# Fox Chase Cancer Center

# Annual Progress Report: 2008 Formula Grant

**Reporting Period**

July 1, 2009 – June 30, 2010

**Formula Grant Overview**

The Fox Chase Cancer Center received $3,131,563 in formula funds for the grant award period January 1, 2009 through December 31, 2011. Accomplishments for the reporting period are described below.

**Research Project 1: Project Title and Purpose**

*A Growth-Regulating Protein Tyrosine Phosphatase* - We seek to understand how an enzyme called PTP1B is regulated and how it, in turn, regulates other proteins that control blood sugar, body weight, and, in women, the development of breast cancer. The knowledge we gain from these studies will help us better understand these processes and could also lead to the development of better drugs to treat diabetes, obesity, and breast cancer.

**Anticipated Duration of Project**

1/1/2009 - 12/31/2010

**Project Overview**

The phosphorylation and dephosphorylation of proteins at tyrosine residues represents one of the most important means of regulating signal transduction in higher organisms. This process is governed by protein tyrosine kinases and protein tyrosine phosphatases. How the latter are regulated, and how they select their substrates, is not well understood. We have recently discovered that protein tyrosine phosphatase 1B (PTP-1B) - an enyzme that targets insulin, ErbB2, and leptin signaling pathways - is regulated by an unusual mechanism: enzymatic inhibition by sumoylation. This process occurs in an insulin-sensitive manner and is rapidly reversible, suggesting that sumoylation machinery is transiently engaged downstream of the insulin receptor to inhibit PTP-1B. We have also found that PTP1B regulates growth factor signals by at least two distinct mechanisms: PTP-1B targets receptor protein tyrosine kinases such as the insulin receptor by an N-terminal di-tyrosine binding motif; in contrast, it targets src-homology 3 containing substrates such as Src via a C-terminal proline-rich sequence. These putative targets for PTP-1B are key elements in growth factor-mediated signal transduction, and might account for PTP-1B's complex effects on cell proliferation and metabolism. Our overarching goals in this project are to identify the molecular mechanisms by which PTP-1B is regulated by growth factors, and to determine how this phosphatase, in turn, regulates signal transduction pathways relevant to cell metabolism and neoplastic transformation.

*Specific Aim 1: Define the mechanism(s) by which SUMO (small ubiquitin-like modifier) modification regulates PTP1B activity.*

*Specific Aim 2: Determine the role(s) of PTP1B in ErbB2 signaling.*

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Stevan Hubbard, PhD - employed by New York University

**Expected Research Outcomes and Benefits**

The enzyme PTP1B is a key regulator of the insulin receptor, the leptin receptor, and ErbB2, a protein that is frequently activated in human breast cancer. When the gene for PTP1B is knocked out in mice, these mice become resistant to diabetes, remain lean even when fed high-fat diets, and resist getting breast cancer in mouse models that are prone to this disease. For these reasons, there is intense interest in PTP1B as a therapeutic target in diabetes, obesity, and cancer. Our studies will help clarify how PTP1B regulates these processes, and, as such, could help guide the development of anti-PTP1B drugs. Such agents could impact a large number of people, as diabetes, obesity, and breast cancer are all important health problems in need of better treatments.

**Summary of Research Completed**

We have made considerable progress on the first specific aim, as detailed below.

*Specific Aim 1: Define the mechanism(s) by which SUMO (small ubiquitin-like modifier) modification regulates PTP1B activity.*

We had previously shown that PTP1B is sumoylated on at least two sites (K335 and K347) and that sumoylation inhibited PTP1B activity. We have subsequently used a variety of cell biology methods to determine the location of sumoylated PTP1B, which, in turn, has given clues as to its function.

Localization of PTP1B at the inner nuclear membrane (INM). Most sumoylated proteins are found within the nucleus or nuclear envelope. However, PTP1B is attached to the outer leaflet of the endoplasmic reticulum (ER) leading us to wonder if sumoylated PTP1B accumulated at the

outer nuclear membrane (ONM), a structure contiguous with the ER. We therefore studied the localization of PTP1B and GFP-SUMO in mouse embryo fibroblasts (MEFs). As expected, endogenous PTP1B was found on reticular structures throughout the cytosol as well as surrounding the nucleus (Fig. 1). This localization reflects authentic PTP1B distribution, as no staining was seen in PTP1B$^{-/-}$ MEFs. GFP-SUMO was seen mostly in the nucleus, but also in the nuclear envelope, where it co-localized with PTP1B.

Next we stained cells with antibodies against endogenous lamin A/C and PTP1B. Lamin is located in the nuclear lamina, just within the INM, and is therefore not expected to co-localize with ER proteins. To our surprise, we found that lamin A/C and PTP1B were also co-localized by immunofluorecence (Fig. 2A). Similar finds were obtained with exogenous WT or KR (sumoylation-resistant) PTP1B (not shown), the localization of PTP1B to the nuclear envelope is not regulated by sumoylation. These unexpected findings prompted us to examine the subcellular distribution of PTP1B in greater detail. We asked whether PTP1B had the same cellular distribution as typical ER-resident proteins, by staining MEFs with anti-KDEL (antibody against a resident ER protein), anti-PTP1B, and anti-lamin antibodies. KDEL and PTP1B co-localized throughout the cytosol in a typical ER pattern, except at the nuclear envelope, where PTP1B staining was much more pronounced than KDEL. Lamin A/C was exclusively found in a tight band around the nucleus, overlapping the nuclear envelope signal of PTP1B. No overlapping signal was seen between lamin A/C and KDEL.

To supplement these findings, we fractionated purified nuclear envelopes into outer and inner nuclear membranes. Treatment of nuclear envelopes with 1% Triton X-100 is reported to solubilize ER and ONM proteins, leaving the INM and nuclear lamina intact. In contrast, treatment of nuclear envelopes with chaotropic reagents such as urea has the opposite effect, solubilizing the INM and lamina, while leaving the ER and ONM intact. We used this method to assess the location of PTP1B in the nuclear envelope. As expected, a typical ER protein, calnexin, was solubilized by Triton X-100 and not by urea, whereas lamin A/C was solubilized by urea, but not Triton X-100 (Fig. 2B). In contrast to either of these proteins, PTP1B was not fully solubilized by either treatment. These findings are consistent with the idea that PTP1B resides both in the ER/ONM and also in the INM.

<u>Sumoylated PTP1B is enriched at the inner nuclear membrane</u>. Because previous studies from our lab suggested that sumoylated PTP1B accumulates in the vicinity of the nuclear envelope, we wondered whether this form of PTP1B was distributed throughout the nuclear envelope, or was restricted to either the ER/ONM fraction or the INM fraction. T7-tagged SUMO-1 was expressed in HeLa cells, which were then fractioned into ER/ONM and INM as described above. Interestingly, all of the sumoylated PTP1B was found in the Triton X-100 insoluble fraction, representing the INM (Fig. 2C). These results indicate that sumoylated PTP1B has a limited intracellular distribution, and suggests a function for PTP1B at the INM.

<u>PTP1B interacts with the inner nuclear membrane proteins emerin</u>. Because we found PTP1B at the INM, and immunofluorescence experiments showed apparent co-localization of PTP1B and lamin A/C, we asked whether PTP1B physically associates with INM proteins. We focused in particular on emerin, a lamin binding protein that is associated with the X-chromosome-linked Emery-Dreifuss muscular dystrophy (EDMD). Several tyrosine phosphorylation sites on emerin

have been identified. One tyrosine residue, Y95, is of particular interest, as small deletions that include this tyrosine residue (Δ95-99) have been found in emerin in human EDMD. To ask if PTP1B interacts with emerin, PTP1B was immunoprecipitated from a HeLa cell lysate and probed with anti-emerin antibodies. Using MEFs, we found that emerin co-immunoprecipitates with PTP1B and with a known partner, lamin A/C, but not with control IgG (Fig. 3A). Interestingly, the same degree of binding was observed in pervanadate-treated cells and in cells transfected with catalytic inactive/ substrate trapping mutant of PTP1B (PTP1B-C215S mutant, data not shown). These results demonstrate that PTP1B and emerin interact and that this interaction requires neither phosphatase activity of PTP1B nor tyrosine phosphorylation of emerin.

To examine whether PTP1B regulates the tyrosine phosphorylation of emerin, PTP1B-WT MEFs were transfected with GFP-emerin plus empty vector or the inactive mutants PTP1B-CS or PTP1B-DA. Phosphotyrosine levels of emerin were higher in cells expressing inactive forms of PTP1B, suggesting that endogenous PTP1B influences emerin tyrosine phosphorylation (Fig. 3B, left panel). In agreement with this interpretation, siRNA-mediated knockdown of PTP1B increased emerin tyrosine phosphorylation (Fig. 3B, right panel).

<u>Sumoylation of PTP1B is regulated in a cell-cycle dependent manner.</u> Given that SUMO modification correlates with various aspects of progression through mitosis, we asked whether PTP1B is transiently regulated by sumoylation during the cell cycle. To address this issue, we transfected *PTP1B*-null MEFs with PTP1B-WT and T7-SUMO-1, either alone or together, and synchronized the cell cycle using thymidine treatment, followed by a release to overnight nocodazole block to achieve G2/M arrest. The mitotic blocked cells were then washed and released into nocodazole-free media for indicated time, and cell lysates were subjected to anti-PTP1B immunoprecipitation followed by anti-T7 immunoblot. We found that PTP1B sumoylation markedly increases as the cells enter mitosis (Fig. 4, lane 5). Upon release from mitotic block, PTP1B sumoylation levels drop to a low level within 30 min (Fig. 4, lanes 8 & 9). These results show that PTP1B sumoylation is temporally regulated *in vivo* in a cell cycle dependent manner.

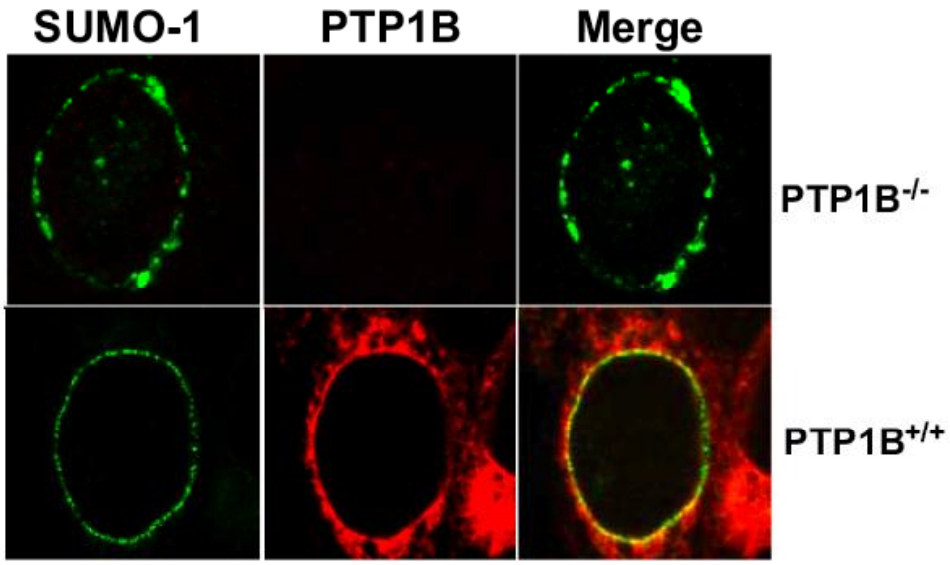


Fig. 1. Co-localization of SUMO-1 and PTP1B at the nuclear envelope. *PTP1B*-null or –WT MEFs were transfected with RFP-SUMO-1 and immunostained with anti-PTP1B antibodies.

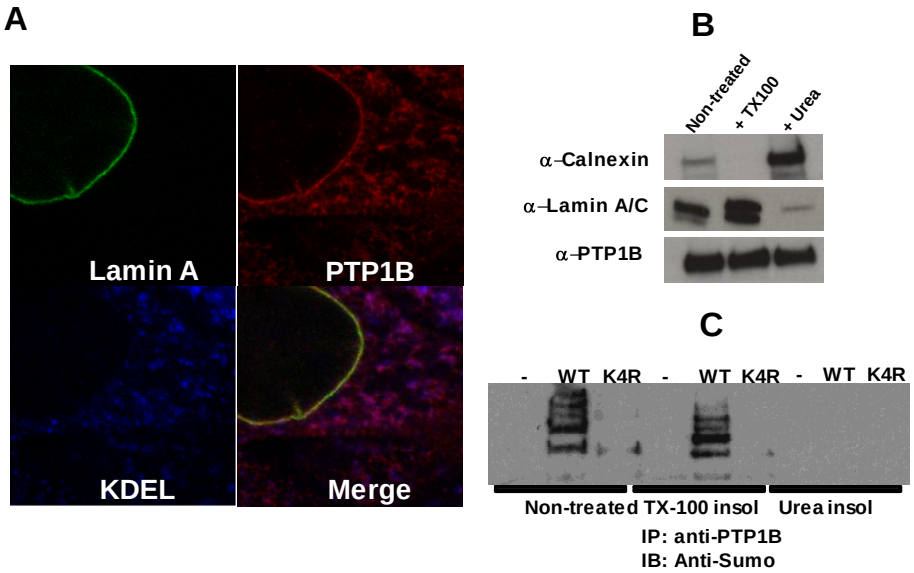


Fig. 2. Localization of sumoylated PTP1B at the inner nuclear membrane. **(**A) PTP1B-WT MEFs were stained with anti-lamin A, -PTP1B, or KDEL antibodies. Confocal images are shown. (B) Nuclear envelopes were prepared from HeLa cells and further fractionated using either 0.5 % Triton X-100 or 6M Urea to purify the inner nuclear membrane (INM) and the endoplasmic reticulum/outer nuclear membrane (ER/ONM) respectively. The chaotrope-resistant fraction representing the ER/ONM, and the Triton-resistant fraction representing the INM/lamina were immunoblotted with antibodies against the ER-resident protein calnexin, the INM/lamina-resident proteins emerin and lamin A/C, and against PTP1B. (C) *PTP1B* –null MEFs were transfected with HA-PTP1B-WT together with T7-SUMO-1. Nuclear envelopes were prepared and fractionated into ER/ONM and INM/lamina fractions as above. HA-PTP1B was immunoprecipitated, followed by immunoblot with anti-T7-SUMO-1 antibodies.

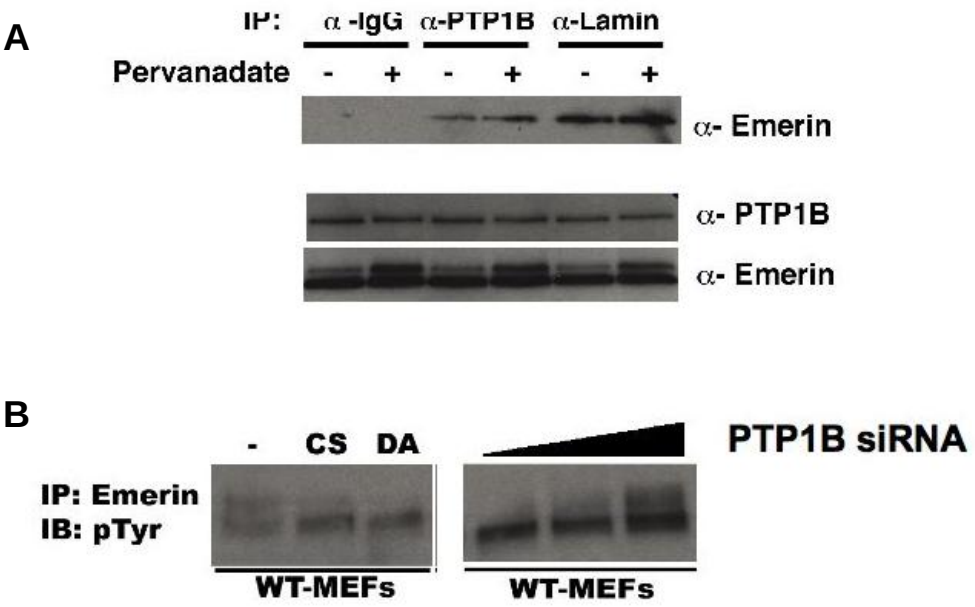


Fig. 3. PTP1B binds to emerin and regulates its tyrosine phosphorylation**.** (A) Endogenous lamin A/C and PTP1B were immunoprecipitated from HeLa cells and the immunoprecipitates were examined by immunoblot for the presence of emerin. To ensure equal loading, the bottom panels show the whole cell lysate protein expression of PTP1B and emerin from Hela cells. (B) *PTP1B*-WT or *PTP1B*–null MEFs were transfected with GFP-emerin together with catalytic inactive mutants of PTP1B (PTP1B-C215S or PTP1B-D181A) or GFP-Emerin was transfected together with siRNA against PTP1B in *PTP1B*-WT MEFs. Tyrosine phosphorylation status of Emerin was examined by immunoblot analysis using catalytically dead mutants (CS), cysteine to serine mutation, and (DA) aspartate to alanine mutation.

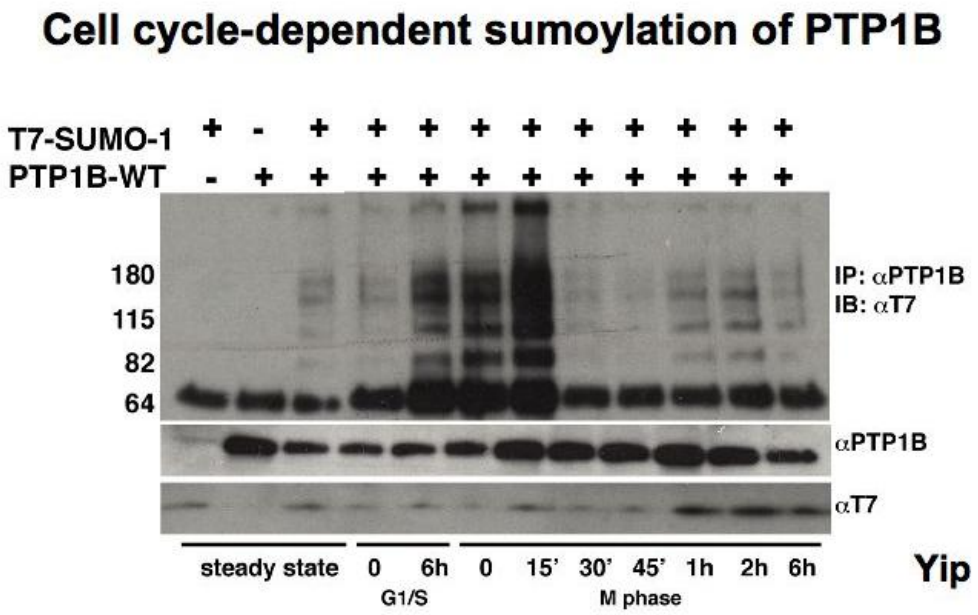


Fig. 4. Cell cycle dependent sumoylation of PTP1B. *PTP1B*-null MEFs were co-transfected with PTP1B-WT and T7-SUMO-1 and the transfected cells were synchronized by thymidine treatment followed by nocodazole block. The mitotic synchronized cells were released from metaphase at the indicated time point and lysed in the presence of NEM. Anti-PTP1B immunoprecipitation and anti-T7 immunoblots were performed to evaluate the sumoylation level of PTP1B throughout the cell cycle.

## Research Project 2: Project Title and Purpose

*Characterization of the Role of MTAP Gene in Tumorigenesis* - Methylthioadenosine phosphorylase is a highly evolutionarily conserved metabolic enzyme involved in methionine and adenine metabolism that is expressed in all tissues throughout the body. Deletion of the gene for methylthioadenosine phosphorylase (*MTAP*) is one of the most frequent genetic changes observed in many different types of human tumors. Because *MTAP* is located near the *CDKN2A/ARF* tumor suppressor gene, it is unclear if loss of *MTAP* plays a primary role in tumorigenesis or if it is lost simply due to proximity to *CDKN2A/ARF*. The purpose of this research project is to confirm that loss of *MTAP* directly affects tumorigenesis and to identify potential oncogenic mechanisms affected by loss of *MTAP* expression.

### Anticipated Duration of Project

1/1/2009 - 12/31/2010

## Project Overview

Methylthioadenosine phosphorylase (MTAP) is a key enzyme in the methionine salvage pathway whose function is to convert 5′-deoxy-5′-methylthioadenosine (MTA) into methionine. Inactivation of *MTAP*, generally by homozygous deletion, is found in both solid and hematologic malignancies at a high frequency. Because *MTAP* is located near the *CDKN2A/ARF* tumor suppressor gene, it is unclear if loss of *MTAP* plays a primary role in tumorigenesis or if it is lost simply due to proximity to *CDKN2A/ARF*. In published work and our preliminary data, we have shown that reintroduction of *MTAP* into *MTAP*-deleted tumor cell lines significantly suppresses tumorigenicity and affects processes involved in cell migration and invasion *in vitro*. We also found that mice heterozygous for an *MTAP* null allele ($Mtap^{lacZ}$) die prematurely of a lymphoproliferative disease resembling T-cell lymphoma. Taken together, these data suggest that *MTAP* loss plays a functional role in the development of cancer. The overall goal of this project is to determine how MTAP loss contributes directly to the development of cancer. There are three specific aims. In the first aim we will characterize MTAP-deficient tumor cells from two different systems, mouse lymphoma cells and human fibrosarcoma cells. In the second aim we will create a Tet-inducible MTAP expressing cell line to facilitate the study of how MTAP expression alters gene regulation. In the third aim we will use the Tet-inducible system created in Aim 2 to perform kinetic analysis of MTAP regulated transcripts in response to MTAP expression. These studies are significant because an understanding of the role that *MTAP* deletion plays in tumorigenesis may lead to novel therapeutic strategies for *MTAP*-deleted tumors. In addition, these studies will establish how a "housekeeping" metabolic enzyme can have a novel role as a tumor suppressor gene.

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## Expected Research Outcomes and Benefits

Deletion of the gene for methylthioadenosine phosphorylase (*MTAP*) is one of the most frequent genetic changes observed in many different types of human tumors. This project is relevant to human health because the identification of *MTAP* as a tumor suppressor gene could identify individuals at increased risk of cancer. In addition, understanding of the mechanism by which *MTAP* deletion contributes to tumorigenesis may lead to novel strategies that can selectively target *MTAP*-deleted tumor cells for destruction. This would potentially have relevance to many different kinds of human cancer.

**Summary of Research Completed**

Comparison of growth kinetics of MTAP+ and MTAP- HT1080 subcutaneous tumors using biofluorescent imaging.

In our last progress report, we reported the results on our initial experiment examining the growth of isogenic MTAP+ and MTAP- HT1080 fibrosarcoma cells. In the current reporting period we have repeated and extended this result. A total of 10 mice were injected subcutaneously with either $2x10^6$ HT1080:MTAP+:GFP or HT1080MTAP-:GFP and tumors were monitored weekly using an IVIS Spectrum Bioluminescent/fluorescent imager. We found that after 28 days all five animals injected with HT1080MTAP-:GFP cells had detectable tumor cells, compared to only three of the five animals injected with HT1080MTAP+:GFP (Figure 1a). Furthermore, two of these animals had tumors that were very small relative to those injected with the MTAP- cells (Figure 1a, and 1b).

We further characterized these tumors by excising them and weighing them. The mean weight for the tumors derived from HT1080MTAP-:GFP cells was 536 mg compared to 144 mg for HT1080:MTAP+:GFP cells (P=0.019, two-sided t-test). In addition, we made enzyme extracts from each of the tumors, and measured MTAP enzyme activity (Figure 1, bottom right). The tumor material from the tumors derived from MTAP+ cells had on average 3.4 times more MTAP enzyme activity then tumors derived from MTAP- cells (P=0.003). The residual MTAP activity in the tumors formed from the MTAP- cells is likely to be derived from contaminating stromal cells.

<u>Create a Tet-inducible MTAP expressing cell line.</u>

In order to create a Tet-inducible MTAP expressing cell line, we first needed to construct a plasmid in which the MTAP ORF was cloned downstream of the Tet-regulated promoter (pTRE-Tight:MTAP). This plasmid was created by first digesting pCR3.1:MTAP with BamHI and EcoRV and isolating the 800 bp MTAP-containing fragment. This DNA fragment was then cloned into pTRE-Tight digested with the same enzymes. After ligation and transformation, positive *E. coli* clones were confirmed by restriction nuclease digestion and DNA sequencing.

A single clone of pTRE-Tight:MTAP was then used to transform MCF-7 Tet-on Advanced Cells (obtained from Clontech). These Cells express the Tet-On Advanced reverse transactivator protein that can initiate transcription from the TRE promoter in the presence of tetracycline analog Doxycycline (Dox). Also, these cells contain a homozygous deletion of the endogenous MTAP locus, and thus lack MTAP protein. To create stable tranfectants of these cells, we first linearized pTRE-Tight:MTAP by digestion with XbaI restriction nuclease. After gel purification, the linearized pTRE-Tight:MTAP plasmid was combined with a linear puromycin selection maker at 20 to 1 ratio and transfected into MCF-7 Tet-on Advanced Cells using Lipofectamine Transfection agent (Invitrogen) according to the manufacturer's instructions. The transfected cells were then cultured in DMEM medium supplemented with 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, 250 μg/ml G418,and puromycin (1 μg/ml). After 3 weeks, puromycin resistance clones were selected and cultured in the absence and presence 1 μg/ml of Dox and western analysis was performed (Figure 2a). We found that

seven of the nine clones expressed MTAP only when cultured in the presences of 1 μm/ml Doxycycline (Dox).

We then performed additional analysis on one of these clones (C8). First we examined the kinetics of MTAP induction by harvesting cells after exposing the cells to Dox between 0 and 40 hours (Figure 2b). This experiment demonstrates that induction begins at 8 hours and picks up considerably between 8 and 16 hours, although protein continues to increase up to 40 hours. We also examined the sensitivity of induction by modulating the Dox levels in the media (Figure 2c). The data show that addition of 0.2 ug/ml is sufficient to maximize induction at 40 hours.

This characterization confirms that we have created a tightly controlled dox inducible MTAP-expressing cell line.

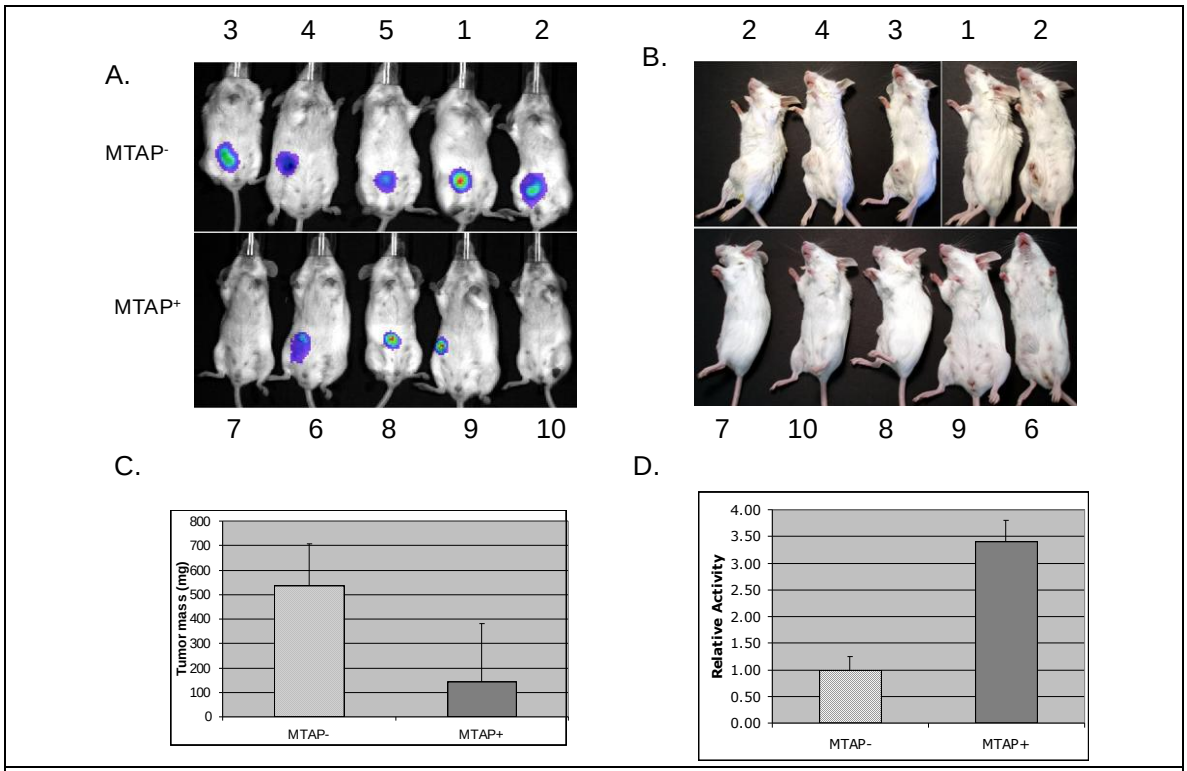


Figure 1. Tumor formation *in vivo* by MTAP+ and MTAP-HT1080 cells. A. SCID mice implanted with either MTAP+ or MTAP- GFP labeled HT1080 cells imaged for GFP at 28 days post-injection. Number above or below each lane is the mouse number. B. Same mice visualized by regular photography. C. Mass of tumor. D. Relative MTAP activity in tumors from MTAP- (n=5) vs MTAP+ cells (n=3).

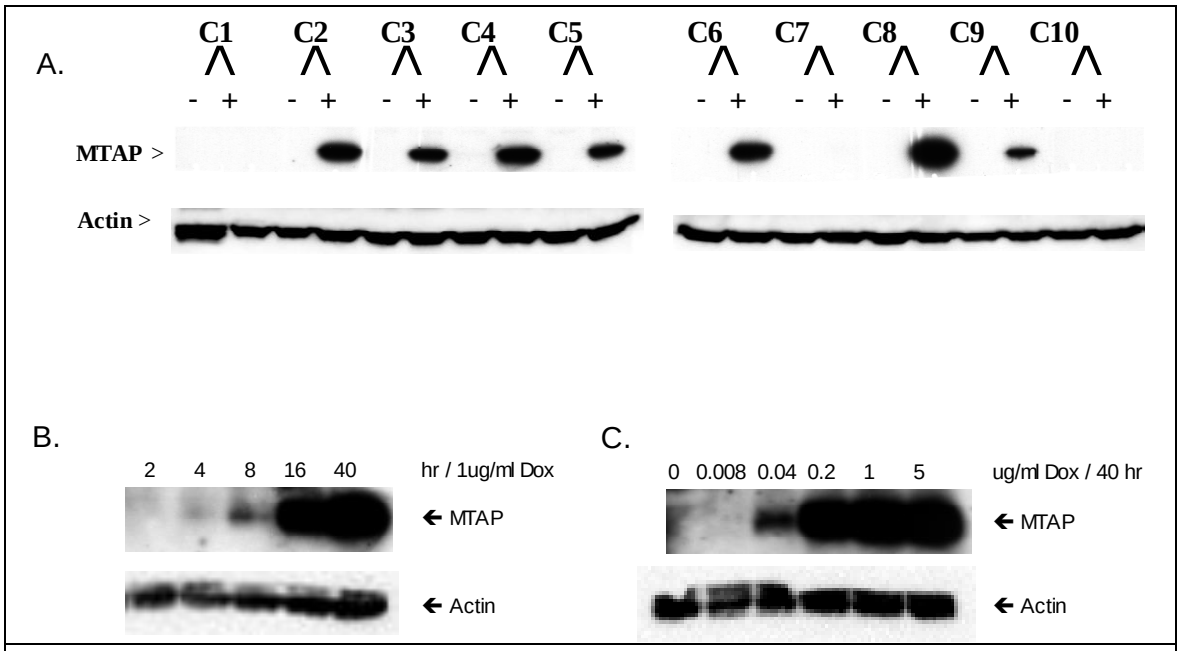


Figure 2. Doxycycline inducible MTAP in MCF-7 cells. A. Examination of MTAP expression in 10 transfected clones for MTAP expression in the presence (+) and the absence (-) of doxycycline (1 μg/ml, 40 hours). Twenty microgram of total protein was used in each lane. B. MTAP levels in clone C8 as a function of time. C. Effect of doxycycline concentration on MTAP expression.

## Research Project 3: Project Title and Purpose

*The ARF Tumor Suppressor and Autophagy* - We have recently discovered that the ARF tumor suppressor (ARF) plays an integral role in a key survival pathway utilized by tumor cells called "autophagy". Autophagy is a self-catabolic process wherein cells exposed to metabolic or hypoxic stress use the lysosome to digest long-lived proteins and organelles in order to release free amino acids and thus survive. The importance of this pathway for cancer cell survival is epitomized by the fact that chloroquine, an autophagy inhibitor, can potently suppress the development of certain tumors. We propose to elucidate the role of ARF and an interacting protein, Hsp70, in autophagy. We will test the hypothesis that a novel autophagy inhibitor discovered in the laboratory of our collaborator, and found by our group to specifically target ARF-dependent autophagy, is an effective new chemotherapeutic agent.

### Anticipated Duration of Project

1/1/2009 - 12/31/2011

### Project Overview

The objective of this research is to improve our understanding of the role of the ARF tumor suppressor (ARF) in the pro-survival pathway of autophagy. We have recently found that ARF

induces autophagy, and that it must traffic to mitochondria in order to do so. Further, we have found that ARF must interact with the cytosolic chaperone protein Hsp70 in order to traffic to mitochondria. Hsp70 is over-expressed in the majority of human tumors, and over-expression of this protein universally correlates with poor prognosis. A long-time collaborator recently identified a novel chemical inhibitor of Hsp70, which we term PAS. We have found that PAS selectively inhibits the ability of ARF to induce autophagy. Moreover, we find that PAS selectively kills cells that over-express ARF. ARF is not expressed in normal, non-transformed cells, and is transcriptionally repressed by the p53 tumor suppressor; therefore, tumors with mutant p53 tend to express high levels of ARF. We predict that PAS will be selectively toxic to tumor cell lines with mutant p53. We propose to test this hypothesis.

Aim 1 To define the domains of ARF necessary for mitochondrial localization, autophagy induction, and interaction with Hsp70
Aim 2 To evaluate the efficacy of a novel Hsp70 inhibitor (PAS) on human tumor cell lines
Aim 3 To test the hypothesis that ARF and Hsp70 silencing are inhibitory to human tumor development in a xenograft model

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**Expected Research Outcomes and Benefits**

The overwhelming majority of chemotherapeutic drugs used in the clinic today preferentially kill tumor cells that contain wild type p53 gene. Sorely lacking in the clinic are drugs that target novel pathways (such as autophagy) and that preferentially target tumors with mutant p53 genes. The goal of this research is to use ARF and autophagy as biomarkers of efficacy of a novel compound, PAS.

The ARF tumor suppressor is not detectably expressed in normal, non-transformed cells. It is however highly expressed in tumors with mutant or inactive p53. We have recently found that tumor cells with mutant p53 and high levels of ARF have a survival advantage over tumor cells with no ARF expression. This is due to the integral role of ARF in the survival pathway known as autophagy. We have recently published that tumors with mutant p53 and high levels of ARF demonstrate increased survival in response to metabolic stress.

Additionally, we have found that silencing ARF in tumor cells with mutant p53 inhibits

autophagy and impedes the progression of these tumors *in vivo*. We have found that a small molecule inhibitor of the chaperone protein Hsp70 inhibits the ability of ARF to traffic to mitochondria, and to induce autophagy. We have shown that this compound is toxic to tumor cells but not normal cells and that it is preferentially toxic to tumor cells with high levels of ARF (that is, those with mutant p53). We expect to learn from this research the mechanism of action and potential anti-cancer function of this unique small molecule. The identification and characterization of a novel small molecule (PAS) with a new mechanism of action (autophagy) are the two most important research benefits of this research.

**Summary of Research Completed**

Aim 1: To define the domains of ARF necessary for mitochondrial localization, autophagy induction, and interaction with Hsp70

The tumor suppressor ARF is a 169 amino acid protein that shares no homology to other proteins, and for which there is limited structural information available. To date ARF's tumor suppressor activity and ability to inhibit MDM2 and stabilize p53 maps to the first 50 amino acids. In order to map the domain of ARF required to induce autophagy, we generated six different deletion constructs of ARF. These include an amino terminal deletion we denote Δ1-50, which lacks the first 50 amino acids, as well C-terminal deletion mutants encoding amino acids 1-100, 1-120, and 1-140, as well as full length ARF (1-169). These inducible mutants were generated by cloning them using the polymerase chain reaction upstream of the CMV promoter and 2x-tetracycline operator in the pcDNA 4/TO plasmid, and stably-trasfected into the doxycycline-inducible human osteosarcoma cell line U2OS-TRex (kindly provided by P. Raychaudhuri, University of Illinois at Chicago).

Each ARF plasmid (10ug) was transfected into U2OS-Trex cells using Fugene-6 transfection reagent (Roche). The transfected U2OS cells were grown in drug-selection media (50 μg/mL zeocin) for 3 weeks. Ten colonies from each transfection were selected and propagated in media + 50 μg/mL zeocin + 50 μg/mL hygromycin B. Four plates of each line were grown to ~80% confluency and examined for ARF expression by western blot. One plate was used for the control (-doxycycline), and the others received 0.1 ug/mL doxycycline for 24, 48 and 72 hours to induce wild type and ARF mutants. Cells were harvested, isolated for protein, and run on a Western blot for ARF expression levels, as well as for the level of p62SQSTM1 and LC3. p62SQSTM1 is degraded by autophagy, and LC3 is processed to a faster mobility species (LC3 II) when autophagy is induced. These cells were also analyzed by electron microscopy in order to confirm the presence of autophagy by visualization of autophagosomes.

<u>Results</u>: As depicted in Figure 1, we were able to generate doxycycline-inducible cell lines for each of our ARF deletion mutants. These have been tested for autophagy induction, mitochondrial localization, and HSP70-binding. As depicted in Figure 1, while full length ARF (1-169) is capable of inducing autophagy, as assessed by decreased expression of the p62SQSTM1 protein, multiple clones of the 1-100 mutant fail to induce autophagy, as assessed by p62SQSTM1 degradation and LC3 II appearance. In contrast, the 1-120 and 1-140 mutants were fully capable of inducing autophagy (not shown). These studies thus map the autophagy-inducing activity of ARF to amino acids 100-120. Notably, these data indicate that the p53-

inducing and autophagy-inducing domains of ARF are distinct; this has been a standing question in the field.

The five deletion mutants described above have been tested for mitochondrial localization (Figure 2). We isolated mitochondria from these inducible cell lines using our established mannitol gradient protocols, and verified the purity and integrity of these mitochondria by probing lysate for GRP75 (a mitochondrial marker) and PCNA (nuclear/cytosolic protein). As shown in Figure 2, we found that the 1-100 mutant of ARF is greatly impaired for mitochondrial localization; therefore, there is a consistent correlation between mitochondrial localization of ARF and autophagy. This has also been a major question in the field. Finally, these deletion mutants were also tested for interaction with Hsp70, a protein that we have shown plays an essential role in autophagy. For these studies we used immunoprecipitation-western blotting to assess the ability of the 1-100 mutant to interact with Hsp70. As shown in Figure 3, the 1-100 mutant, which fails to induce autophagy, also fails to interact with Hsp70. These data implicate the Hsp70-ARF interaction as being potentially critical for autophagy.

Aim 2: To evaluate the efficacy of a novel Hsp70 inhibitor (PAS) on human tumor cell lines

We sought to test the hypothesis that the Hsp70 inhibitor PAS induces cell death in an ARF-dependent manner. Specifically, we identified two cell lines that lack p53, mouse embryo fibroblasts from the p53 knockout mouse, as well as a sarcoma tumor cell line from the p53 knockout mouse. Because p53 normally represses ARF, these two cell lines both express very high levels of ARF. We next generated versions of these in which ARF is silenced using a short hairpin to ARF (shARF), as well as parental cells infected with a non-silencing control (shControl). As depicted in Figure 4, cells with ARF expressed (infected with ShControl) are markedly more sensitive to PAS, as assessed by cell morphology (Figure 4A). In contrast, cells in which ARF is silenced are markedly resistant to PAS. We assessed the IC50 of PAS in these two matched sets of cells using the sulforhodamine blue (SRB) assay, which measures cell viability. These data indicate that the IC50 for PAS is 2 uM in shControl cells, and increases to 7 uM in cells in which ARF is silenced. These data establish ARF as a biomarker for PAS toxicity, and suggest that PAS may be selectively toxic to tumors in which p53 is mutated, and which consequently express high levels of ARF.

The results from Aims 1 and 2 have been submitted for publication.

Aim 3: To test the hypothesis that ARF and Hsp70 silencing are inhibitory to human tumor development in a xenograft model

There are two tandem genes that encode Hsp70 in the mouse. We have obtained mouse embryo fibroblasts (MEFs) from the mouse in which both genes are knocked out by homologous recombination (HSP70 -/- MEFs, obtained from Donna L. George, University of Pennsylvania). We infected these MEFs, as well as wild type MEFs (+/+), with a retrovirus encoding the E1A and Ras transforming genes. These transformed Hsp70 -/- and +/+ MEFs were assessed to have equal levels of E1A and Ras by western blot analysis. These were then infected with a retrovirus encoding the firefly luciferase gene, and injected into the flanks of nude mice. One million cells were injected and allowed to establish as tumors for 3 days. After 3 days (and again at 7 days),

tumor volume was assessed using the Xenogen IVIS luminescence system, available in the Small Animal Imaging Facility at Fox Chase. Six different established +/+ and Hsp70 -/- tumor lines were created and analyzed. Notably, each of the Hsp70 -/- tumors developed more slowly than the +/+ counterparts (not shown). These data establish Hsp70 as necessary for efficient tumor development, at least in this cell line system. We are currently completing this Aim.

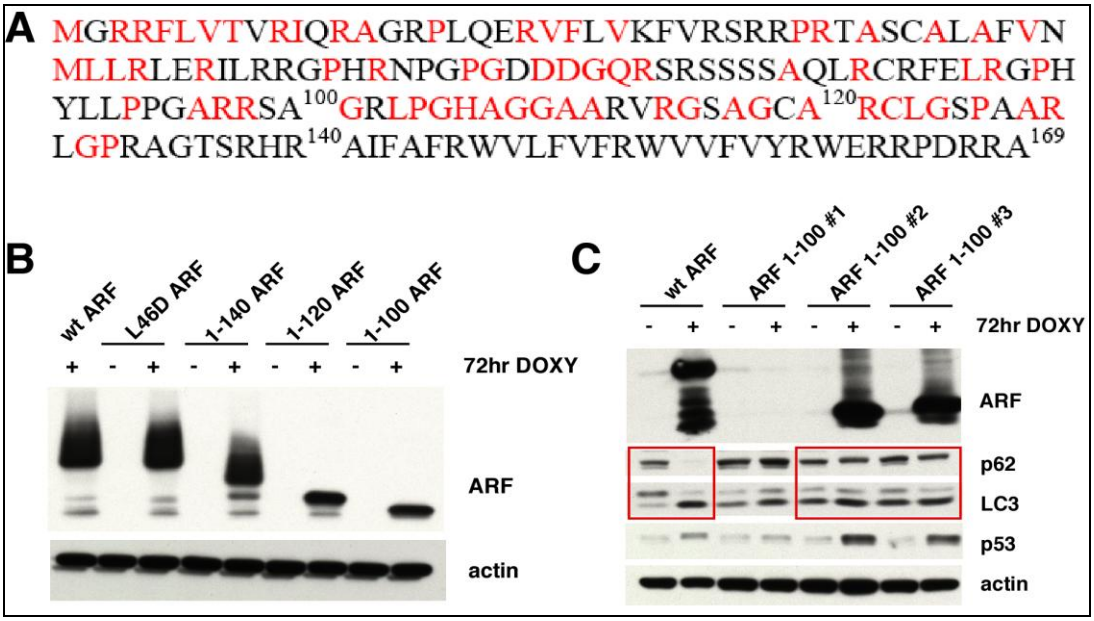


Figure 1. ARF's autophagy-inducing domain maps to conserved C-terminal residues located from amino acids 100-120. (*A*) Protein sequence of the murine ARF protein with numbers indicating amino acid positions, and conserved residues between human and mouse depicted in red. (*B*) Stable doxycycline (doxy)-inducible cell lines for the ARF mutants indicated probed for ARF and actin (*C*) Individual clones of ARF encoding amino acids 1-100 are defective for autophagy after treatment with doxycycline, as assessed by lack of p62 degradation and failure to induce LC3 II. Interestingly, the 1-100 mutants retain the ability to stabilize p53. Clone #1 does not express ARF and serves as a useful doxycycline control.

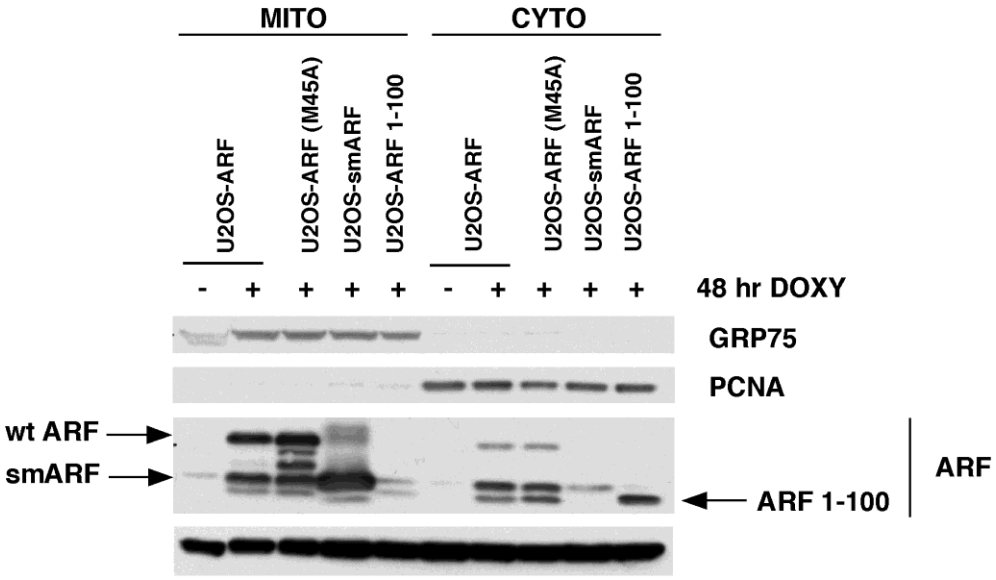


Figure 2. Deletion of the ARF C-terminus impairs mitochondrial localization. Mitochondrial preparations from several stable U2OS-ARF cell lines were verified by western blot. smARF (ARF delta 1-50) is exclusively at mitochondria, while ARF 1-100 C-terminal deletion appears impaired for mitochondrial localization. GRP75 and PCNA are controls for the purity of the mitochondria preparations.

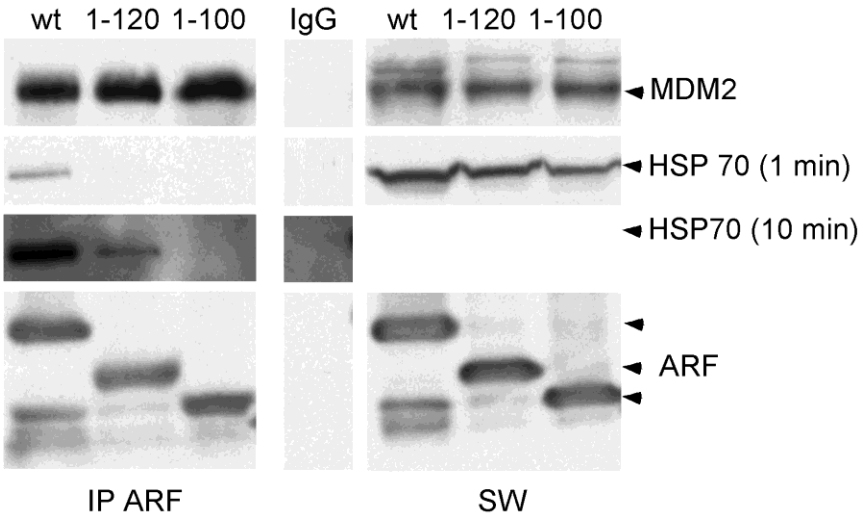


Figure 3. The 1-100 mutant of ARF is impaired for interaction with Hsp70. Co-immunoprecipitation of ARF (wt, 1-120 or 1-100 mutants) with antisera to HSP70 or MDM2 (positive control) or IgG in U2OS-ARF cells treated with doxycycline for 24 hours. SW = straight western of whole cell lysate for ARF mutants, Hsp70 and MDM2.

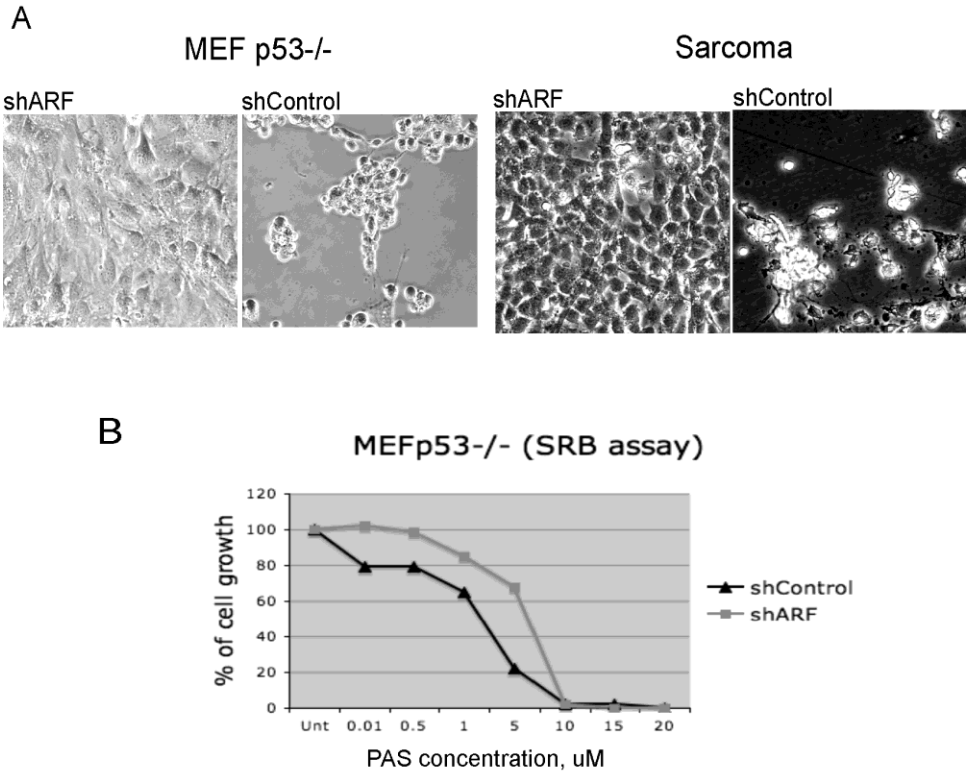


Figure 4. Silencing ARF decreases sensitivity to the Hsp70 inhibitor PAS. (A) Phase-contrast images of the p53-/-MEF or Sarcoma cells infected with shARF or shControl treated with 5uM PAS for 8 hrs. (B) The p53-/-MEF cells infected with shARF or shControl treated with the indicated concentrations of PAS for 72 hrs. A representative SRB assay indicates cell viability in p53-/-MEF cells. Values shown are normalized to the viability of the control (DMSO-treated) cells.

## Research Project 4: Project Title and Purpose

*Anti-Glucose Transporter-1 Antibodies as a Novel Treatment against Human Cancers* - Glucose fuels the rapid growth and metabolism of tumors. Glucose uptake by cells is facilitated by a family of transporter proteins called "Gluts". Tumors frequently over-express the high affinity glucose transporter protein, called Glut-1. Normal cells, on the other hand, require a slow and steady supply of glucose and therefore use lower affinity glucose transporters, Glut-4 or Glut-12. In the studies outlined here we will use antibodies against Glut-1 to decrease the uptake of glucose by the tumor, while sparing glucose uptake by normal cells. This novel and new therapeutic strategy could be applicable to a wide variety of human cancers, including lung cancer, breast cancer, and essentially any cancer that shows increased glucose uptake on a PET scan.

### Duration of Project

1/1/2009 - 1/31/2010

**Project Overview**

Objective: The objective of this project is to inhibit glucose uptake by tumors selectively by the use of anti-Glut-1 antibodies. This could lead to the development of an entirely new class of compounds with potentially wide therapeutic applications in a variety of human cancers, including the two most common human cancers, breast cancer and lung cancer. We have previously shown in human lung and breast cancer lines that blocking membranous Glut-1 by an anti-Glut-1 antibody not only decreases proliferation and enhances apoptosis, but also increases apoptosis induced by chemotherapy, hormonal and targeted agents. We have also shown that this can be achieved by inhibiting intra-tumoral glucose uptake, which, in turn, leads to up-regulation of the proapoptotic 5' adenosine monophosphate-activated protein kinase (AMPK) in its phosphorylated form (pAMPK) and the downregulation of antiapoptotic Akt protein, in its phosphorylated form (pAkt). Studies in xenograft models are currently underway and are funded by the Department of Defense. The work outlined in this project will build on our previous work and lay the foundation to initiate human studies in the future.

Working towards this goal, our specific aims for this project are: Aim 1: In primary human non-small cell lung cancer (NSCLC) specimens, correlate Glut-1 expression with overall survival (OS) and disease free survival (DFS). We will also correlate Glut-1 expression with pAKT and pAMPK expression. *The objective of this specific aim is to confirm in clinical specimens the observations in the laboratory. This may also help us identify subsets of patients who may benefit from anti-Glut-1 antibody therapy.* Aim 2: Generate fully human IgG1 antibodies against the large extracellular loop adjacent to the N-terminus of the Glut-1 transporter protein. We hypothesize that binding of antibodies to this domain will effectively inhibit glucose transport, starving the cancer cells and providing a therapeutic effect. To validate our human IgG1 antibodies we will compare their efficacy with that of the commercially available mouse monoclonal antibody (MAB1418 generated from clone# 202915) against human cancer cell lines. *The objective of this specific aim is to generate an optimal human antibody that will duplicate the results demonstrated by the mouse antibody and which could be used for future human studies in the treatment of any cancer that is glucose avid on the 18-Flourodeoxy glucose positron emission tomography (PET) scan in general and metastatic NSCLC and breast cancer in particular.*

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## Expected Research Outcomes and Benefits

This work could lead to the development of an entirely new class of compounds with potentially wide therapeutic applications in common cancers like lung cancer and breast cancer, especially in the metastatic setting, and these compounds can be used in combination with other chemotherapeutic, hormonal and targeted agents. This treatment strategy could be potentially utilized for any cancer that demonstrates increased glucose uptake in the PET scan. Hence, metastatic prostate cancer, metastatic colorectal cancers, kidney cancer, pancreatic cancer, melanoma, sarcomas and even lymphomas could be potentially amenable to this treatment strategy. Currently there are no good curative or even palliative treatment strategies available for several of these tumors. Regardless of tumor type, treatment could be selectively tailored to tumors that show increased glucose avidity on a PET scan, a widely available diagnostic clinical tool. For additional rigor, immunohistochemistry (IHC) for Glut-1 can be easily performed by a standard pathology laboratory and treatment could be specifically tailored to patients that over express Glut-1.

This work, once completed, will pave the way for the use of anti-Glut-1 antibodies in human cancers. The optimal antibody will be chosen for further study, and the mechanisms of antibody action will also have been worked out. Outside the scope of this work, but funded by a Department of Defense grant, we will study this treatment strategy in xenograft models. Taken together these research projects will pave the way for future human studies.

## Summary of Research Completed

Our research consists of the following components; validating the association between Glut-1 expression levels in lung tumors, correlating survival with the associations between Glut-1, AMPK and pAKT, and associating the expression of these proteins with each other and overall survival.

Several factors have resulted in lack of significant progress toward our goals. First, during this reporting period, the experimental work was disrupted by the departure of both postdoctoral associates working on the project. Secondly, the PI has refocused his career toward clinical care with a concomitant closure of his research laboratory. However, key conceptual aspects for addressing Specific Aim 2 were clarified during the final months of this project.

It became evident to us during execution of a series of repeated experimentation that commercial antibodies obtained from multiple companies were only able to induce cell kill intermittently and unreliably. It was then decided to make our own antibody, which was the thrust of the second specific aim of the project.

One of the rationales of inhibiting Glut-1 with an antibody versus a small molecule was secondary to the fact that Glut-1 is universally expressed on RBCs (Red Blood Corpuscles). We attempted to determine whether the commercially available antibodies both of the MAB1418 clone and the anti-Glut-1 antibody generated against an unspecified epitope on the Glut-1 transporter protein made by R and D Systems would bind to the Glut-1 transporter protein found on the surface of RBCs.

Both the antibodies were able to bind to the Glut-1 on the surface of the RBCs. This raised the question of whether the administered antibody would be exhausted by binding to the RBCs, leaving very little antibody to attack to the tumor.

In order to circumvent this problem, we calculated how much antibody would be needed to saturate the Glut-1 antibodies on the surface of the RBCs while leaving unbound antibody to float freely in the serum to bind to the tumor. A preliminary analysis revealed that micromolar concentrations per kg body wt would suffice to saturate the antibodies on the surface of the RBCs and leave enough antibodies to bind to the tumor to generate cell killing effects.

An alternative option was to generate the new antibody against an epitope that is unique to tumoral Glut-1 rather than to RBS Glut-1. This would require sequencing the Glut-1 on the RBCs versus the tumors and identifying an extracellular epitope whose sequence was unique to tumoral Glut-1. The sequence of RBC glut-1 was available from public databases. However the Glut-1 on lung cancer lines was not available for comparison from RBC Glut-1 sequence. However, sequencing efforts stalled owing to aforementioned events.

As detailed by our previous report, we have worked out the mechanism by which the Glut-1 antibody induces cell kill. Two key proteins in the cell signaling pathway are affected. An oncogenic protein is turned off and another protein with tumor suppressor properties is turned on. This led to experimentation with other molecules demonstrating similar effects, both in our laboratory as well as other labs across the country. If evidence accumulates that these molecules appear to have a greater potential to yield a therapeutic modality of interest, future research will be refocused accordingly. Several such readily available compounds have been identified and will be exploited using other funding sources.