhibitors, Bcl-2 and Bcl-X_L, and displaces pro-apoptotic Bcl-2 proteins Bax (Bcl-2 Associated protein x) and Bak (Bcl-2 Antagonist killer) (10,1). Bax and Bak oligomerize and form pores in the mitochondrial outer membrane leading to the loss of cytochrome C (cyt-C) into the cytosol. Once cyt-C interacts with APAF-1 (Apoptotic Protease Activating Factor-1), the caspase cascade will be activated resulting in cell death (3).

PUMA Mediated Autophagocytosis as an Alternative Cell Death Pathway

Autophagy is a catabolic process consisting of nonspecific degradation of cellular components that can either promote short term cell survival under conditions of nutrient depravation or lead to cell death in cells exposed to genotoxic stress. Autophagy normally occurs at constant low levels in all cells to maintain cellular energy and recycle components of the cell which are damaged or no longer needed (10,13). It is thought that autophagy plays a tumor suppressive role by degrading malfunctioning organelles, removing toxic chemicals, or reducing the rate of genetic mutation (14).

PUMA mediated autophagy is initiated when PUMA expression increases in response to cellular stress signals. The increased concentration of PUMA leads to competitive binding with the BH3 domains of anti-apoptotic Bcl-2 proteins Bcl-2 and Bcl-X_L allowing for the release of Beclin-1, a BH3-only protein required for the initiation of apoptosis or autophagocytosis (10,13). The release and activation of Beclin-1 initiates the formation of an isolation membrane that will be elongated to form a circularized autophagosome upon stimulation by autophagy-related proteins (Atg-7, Atg-5) (15,16). During elongation a soluble microtubule-associated protein, one light chain three (LC3-I), is converted into a phosphatidylethanolamine-conjugated lipated form (LC3-II) which remains associated with the autophagosome until degradation. The conversion of LC3-I to LC3-II is commonly used to quantify autophagosome development within a cell as an indicator of autophagocytic activity.

Finally the autophagosome, containing cellular components, will fuse with a lysosome and is degraded by acid hydrolysis (10).

Suppression of autophagy is a common feature of cancerous cells. In their early stages cancer cells have been shown to decrease autophagy by suppressing or deleting Beclin-1 (14). As tumors mature in the presence of anti-cancer drugs, the cells up-regulate autophagy as a protective function to cope with the increased toxicity and cellular starvation (14). By being able to directly up-regulate the expression of Beclin-1, PUMA could potentially be used to mediate these effects in cancer cells.

Pathways Facilitating Communication between Apoptosis and Autophagy

There has recently been a great interest in understanding the overlap between apoptosis and autophagocytosis pathways mediated by BH3 proteins such as PUMA. PUMA is able to initiate autophagy and apoptosis by competitively binding inhibitor proteins such as Bcl-2 and Bcl-X_L to free Bax/Bak, initiating apoptosis, or Beclin-1, initiating autophagocytosis. Atg-5, a component of the autophagocytosis pathway, can also become activated by calpain cleavage allowing Atg-5 to bind Bcl-X_L and initiate the caspase cascade by releasing Cyt-C from the mitochondria (Figure 2) (10).

The factors which dictate which pathway will be initiated are still unclear, however there is evidence that cells with compromised apoptosis initiation will upregulate autophagocytosis as an alternative means of inducing cell death, while cells with defective autophagy pathways will have upregulated apoptosis function (10,17). In 2010 Hai-Yan et al discovered that autophagy can serve as an alternative pathway for promoting cell death in cancer cells that have adapted the ability to override signals initiating the apoptosis pathway (11). While mild levels of autophagy resulted in prolonged short term cell survival, cells incapable of initiating apoptosis have demonstrated massive increases in autophagosome development leading to cell death (10,11).

In 2014 a study by J Thorburn and colleagues showed that autophagy is also capable of modulating the rate by which apoptosis occurs through the activation of PUMA. Thorburn found that p62-dependent autophagy resulted in reduced levels of PUMA expression and desensitized cells to apoptosis initiation. She also demonstrated that autophagy can delay apoptotic cell death by interacting with factors that affect the efficiency of MOMP (18).

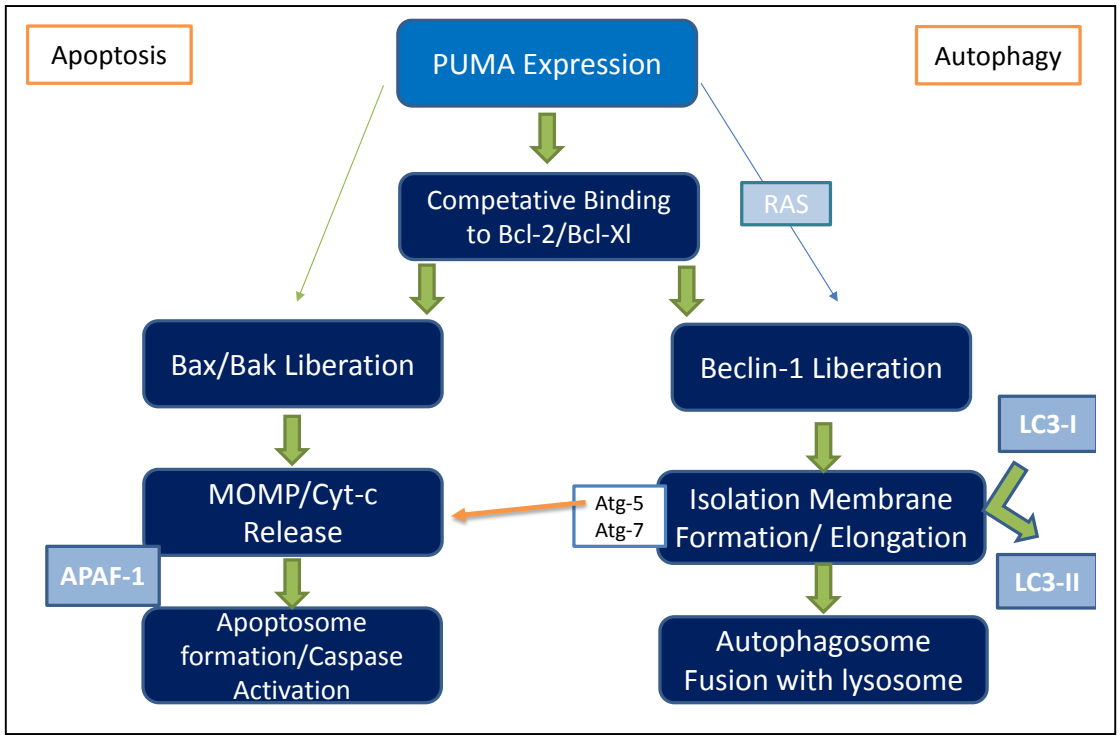


Figure 2 Simplified Overview of PUMA Mediated Crosstalk between Autophagy and Apoptosis Pathways

Post-Translational Regulation of PUMA

By conducting immunoprecipitation assays using radioactively labeled ^{32}P-orthophosphate Fricker and colleagues discovered that PUMA was post-translationally regulated by the phosphorylation of select amino acid residues (2). Further investigation using 1D-TLC (one-dimensional thin-layered chromatography) revealed eleven serine residues

within PUMA as potential phosphorylation sites (Figure 3). Serine to alanine non-phosphorylation point mutants were made at each serine position to pinpoint which residues were the sites of phosphorylation. The mutant expression vectors were over expressed in HeLa cells labeled with ^{32}P-orthosphosphte and analyzed by protein immunoblotting revealing three serine residues which were being phosphorylated, Serine-10, -96 and -106 (2).

Phosphorylation was shown by the western blot as two bands, a stronger lower band which was the result of serine-10 phosphorylation and a more subtle upper band which was a result of serine- 96 and -106 phosphorylation (2). This data led to the conclusion that serine-10 was the primary site of phosphorylation in PUMA and so Ficker et al. continued to study its regulatory function in post-translational regulation of PUMA. It is important to note that elimination of the upper phosphorylation band was only seen when serine-96 and -106 were simultaneously mutated in the PUMA S96,106A construct while results from individual S96A and S106A mutations were inconclusive (2).

1 MARARQEGS**S**PEPVEGLARDGPRPFPLGRLVPSAVSCGLCEPGLAAAPAA
51 PTLLPAAYLCAPTAPPAVTAALGGSRWPGGPRSRPRGPRPDGPQP**S**LSLA
101 EQHLE**S**PVPSAPGALAGGPTQAAPGVRGEEEQWAREIGAQ**LRRMADDLN**A
151 QYERRRQEEQQRHRPSPWRVLYNLIMGLLPLPRGHRAPEMEPN

Figure 3 PUMA amino acid sequence: The figure above shows the complete amino acid sequence of PUMAα with the three serine phosphorylation sites underlined, Serine 10, 96, and 106. The BH3 domain is also shown in bold between residues 140-149.

Fricker analyzed the protein stability of PUMA wt in comparison with the PUMA S10A mutant by overexpressing each construct in HeLa cells and treating them with cyclohexamide, an inhibitor of protein biosynthesis. Results showed that levels of PUMA in the S10A mutant cell line were much more stable than that of PUMA wt (2). Later it was shown that the S10A mutant expressing cells underwent apoptosis more frequently than cell lines expressing PUMA wt as a consequence of its increased protein stability. This study

concluded that the phosphorylation of serine-10 regulated proteasomal degradation of PUMA and promoted cell survival by suppressing apoptosis as a result of decreased protein stability (2).

Next Sandow and colleagues identified the stimuli and kinase that regulated serine-10 phosphorylation. Phosphorylation was induced in FDM (factor-dependent myeloid cells) cells by IL-3 (interleukin-3) stimulation in the presence of a variety of kinase inhibitors identifying IKK1 (IκB Kinase-1) as the kinase responsible for serine-10 phosphorylation (19). A co-immunoprecipitation assay showed IKK1 is capable of directly binding and phosphorylating overexpressed PUMA (19). This study identified IL-3 as a stimulus for PUMA serine-10 phosphorylation by IKK1 and provided more evidence to Fricker's finding that phosphorylation of PUMA regulated protein activity by targeting it for degradation.

In 2013, PUMA was found to also be post-transcriptionally regulated by the phosphorylation of tyrosine residues mediated by HER2. HER2 is overexpressed in 20% of all incidents of breast cancer and can be permissive to breast cancer development by decreasing cellular apoptosis. Western blot/ immunoprecipitation assays (WB/IP), using breast cancer cell lines SK-BR3 and BT-474, showed that overexpressed HER2 interacted with PUMA (20). Further analysis using WB/IP and site-directed mutagenesis eliminating phosphorylation revealed that HER2 directly phosphorylates three tyrosine residues, Y-58 -152 and -172 (20). A protein stability assay using the MDA-MB-453 breast cancer cell line treated with cyclohexamide over a period of 16 hours revealed that phosphorylation of PUMA by HER2 destabilizes the PUMA protein. HER2 is able to promote the growth and survival of breast tumors by increasing PUMA phosphorylation and evading apoptotic pathways necessary to target tumor cell proliferation (20). This result is consistent with the discovery made by Fricker which demonstrated that the phosphorylation of PUMA leads to protein destabilization and a reduction in apoptotic activity.

The involvement of PUMA in multiple cell death pathways makes it a powerful tool in cancer treatment. Most anti-cancer therapies work by inducing large amounts of DNA damage in hopes of activating tumor suppressors, such as p53, which will respond by initiating apoptosis. One of the biggest challenges limiting the success of such treatments is that more than 50% of all cancer cells have acquired mutations in p53 or have degraded the proteins necessary to initiate cell death pathways, including p53 (21,22). In other cases cancer cells may become resistant to chemical treatments by pumping out or degrading any toxic chemicals they take up by autophagocytosis (21). We could resensitise cells which have developed the ability to remove toxic agents or block apoptotic pathways by using PUMA to promote cell death and proliferation.

Despite thorough characterization of the S-10 phosphorylation site and its impact on PUMA function, little is understood about the regulatory function of S-96 and S-106. The aim of this study is to gain a better understanding of how the S-96 and S-106 phosphoryaltion sites contribute in the post-translational regulation of PUMA. We will achieve this goal by first developing PUMA wt and PUMA S96,106A point mutation expression vectors eliminating phosphorylation at these positions. Next, these expression vectors will be used to test for functional changes in PUMA driven apoptosis, autophagocytosis, and protein stability. Understanding how PUMA is post-translationally regulated will make it more possible to use PUMA successfully to improve cancer treatment.

Materials and Methods

Generating PUMA wt and PUMA S96,106A Constructs

For the purpose of this study, we generated a PUMA mutant construct with point mutations substituting serine to alanine at amino acid positions 96 and 106 (S96,106A). PUMA wt and S96,106A were cloned into mammalian expression vectors, pcDNA3 (Invitrogen™) pmCherryN1 (Clontech, Inc.) (Figures 4a-b) and FUY-tetO-hMYC (Addgene) (Figure 5a). The tetO vector was used to create an inducible cell line (explained later in this section), while pmCherryN1 and pcDNA3 were used to generate cell lines which overexpressed PUMA wt and PUMA S96,106A. Wild-type cell lines were made to serve as a positive control for PUMA function.

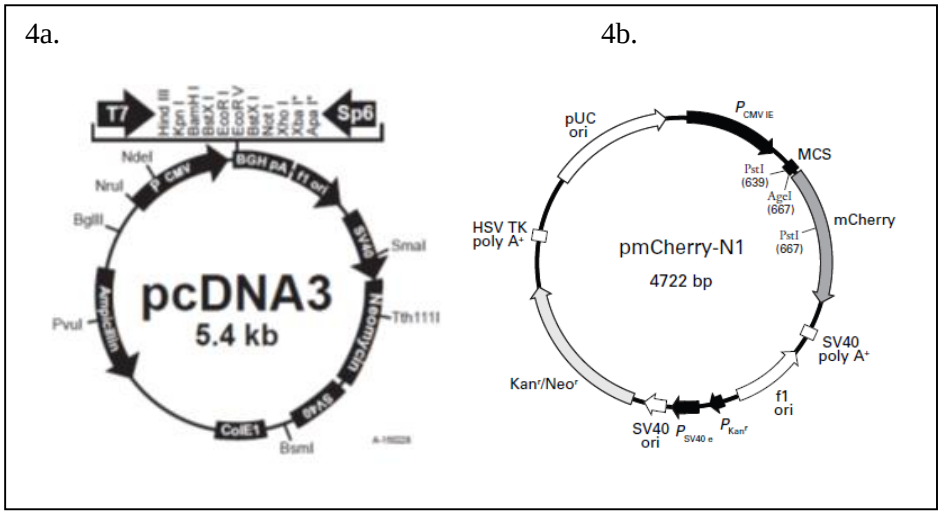


Figure 4 Plasmids used to make PUMA wt and PUMA S96, 106A expression constructs: **a.** pcDNA3 (Invitrogen™) **b.** pmCherry-N1 (Clontech, Inc.).

Wild-type PUMA construct were generated using primers 5'-tataagcttatggcccgcgcacgccag -3'(F) and 5' -attgggctccatctcggggg -3'(R) for the pcDNA3 constructs and 5'-tataagcttatggcccgcgcacgccag -3' (F) and 5' -ttaggatccggattgggctccatctcggggg -3' (R) for the pmCherryN1 constructs shown in Table 1. To create the PUMA S96,106A

mutant construct a similar protocol was followed with the addition an additional overlap PCR amplification. The primers used to create the serine to alanine point mutations were 5'-cagccc**TCG**ctctcgctggcggagcagcacctggag**TCG**cccgtg-3' (F) and 5' -cacgggcgactccaggtgctgctccgccagcgagagcgagggctg- 3' (R) with the new alanine codons shown in bold at positions 96 and 106 (Table 1).

After PCR amplification we used a QIAquick Gel Extraction Kit (QIAGEN), following the manufacturer's protocol, to purify the DNA products. A restriction digest using BamHI and HindIII restriction endonucleases (New England BioLabs, Inc.), for pmCherryN1 and pcDNA3 constructs, and a second DNA purification by gel extraction was done in order to isolate our insert. Each insert was ligated into the pcDNA3 and pmCherryN1 vectors using Quick T4 DNA Ligase (New England BioLabs Inc).

The ligated vector insert constructs were transformed into DH5-alpha E. coli cells, a competent cell line designed to generate high plasmid yield. The transformed DH5-alpha cells were grown overnight at 37°C on LB Agar plates treated with antibiotics, Kanamycin and Ampicillin, to select for colonies containing the desired plasmid. After 24 hours DNA was extracted from colonies that had formed. Extracted DNA was digested using BamHI and HindIII and fragments were separated by gel electrophoresis using a 1% agarose gel to verify the presence of our PUMA wt and S96,106A. Finally, colonies containing a plasmid were sequenced by Eton Bioscience, Inc. to confirm its sequence.

In addition to the mutant and wild-type constructs pcDNA3 and pmCherryN1 vectors were also transformed into DH5-alpha cells so that they could be used as a negative control in our experiments. Colonies with the desired expression constructs were stored at -80°C in 30% glycerol.

Table 1 List of primers used as stated in the Materials and Methods:

Primer Name	Primer Sequence
3xFlagpcDNA3- wt sense	5'-tataagcttatggcccgcgcacgccag -3'
3xFlagpcDNA3- wt antisense	5' -attgggctccatctcggggg -3'
mCherryN1 - wt sense	5'-tataagcttatggcccgcgcacgccag -3'
mCherryN1 - wt antisense	5' -ttaggatccggattgggctccatctcggggg -3'
S96,106A sense	5'-cagccc**TCG**ctctcgctggcggagcagcacctggag**TCG**cccgtg-3'
S96,106A antisense	5'-cacggg**CGA**ctccaggtgctgctccgccagcgagag**CGA**gggctg-3'

Cell Culture

HEK 293 cells, derived from human embryonic kidney, are a commonly used as an expression model for their ease of reproduction, maintenance, reliable protein production, and high transfection efficiency. 293 cells are especially advantageous when transfecting vectors under the control of a constitutive mammalian promoter such as cytomegalovirus (CMV) (23). Upon transfection with a CMV driven vector, such as pcDNA3 and pmCherryN1, the cellular machinery strongly promotes translation of plasmid genes (23).

All cells were cultured in DMEM (Gibco®) with 1% pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS) (Gibco®) filtered through a 0.1 µm vacuum filter. Cell culture media was kept refrigerated at 4°C and all cells were incubated at 37°C in 5% CO_2.

Generating stable cell lines

Stable cell lines were generated, using HEK 293 cells, which continuously overexpressed pmCherryN1 and pcDNA3 cell lines. 4µg of DNA was transfected into HEK 293 cells split in a 6-well tissue culture dish using Lipofectamine® 2000 (Life Technologies) transfection agent. After 48 hours of incubation cell populations were diluted 1:10 followed by cell selection using 3ug/ml G-418 aminoglycoside antibiotic treatment.

Once the selection process was complete, a colony was selected from each cell line and grown in a 96-well tissue culture dish using DMEM with 1% pyruvate, 1% nonessential

amino acids, 1% penicillin-streptomycin, and 10% fetal bovine serum. Stable cell lines were stored in freeze medium containing 10% DMSO and 90% FBS at -80°C.

Transient Transfection and Apoptosis Assay

To measure apoptosis by flow cytometry, DNA from the pmCherryN1 constructs, pmCherryN1-PUMA wt, pmCherryN1-PUMA S96,106A and pmCherryN1, were transiently transfected into HEK 293 cells. HEK 293 cells were split into a 24-well tissue culture dish at a cell density of $5x10^4$ cells per well. Following 24 hours of incubation the wells were replaced with fresh media two hours prior to transfection. Cells were transfected with a mixture of Opti-MEM™ (Gibco®), 2ug DNA and 4x PEI (Polyethylenimine) transfection reagent and incubated overnight.

Transfected cells were washed with cold PBS and resuspended in 1x Annexin V Binding Buffer (BD Pharmingen™). To ensure that all cells, including apoptosed cells, would be accounted for during FACS analysis, cells found in the supernatant were also pelleted and resuspended in Binding Buffer. 50µl of the cell resuspension was transferred to FACS tubes (BD Falcon™) in addition to 2µl of PE Annexin V (BD Pharmingen™) and 10µg DAPI (to a final concentration of 200ng/ml). Each sample was gently vortexted and incubated in the dark for 15 minutes at 25°C. Samples were analyzed by FACS analysis using the BD™ LSR II flow cytometer. During FACS analysis we gated for mCherry high to ensure that we were only measuring cell populations with comparable levels of PUMA expression.

Autophagosome Quantification by Flow Cytometry

Flow cytometry was used to quantify autophagosome development in stable cell lines expressing pmCherryN1-PUMA wt, pmCherryN1-PUMA S96,106A and pmCherryN1. Cell lines were split into a 24-well tissue culture dish at a density of $5x10^4$ cells per well. After 24 hour incubation, autophagic vacuoles were stained with 0.05mM/L Monodansylcadaverine

MDC (Sigma-Aldrich, Inc.) in DMEM at 37°C 5% CO_2 for 10 minutes. After incubation cells were washed with PBS, treated with Trypsin(Gibco®), and resuspended in 1ml PBS. Samples were analyzed immediately after by BD™ LSR II flow cytometer. The fluorescence of MDC was measured at UV configuration. Samples were gated for mCherry high to ensure that only cell populations with comparable levels of PUMA expression were being included in the analysis.

Confirming Autophagy Results by Western Blot

Data obtained by FACS analysis was confirmed by quantifying autophagosome development through the conversion of LC3-I to LC3-II by protein immunoblotting. For this assay we seeded pcDNA3-PUMA wt, pcDNA3-PUMA S96,106A and pcDNA3 cell lines in a 24-well tissue culture plate at a density of $5x10^4$ cells per well. PUMA wt and PUMA S96,106A pCDNA3 cell lines were tagged with a 3xFlag fusion protein to allow for detection of PUMA by western blot. After 24 hour incubation cells were harvested in 40µl 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.)

Samples were prepared for western blot by first sonicating each sample at 4°C for 10 seconds. Next, Samples were boiled in a sand bath at 96°C and centrifuged at 14,000x RPM at room temperature for ten minutes. 4µl of each prepared sample was loaded onto a 15% SDS PAGE gel and run at a current of 80V. The gel was transferred over night at 4°C onto a nitrocellulose membrane at 30V.

The transferred membrane was incubated with 5% Blocking Buffer (Bio-Rad Laboratories, Inc.) for 1 hour. Proteins were detected at room temperature by 1 hour incubation with Monoclonal Anti-LC3 antibody produced in rabbit (Sigma-Aldrich, Inc.) at a 1:1,000 dilution in 3% Bovine Serum Albumin BSA (Sigma-Aldrich, Inc.). The membrane was also incubated for one hour with Monoclonal Anti-α-Tubulin antibody as well as Monoclonal Anti-Flag M2 antibody produced in mouse (Sigma-Aldrich, Inc.) and diluted

1:10,000 in 3% BSA. After membranes were incubated with the primary antibody they were washed three times for five minutes each time with TBST and incubated with their respective secondary antibodies at a 1:10,000 dilution in 5% milk (Sigma-Aldrich, Inc.). Lastly, membranes were treated with DURA chemiluminescent substrate (Thermo Scientific). Membranes detected with LC3 primary antibody were exposed for 3 minutes while membrane detected with Flag and Tubulin were exposed for 30 seconds.

Creating Inducible Cell Lines and Quantifying GFP-LC3 Puncta

To create the inducible cell line, PUMA wt and PUMA S96,106A inserts were first cloned into the FUY-tetO-hMYC expression vector (Addgene) (Figure 5a). First, the c-MYC insert was removed by digestion with EcoRI followed by treatment with T4 DNA Polymerase in order to generate blunt ends and 50µg/ml CIP (Calf Intestine Phosphatase). Each insert was teated with T4 DNA Kinase and ligated into the tetO vector using Quick T4 DNA Ligase (New England BioLabs Inc).

The tetO vector was co-transfected with the FUW-M2rtTA vector into HEK 293 cells using the protocol outlined by Hockemeyer and colleagues (Figure 5b) (24). Treatment with 1µg/ml Doxycycline changes the conformation of the transcriptional activator, FUW-M2rtTA, causing it to bind the promoter of the TatO construct and transcribe our inserts within the cell. Finally, each 6-well plate was transiently transfected with 4µg/ml GFP-LC3 DNA and visualized by fluorescence microscopy 24 hours later.

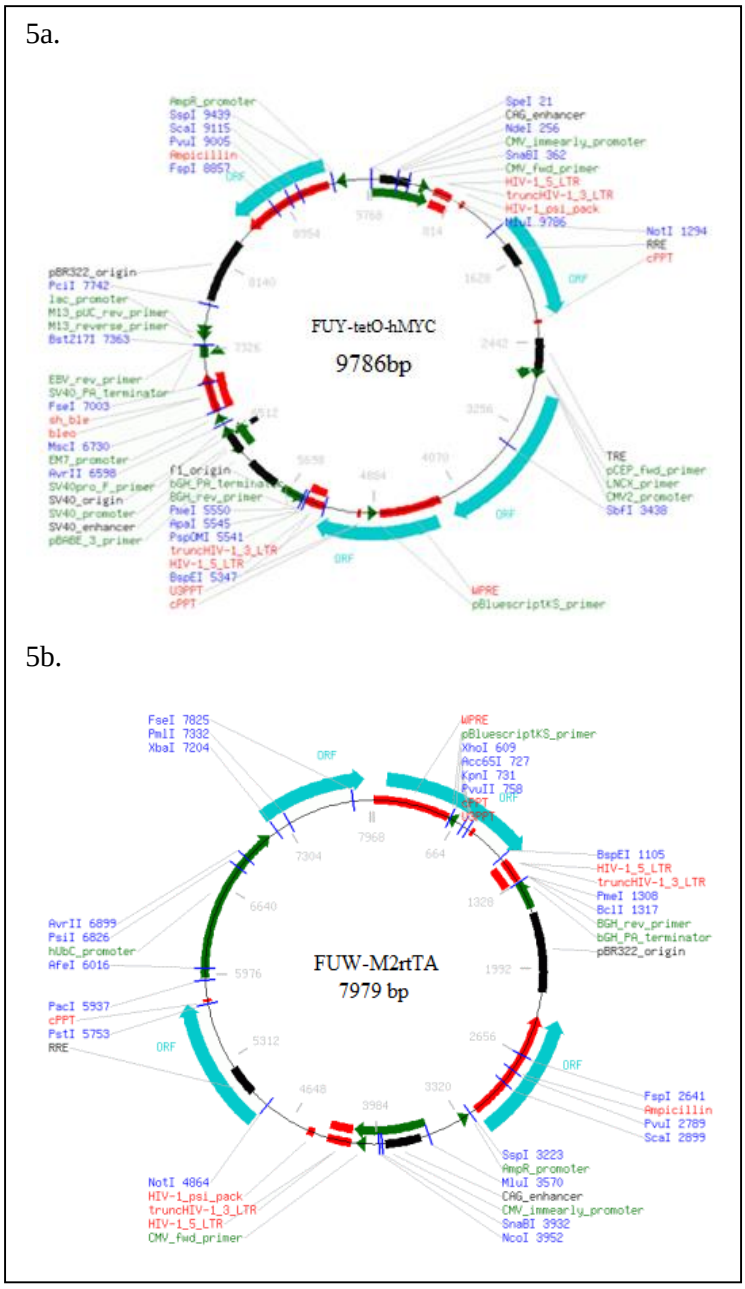


Figure 5 Inducible Cell Line Expression Vectors: **a**. FUY-tetO-hMYC **b**. FUW-M2rtTA

Protein Stability of PUMA wt and PUMA S96,106A Products

Differences in protein stability between PUMA wt and PUMA S96,106A were analyzed by protein immunoblotting analysis. To do this cells expressing 3xFlag tagged

pcDNA3-PUMA wt and pcDNA3-PUMA S96,106A were split into 4 wells of a 24-well tissue culture plate at equivalent cell densities. Once confluent, each well was treated with 20µg/ml CHX (cyclohexamide), an inhibitor of protein biosynthesis. To measure the changes in protein levels we collected samples at a range of time points beginning at 0hr, 3hr, 6hr and lastly 9hr.

To collect each sample we aspirated CHX containing medium, washed the cells with PBS, and suspended them in 35µl 2x Laemmli Sample Buffer (Bio-Rad). Preparation of samples and western blot protocol is exactly as described in the autophagy western blot assay. Once the membrane was transferred we incubated the membrane with Monoclonal Anti-α-Tubulin antibody as well as Monoclonal Flag M2 antibody produced in mouse (Sigma-Aldrich, Inc.) diluted 1:10,000 in 3% BSA. After incubation with secondary Anti-Mouse antibody, DURA substrate (Thermo Scientific) was used to visualize our proteins. Membranes incubated with Flag and Tubulin primary antibodies were exposed for 30 seconds.

Results

Confirming the Function of PUMA Expression Constructs and Cell Lines

Our first approach to understanding the regulatory role of Serine -96 and -106 phosphorylation sites in PUMA function was to generate constructs expressing wild-type and S-96,106 non-phosphorylation versions of PUMA. The PUMA mutant was created by generating two point mutations substituting serine-96 and -106 with alanine (S96,106A) thus eliminating phosphorylation at these positions. Each insert was cloned into three mammalian expression vectors, pmCherryN1, used for all flow cytometry assays, and 3xFlag-pcDNA3, used in all western blot assays, and FUY-tetO-hMYC, used to create inducible cell lines (Figure 4a,b & 5). pmCherryN1 and pcDNA3 constructs were used to generate stable and transient cell lines in HEK 293 cells which overexpressed PUMA wt and PUMA S96,106A. Before these cell lines could be used in our assays to study post-translational regulation, we needed to confirm the validity of our expression models by confirming that they expressed functional and complete copies of our constructs.

To confirm successful elimination of phosphorylation at positions S-96 and S-106 in the mutant construct, HEK 293 cells expressing pcDNA3-PUMA wt, pcDNA3- S96,106A and pcDNA3 were harvested from a 24-well tissue culture dish using 40µl 2x Laemmli Sample Buffer per well (Bio-Rad). Each sample was prepared for western blot (protocol outlined in the Materials and Methods section) and run on a 15% SDS PAGE gel. Proteins were detected using Monoclonal Anti-FLAG antibody as well as Monoclonal Anti-α-Tubulin (Sigma-Aldrich, Inc.) followed by incubation with mouse secondary antibody. Based on the understanding that phosphorylated proteins migrate slower than non-phosphorylated proteins due to a slightly higher molecular weight, if PUMA S96,106A migrates further than PUMA wild-type we can confirm a loss of phosphorylation at the mutated residues (25).

The results show that PUMA wt has migrated to 23 kDa, as expected, while PUMA S96,106A has migrated slightly further to about 21kDa, confirming the elimination of phosphorylation at residues S-96 and S-106 in PUMA S96,106A (Figure 6). We also did not detect any Flag expression in the pcDNA3 control which tells us that the Flag expression detected in PUMA wild-type and mutant samples was a valid way to detect PUMA expression and not an inherent property of the vectors we were using.

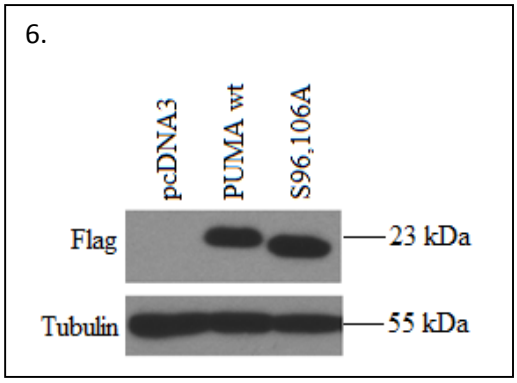


Figure 6 Confirming elimination of S96 and S106 phosphorylation: In this western blot PUMA S96,106A migrated further than the wild-type sample indicating a loss of phosphorylation at the mutated alanine -96 and -106 residues. The absence of a Flag band under the pcDNA3 control sample lane confirms that detection of Flag is associated with PUMA expression and not the pcDNA3 vector alone.

Next, HEK 293 cells were transiently transfected with pmCherryN1-PUMA wt and S96,106A in order to confirm that the cell lines expressed a complete and functional copy of PUMA wt and S96,106A. Given that PUMA has a Mitochondrial Localization Signal (MLS) at its C-terminus, successful cloning of the entire PUMA gene means that PUMA proteins should be found in the mitochondria of all transfected cells.

To determine whether or not the constructs used express complete and functional copies of PUMA wt and S96,106A, fluorescence microscopy was used to visualize pmCherryN1 expression and localization in the cell. To study this, a 6-well tissue culture dish

was seeded with stable HEK 293 cell lines expressing pmCherryN1 PUMA wt, S96,106A, and pmCherryN1 at a density of $3x10^5$ cells per well and incubated them over night. Prior to visualization cells were stained with 200pM MitoTracker Green FM (Life Technologies), a green fluorescent mitochondrial stain, diluted in 2ml media and incubated for 15 minutes at 37°C in 5%CO_2. Using a mitochondrial stain allows us to detect colocalization between mCherry red fluorescing proteins and the green fluorescing MitoTracker. Once staining was complete the medium in each well was replaced with PBS and the samples were visualized using a fluorescence microscope.

When visualizing the control pmCherryN1 cell line, which did not express any form of PUMA, there was a homogeneous distribution of mCherry fluorescence throughout the cell (Figure 7a). We also did not detect any colocalization between mCherry red in the control cell line with the MitoTracker green fluorescence indicating that the pmCherryN1 vector did not localize in the mitochondria independently of PUMA. When visualizing the pmCherryN1 PUMA wt and S96,106A cell lines there was a dark circular clearing at the center of the cell which is expected to be the nucleus (Figure 7b, c). We also detected colocalization of mCherry and MitoTracker fluorescence indicating that PUMA wt and S96,106A proteins were localized in the mitochondria. From these data we can conclude that the complete PUMA and PUMA mutant proteins were successfully cloned into our expression constructs, including the MLS signal, and these constructs are being expressed in our transfected cell lines. In addition, we can say that the point mutations created at serine-96 and -106 do not change PUMA's ability to localize in the mitochondria as it demonstrated a similar localization pattern as PUMA wt.

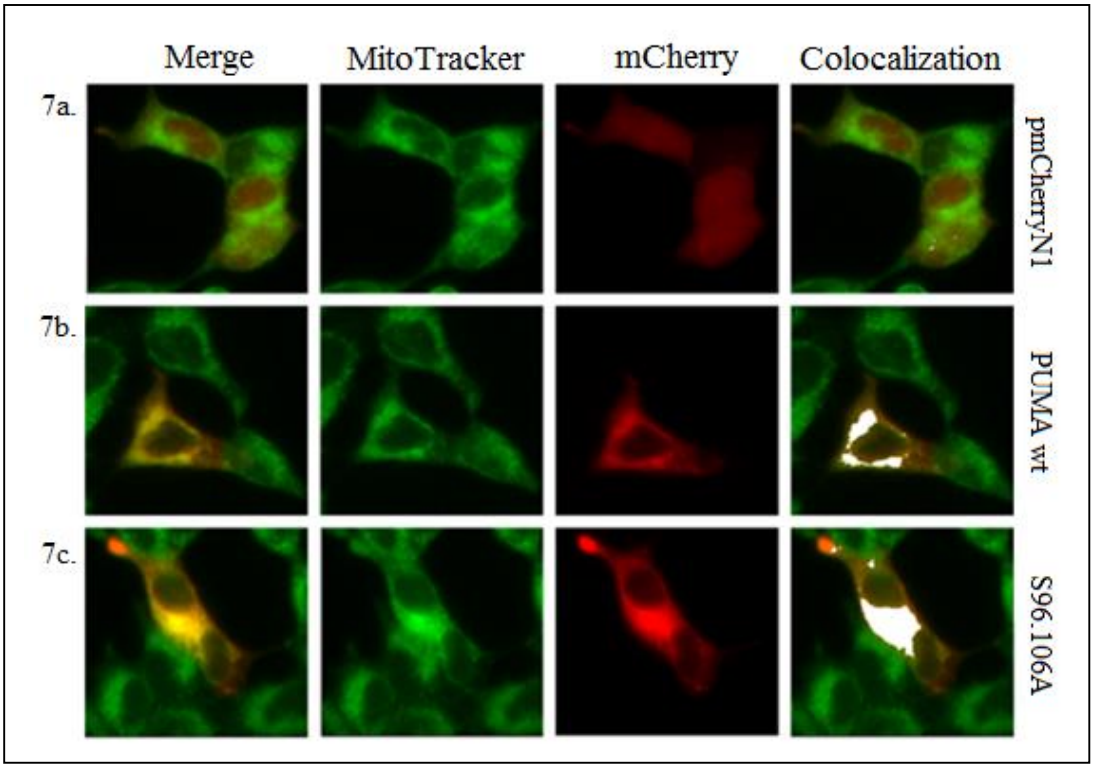


Figure 7 Confirming PUMA function in expression models: **a**. Fluorescence images of control cell line pmCherryN1, showing that mCherry fluorescence is distributed evenly throughout the cell and is not co-localized within the mitochondria. **b**. Images of PUMA wt and **c**. PUMA S96,106A pmCherryN1 cell lines demonstrate similar patterns of mitochondrial localization. The yellow overlap region under 'Merge' illustrates areas of co-localization between the MitoTracker and mCherry fluorescence. Co-localization is also confirmed in the last column where the white patch seen is indicative of PUMA mCherry fluorescence overlapping with MitoTracker Green.

Characterizing the Function of S96 and S106 Phosphorylation Sites in Apoptosis

After establishing our constructs we sought out to determine whether or not phosphorylation sites S-96 and S-106 contribute to PUMA mediated apoptosis using FACS analysis. Since its discovery, overexpression of PUMA has been associated with increased cellular apoptosis and the regulatory mechanisms behind this process are slowly being understood (1,2,3). As mentioned earlier, studies done by Fricker and colleagues have shown that phosphorylation of serine-10 decreases apoptosis by promoting PUMA protein degradation (2).

To quantify cell death HEK 293 cells were split into a 24-well tissue culture dish amd transiently transfected with 2µg of DNA, including pmCherryN1-PUMA wt, pmCherryN1-PUMA S96,106A and pmCherryN1. After 24 hours cells were resuspended in 1X AnnexinV Binding Buffer (BD Pharmingen™) and 50µl of each sample was incubated with 2µl of PE AnnexinV (BD Pharmingen™) and 10µg DAPI (4',6-diamidino-2-phenylindole) in the dark for ten minutes. AnnexinV staining was used to detect cellular markers of apoptosis, including Phosphatidylserine (PS) and Phosphatidylethanolamine (PE), while DAPI was used to distinguish between early and late apoptosis. By quantifying cells double positive for AnnexinV and DAPI the relative amount of apoptosis in each sample could be determined. Lastly, all samples were gated for mCherry high expression to ensure that all cells being counted had comparable levels of PUMA expression.

Results, obtained from three individual samples, showed a fivefold increase in apoptosis in the PUMA wt sample when compared to the pmCherryN1 control, confirming that overexpression of PUMA in our constructs accurately modeled the expected role of PUMA function in apoptosis. The data also show that there was no significant difference in apoptosis levels when comparing cell lines overexpressing PUMA wt and PUMA S96,106A (Figure 8). Both wild-type and mutant constructs showed a significant increase in apoptosis when compared to the control. With these data we can say that the PUMA S96,106A phosphorylation mutant is demonstrating similar apoptotic function to that of PUMA wt. Moreover, we conclude that since the removal of S-96 and S-106 phosphorylation did not impact apoptosis, these phosphorylation sites are not involved in regulating PUMA mediated apoptosis.

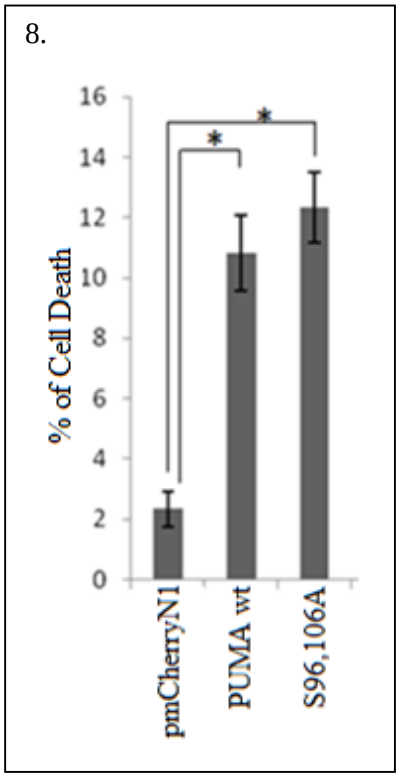


Figure 8 Quantification of apoptosis by FACS analysis: The results from three individual samples (n=3) revealing an insignificant difference in apoptosis in cell lines overexpressing PUMA wt and PUMA S96,106A (p=0.068). Both wild-type and mutant cell lines demonstrated a significant increase in apoptosis when compared to the pmCherryN1 control cell line (p=0.0095 for the wild-type sample) indicating that apoptosis was not effected by the elimination of serine phosphorylation at positions S-96 and S-106.

Characterizing the Function of S96 and S106 Phosphorylation Sites in Autophagocytosis

Next we were curious to see if the serine-96 and -106 phosphorylation sites regulated PUMA mediated autophagy. It is known from previous research that upregulation of PUMA results in an increase in cellular autophagy, however what regulated PUMA to carry out this function is yet to be understood (10,25). To explore this question we used pmCherryN1-PUMA wt, pmCherryN1-PUMA S96,106A and pmCherryN1 stable cell lines to quantify autophagy by FACS analysis.

To prepare samples for FACS analysis pmCherryN1-PUMA wt, pmCherryN1-PUMA S96,106A and pmCherryN1 cell lines were split into a 24-well tissue culture dish. After a 24 hour incubation, cells were stained with 0.05mM/L Monodansylcadaverine MDC (Sigma-Aldrich, Inc.), a dye that labels autophagic vacuoles, in DMEM at 37°C 5% CO_2 for 15 minutes. (26). After staining, each well was treated with Trypsin (Gibco®) and resuspended in 1ml PBS. During FACS each sample was gated for mCherry high to ensure that we were only measuring cell populations with comparable levels of PUMA expression. This process was repeated using six individual samples.

Results indicate that autophagy in PUMA wt cells was significantly higher in comparison with the pmCherryN1 control, confirming that the expression model function was consistent with what is already known about PUMA. When comparing the S96,106A cell line with PUMA wt, there was a significant decrease in the number of autophagosomes observed in the S96,106A sample (Figure 9). It is important to note that there was no significant difference in autophagy when comparing the pmCherryN1 control with PUMA S96,106A. In conclusion, these data show that the serine-96 and -106 phosphorylation sites play a role in regulating PUMA's function in autophagy.

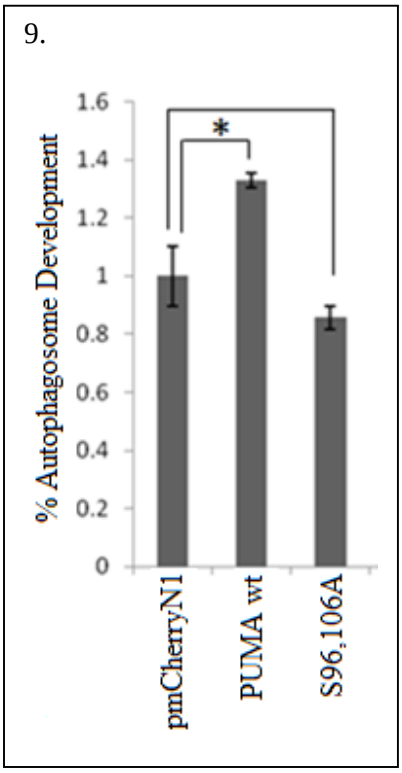


Figure 9 Quantification of autophagy by FACS analysis: Data averaged from 6 individual samples (n=6) show a significant increase in autophagy in PUMA wt when compared wo the control (p=.0003) and mutant (p=.0002) cell lines. The data also demonstrates that there is no significant difference between the S96,106A mutant in comparison with the control sample (p=0.8). These results show that the PUMA serine-96 and -106 residues are involved in the regulation of PUMA mediated autophagy.

To confirm our results we did a western blot to track the conversion of LC3-I to LC3-II as a way to quantify autophagosome formation. During the initiation of autophagy Atg-7 causes soluble LC3-I to embed in the membrane of the developing autophagosome. As autophagy progresses LC3-I becomes converted into its lipated form LC3-II, critical to the autophagosome lipidation process (27). The conversion of LC3-I to LC3-II was measured using the 3xFLag-pcDNA3 stable cell lines, PUMA wt and PUMA S96,106A. Each sample was collected in 40µl 2X Laemmli Sample Buffer and prepared for western blot following the

procedures described by the Materials and Methods section. 4µl of each sample was loaded on a 15% gel and run in room temperature at 80V.

The conversion of LC3-I, 17kDa, to LC3-II, 13kDa, was detected using the Anti-LC3 antibody (Sigma-Aldrich, Inc.) at a 1:1,000 dilution in 3% BSA. We then measured the band intensities using ImageJ to obtain a ratio of LC3-II:LC3-I to determine which sample had increased autophagosome development when compared with the control pcDNA3 sample. Samples with higher ratios are indicative of increased autophagosome development and, as a result, increased levels of autophagy.

The data shown in Figure 10a show a higher conversion of LC3-I to LC3-II in the PUMA wt sample than the PUMA mutant and control samples indicated by the darker lower band in the middle lane (Figure 10a). Data collected from three different samples were collected and LC3-II:LC3-I ratios were normalized to 1 by dividing each sample by ratio taken from the control. The average of data collected from all three samples resulted in a 2.5 fold increase in LC3-I to LC3-II conversion in PUMA wt in comparison with the control and mutant samples (Figure 10b). We also report no difference in the LC3-I to LC3-II ratios between the PUMA mutant and control samples confirming our previous finding that the serine-96 and -106 phosphorylation sites participate in regulating PUMA driven autophagy and elimination of these phosphorylation sites results in a loss of PUMA's capacity to upregulate autophagy.

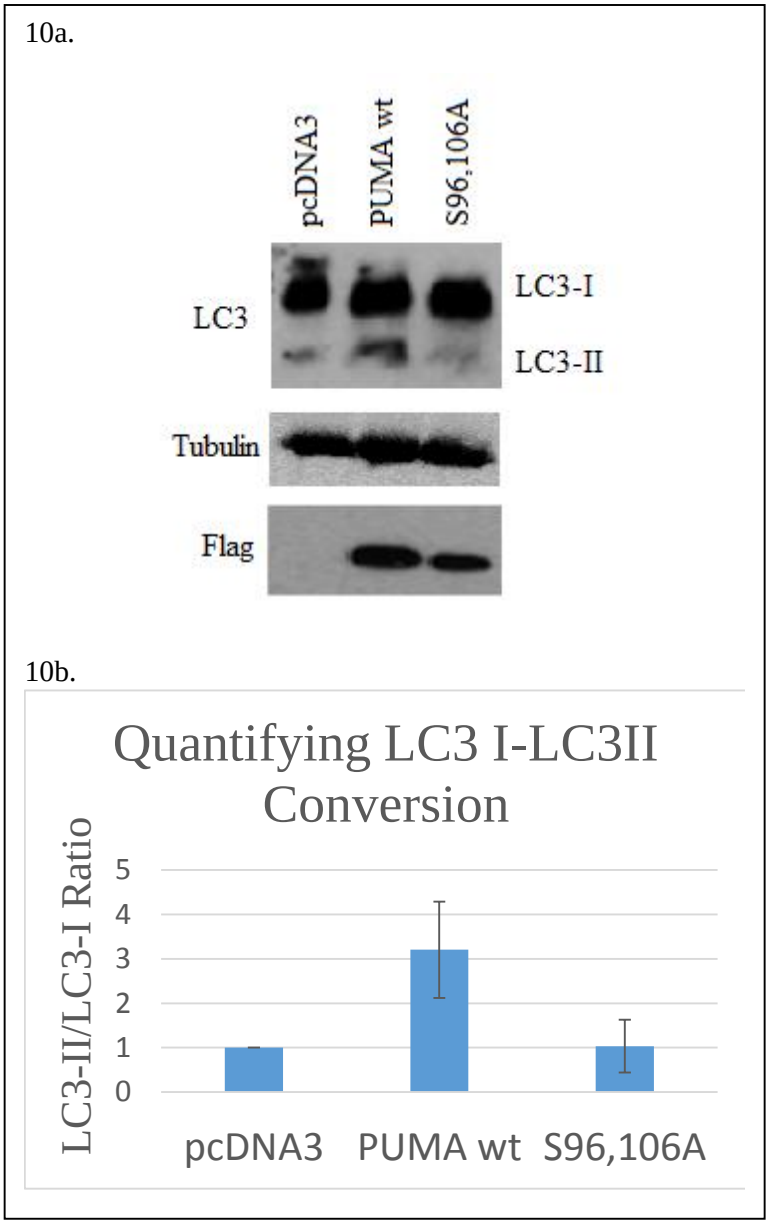


Figure 10. Detecting conversion of LC3-I to LC3-II in western blot analysis of autophagy: **a**. The first membrane was incubated with Anti-LC3 antibody and shows the conversion of LC3-I (upper band) to LC3-II (lower band). The increased LC3-II band intensity for the PUMA wt sample and not in the control or mutant samples is an indication that the wild-type sample underwent autophagy more frequently. The third membrane in this figure is a control detected by Anti-Flag antibody confirming PUMA expression in each sample. The second blot in this section is a tubulin loading control. **b**. These values represent the average ratio of LC3-II/LC3-I quantified by ImageJ. In the third row we calculated the LC3-II/LC3-I ratios to quantify autophagy in each sample. We show that PUMA wt had the highest ratio while the mutant sample did not differ much from the control consistent with our conclusion that the S-96,106 phosphorylation sites affect PUMA mediated autophagy.

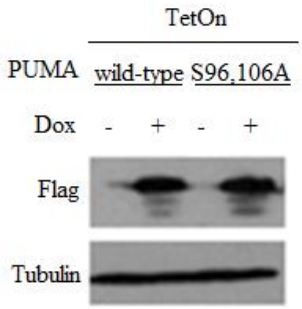
To test these results in a different expression model, inducible cell lines were used to quantify GFP-LC3 puncta using fluorescence microscopy. Counting the number of GFP-LC3-II puncta is a common method used to quantify autophagy (28). To create the inducible cell lines, HEK 293 cells were transfected with two vectors, FUY-tetO-hMYC, expressing 3xFlag tagged PUMA wt and PUMA S96,106A, and FUW-M2rtTA, a transcriptional activator (protocol outlined in the Materials and Methods section). Upon treatment with 1µg/ml Doxycyclin, the M2rtTA vector will change conformation and induce the transcription of our inserts in the tetO vetor to achieve overexpression of our desired gene products in each cell line (Figure 5).

Before using them in functional assays, PUMA wt and S96,106A expression in the inducible cell lines was confirmed by western blot analysis. We collected samples from induced and non-induced wild-type and mutant cell lines and used them to detect Flag expression (Figure 11a). In the non-induced cell lines PUMA expression was not detected, while the induced cell lines showed clear bands at about 23 kDa indicating expression of PUMA wt and S96,106A in each cell line.

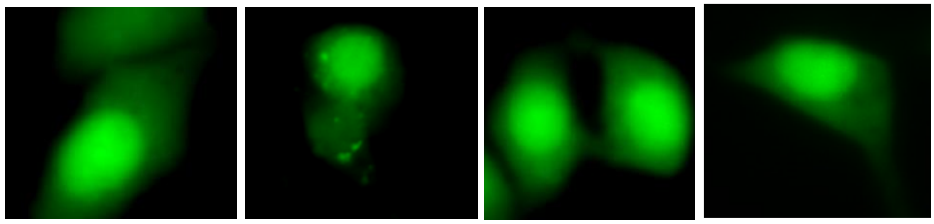
Next, the inducible cell lines were transfected with 2 µg/ml GFP-LC3 into a 6-well tissue culture plate and incubated the samples overnight at 37°C in 5% CO_2. 24 hours later we replaced the cell culture media with PBS and visualized each sample using a fluorescence microscope. By randomly selecting 25 cells from each sample and counting the LC3 puncta in each of them, when looking at the uninduced samples, we counted 6 puncta in the PUMA wt sample and 3 in the PUMA S96,106A samples (Figures11b & d). After inducing protein expression by treatment with Doxycycline 22 puncta were counted in the PUMA wt sample and 5 puncta in the PUMA S96,106A sample (Figures (11c & e). These data are consistent with previous results revealing that phosphorylation of serine -96 and -106 is involved in the regulation of PUMA's activity in autophagocytosis. The scatter plot shorn in figure 11f is a

representation of the number of puncta counted from 25 cells randomly selected from each inducible cell line.

11a.



11b 11c. 11d 11e



11f.

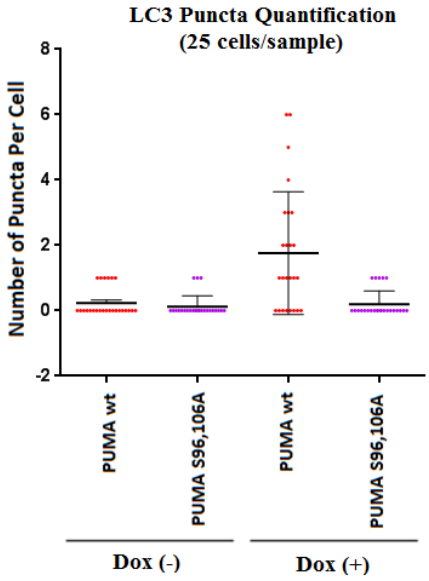


Figure 11 Detecting LC3 puncta in inducible cell lines: **a.** Western blot done to confirm PUMA expression in the inducible cell lines. **b**. No treatment PUMA wt and **d.** No treatment PUMA S96,106A show few GFP-LC3 puncta when visualized by fluorescence microscopy. **c.** Doxycycline induced PUMA wt cell lines showed a drastic increase in visible GFP-LC3 puncta **e.** Doxycycline induced PUMA S96,106A showed no difference in the development of puncta from the uninduced cell lines. **f.** Scatter plot showing overall number of puncta counted in each cell. A total of 25 cell were analyzed in each induced and uninduced cell line.

The Role of Serine -96 and -106 Phosphorylation Sites in PUMA Protein Stability

Lastly, we analyzed how serine -96 and -106 phosphorylation regulates PUMA protein stability. As previously mentioned, a study by Fricker and colleagues showed that elimination of the serine-10 phosphorylation site resulted in a decrease in PUMA proteasomal degradation and increased apoptosis as a result.

To test this function we seeded pcDNA3-PUMA wt and pcDNA3-PUMA S96,106A cell lines in a 24-well tissue culture dish at equal cell densities. After cells had grown to confluence a baseline sample well was collected from each cell line. The remaining wells were treated with 20µg/ml Cyclohexamide (CHX) diluted in media and incubated at 37°C in 5% CO_2. CHX is a chemical which inhibits protein biosynthesis, by preventing any further protein development we can measure the rate at which PUMA is being degraded in the cell over time. Cell samples were collected at three time points, 3 6 and 9 hours, following CHX treatment and PUMA protein expression was detected in each sample by western blot. PUMA expression was detected using Anti-Flag antibody and also detected tubulin levels in each sample in order to normalize the quantity of protein loaded in each well.

Although PUMA S96,106A seemed to be degraded slightly faster than PUMA wt, the serine -96 and -106 phosphorylation sites do not play as strong of a role in regulating PUMA stability as serine-10. Four individual samples were analyzed by western blot and revealing that the differences in degradation between the wild-type and mutant samples were insignificant (Figure 12a). The data collected were used to plot relative protein degradation over time in the wild-type and mutant cell samples and used this data to calculate the half-life of PUMA (12b,c). From these data we conclude that although the S96,106A PUMA protein was slightly less stable, we cannot conclude that the S-96 and S-106 phosphorylation sites had a direct and significant impact on PUMA protein degradation.

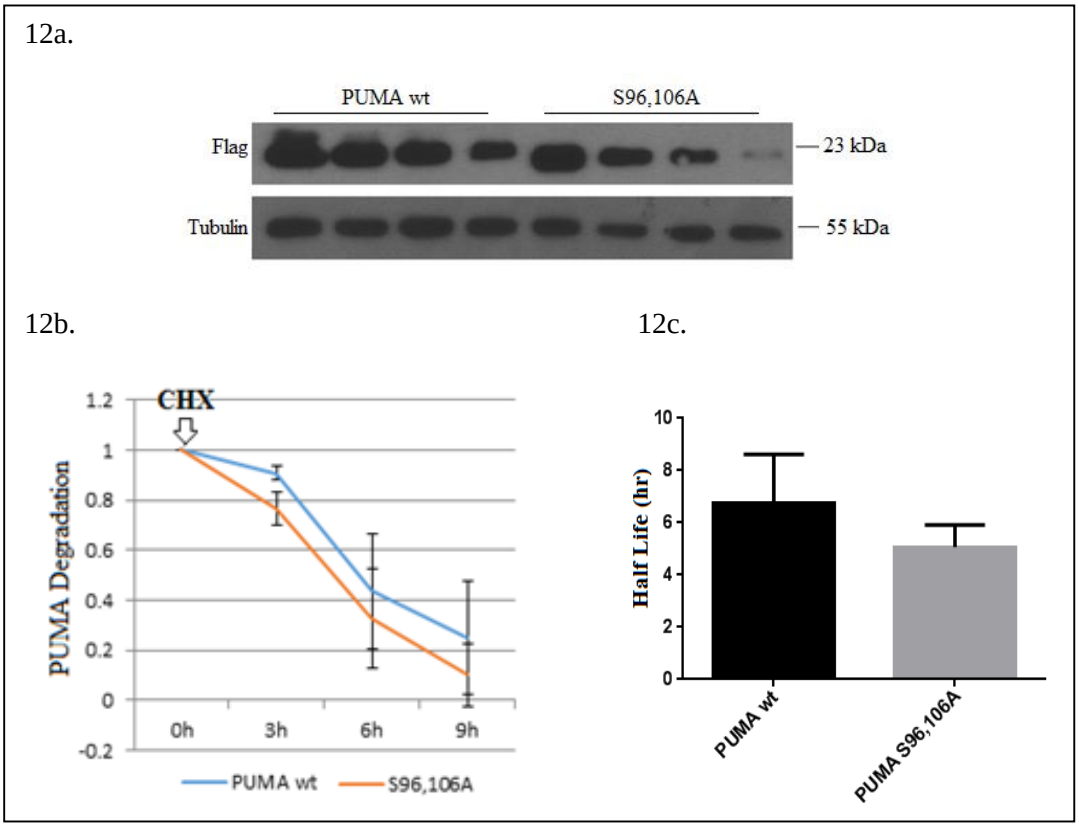


Figure 12 Analyzing PUMA stability in PUMA wt and PUMA S96,106A cell lines: **a**. pcDNA3 PUMA wt and S96,106A cell lines were treated with CHX and tracked over multiple time points to measure the rate of PUMA degradation in 4 individual samples (n=4). Western blot analysis of mutant and wild-type samples show that PUMA S96,106A was degraded slightly more quickly than PUMA wt. After factoring in differences in tubulin, the difference in protein stability was determined to be negligible. **b**. This graph represents the average protein degradation taken from four sample sets. Measurements were standardized by dividing the band intensities measured from the protein degradation by the tubulin control for each sample (Band intensities quantified using ImageJ). **c**. Average half-life of PUMA and PUMA S96,106A from 4 individual samples.

Discussion

Post-translational regulation of PUMA by phosphorylation is a relatively new area of study. The sudden interest in learning more about this fascinating protein stems from our realization of its implications in many human pathologies and processes including but not limited to cancer, multiple sclerosis, alzheimer's, ulcerative colitis, and embryonic development. While we have started to determine the regulatory function of some PUMA phosphorylation residues, until now the function of serine-96 and -106 phosphorylation has never been studied. Understanding how PUMA is regulated opens doors to using PUMA to treat serious diseases including promoting the resensitization of cancer cells to anti-cancer drugs or prevent neuronal cell death.

Up until now only three studies have been done which describe the post-translational regulation of PUMA. Our focus stems from the first of these studies done by Fricker and colleagues which identified that post-translational regulation of PUMA was being carried out by phosphorylation and identified three serine residues as targets for phosphorylation, serine -10 -96 and -106 (2). By over expressing several different PUMA point mutation constructs labeled with ^{32}P-Orthophosphate, they found serine-10 to be the most abundantly phosphorylated site. They chose to focus their studies on understanding the functional role of the serine-10 phosphorylation site and concluded that when phosphorylated it promotes proteasomal degradation of PUMA by decreasing the frequency of apoptosis (2). In 2012 Sandow and colleagues discovered that the kinase responsible for the phosphorylation of serine-10 was IKK1(2). Further studies are still needed to explain the functional effects of phosphorylation at serine-96 and -106. Our aim is to understand the functional role of serine-96 and-106 phosphorylation in PUMA function.

The first step in this study was to create constructs expressing PUMA wt and S96,106A, where phosphorylation was eliminated at residues -96 and -106. This mutation was

introduced by substituting serine residues at positions 96 and 106 to alanine (S96,106A) similar to the mutant created by Fricker and colleagues (S10A) (2). We cloned this DNA into pmCherryN1 and 3xFlag-pcDNA3 vectors and overexpressed each construct in HEK 293 cells to study the differences in PUMA wt and PUMA S96,106A function.

Next, we measured apoptosis in cell lines transiently transfected with pmCherryN1-PUMA wt, pmCherryN1 S96,106A and pmCherryN1. Previously, Fricker noted that by overexpressing PUMA S10A, the PUMA protein was more rapidly being degraded resulting in a decrease in apoptosis (2). After quantifying PUMA driven apoptosis in our constructs using flow cytometry we did not detect a significant change in the level of apoptosis between the PUMA wild-type and mutant cell lines. These results were an indication that the S-96 and S-106 phosphorylation sites do not participate in regulating apoptosis. This finding could explain why phosphorylation at the seine-96 and -106 positions was seen in less abundance than phosphorylation at serine-10.

We went on to studying PUMA wt and PUMA S96,106A function in autophagy by analysis of the pmCherryN1 stable cell lines using flow cytometry. Recent studies have begun to highlight PUMA's role in initiating autophagy as an alternative cell death pathway however what regulates this process is largely unknown. Our results showed that PUMA wt had a significant gain in autophagic activity when compared to the control, however the PUMA S96,106A mutant exhibited similar levels of autophagy as the control sample. We now can say that there is a likelihood these two phosphorylation sites together regulate PUMA's function in autophagy. The decrease in autophagy measured in the PUMA mutant could be an indication that phosphorylation at serine-96 and -106 altars PUMA in a way such that it promotes binding to Beclin-1 and loss of this phosphorylation somehow disrupts this interaction.

We wanted to confirm our findings using a different assay to confidently be able to state that the S-96 and S-106 phosphorylation residues were mediating autophagy in by PUMA using western blot. A similar technique was used by KS Yee and colleagues in their discovery that PUMA increases autophagic activity in the presence of apoptosis inhibitors (10). The Flag tagged pcDNA3 cell line was used to analyze the conversion of LC3-I to LC3-II and saw a greater conversion of LC3-I to LC3-II in PUMA-wt in comparison with the control and mutant samples. We also detected the autophagosome development in inducible cell lines confirming that the S96,106A mutant was involved in regulating PUMA driven autophagy.

Finally, we were interested in studying how S-96 and S-106 affected the protein stability of PUMA by western blot. Four individual samples of Flag tagged PUMA wt and S96,106A were treated with CHX to allow for measurement of PUMA protein levels over 9 hours. The results showed that PUMA S96,106A was being degraded only slightly faster than PUMA wt. Since the difference were so minor, we cannot say for sure that there is a significant effect on protein degradation as a result of S-96 and S-106 phosphorylation. This finding is the opposite of the conclusion made by Fricker who saw a dramatic decrease in PUMA degradation in the S10A mutant. We conclude from this assay that the S-96 and S-106 phosphorylation sites are not likely to be direct regulators of protein stability.

This study raises a couple question regarding the function of Serine-96 and -106 phosphorylation sites in PUMA function. One area of study which should be further explored is the independent function of these two phosphorylation sites to determine if it is necessary that both phosphorylation sites be eliminated to alter PUMA mediated autophagy. This could be done by generating constructs with single point mutations S96A and S106A and conducting functional tests as we have done in this study to examine their individual effects.

The next significant stage in this research would be to determine how phosphorylation of S-96 and S-106 affects PUMA's ability to bind Beclin-1. This will begin to uncover how phosphorylation enables PUMA to selectively choose alternative cell death pathways such as apoptosis or autophagocytosis. Such research will also explain why the elimination of S-96 and S-106 phosphorylation sites leads to a decrease in PUMA mediated autophagy. This research can be done using a technique called FRET analysis to study the interaction between PUMA and Beclin-1 under various cellular conditions.

Finally, it would be worthwhile to investigate the conditions under which Serine-96 and -106 residues become phosphorylated. To do this we would first need to generate antibodies to both serine residues which will allow us to detect phosphorylation at each sites under different conditions. Under some circumstances cells rely on autophagy to promote short-term survival under conditions of cell starvation. In such situations when the cells undergoing autophagy to promote survival are cancerous, it would be interesting to see if PUMA becomes dephosphorylated at residues -96 and -106 to downregulate autophagy and induce apoptosis instead. By understanding the conditions under which PUMA becomes phosphorylated and at what residues, we could use that knowledge to our advantage to help promote the activation of cell death pathways in tumor cells.

There is increasing interest in using this drug in combination with treatments such as chemotherapy to approach cancer treatment with a more dynamic and aggressive approach in hopes that it will one day increase chances for survival in even the most advanced cancers.Recent studies have shown that PUMA plays an important role in suppressing tumor development. A study in 2007 exploring ways to overcome chemosensitization in head and neck squamous cell carcinoma (HNSCC), chemotherapeutic agents such as 5FU and Adrcamycin resulted in decreased cellular levels of PUMA contributing to the tumor's ability to become resistant to treatment by decreasing their aptitude for undergoing apoptosis. By

infecting HNSCC's in-vivo with adenovirus expressing PUMA (AD-PUMA), PUMA was able to induce caspase -3 and -9 and restore apoptosis. Restoration of the caspase cascade and apoptosis restored advanced stage HNSCC tumor sensitivity to chemotherapy treatment (29).

A second study published in 2013 by Lifeng Feng and colleagues highlights the importance of understanding PUMA function for the sake of improving cancer treatment. Feng found that patients with decreased PUMA expression in cancer cells had a significantly decreased chance for survival. When Trichostatin A (TSA) treatment was applied to these cells to promote PUMA expression, it restored the cell's capacity to initiate apoptosis and improved prognosis (22).

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