AWARD NUMBER: W81XWH-17-1-0601

TITLE: Stathmin Phosphorylation as a Target for Blocking Metastasis in Prostate Cancer

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REPORT DATE: October 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2018	Annual	30 Sep 2017-29 Sep 2018

4. TITLE AND SUBTITLE

Stathmin Phosphorylation as a Target for Blocking Metastasis in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0601

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRAA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Metastasis is a primary cause of cancer-related deaths, yet this process remains poorly understood. Stathmin (Stmn1) is an oncoprotein over-expressed in many cancers, including prostate cancer (PCa). While increased Stmn1 correlates with disease progression and poor prognostic outcome, however its role in metastasis is still being elucidated. Stmn1 activity is controlled by four serines (S16, S25, S38, and S63) which are differential phosphorylation by 4 different pathways. Therefore the purpose of this study is to determine which one of these serines (and associated pathway) regulates proliferation and which promotes metastasis. The **hypothesis** is that *the first serine, S16, is the predominant serine that regulates PCa cell proliferation and acts as a gatekeeper to inhibit a cascade leading to metastatic PCa*. To address this hypothesis, *Specific Aim 1* will determine the function of Stmn1 S16 and the inter-relationship between S16, S25, S38 and/or S63 phosphorylation in regulating cell proliferation and a malignant phenotype, *Specific Aim 2* will determine the impact of Stmn1 phosphorylation on metastasis using a zebrafish xenograft model and *Specific Aim 3* will determine the clinical relevance of the different phospho-Stmn1s by analyzing human Tissue Microarrays representing the range of prostate cancer progression from benign to metastatic cancer. This approach will identify the major Stmn1 phospho-forms expressed during the different stages of prostate cancer progression and determine whether a specific isoform could serve as a biomarker for prostate cancer progression.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		**19b. TELEPHONE NUMBER** ***(include area code)***

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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Subject: Metastasis is a primary cause of cancer-related deaths, yet this process remains poorly understood. Stathmin (Stmn1) is an oncoprotein over-expressed in many cancers, including prostate cancer (PCa). While increased Stmn1 expression correlates with disease progression and poor prognostic outcome, it is not known whether Stmn1 overexpression correlates with biological activity. **Purpose**: Our previous work demonstrated that eliminating Stmn1 protein expression only modestly decreased PCa cell proliferation; instead, loss of Stmn1 protein greatly induced metastasis. Therefore, it is essential to determine how Stathmin activity can be selectively manipulated to block PCa cell growth without increasing the risk of more aggressive metastasis. This knowledge is critical for the development of targeted new therapies that block tumor progression and kill tumor cells. Since Stmn1 activity is controlled by four serine residues (S16, S25, S38, and S63) which are differentially phosphorylated by 4 different pathways, the purpose of this study is to determine which one of these serines (and associated pathway) regulates proliferation and which promotes metastasis. **Scope**: Our **hypothesis** is that *the first serine, S16, is the predominant serine that regulates PCa cell proliferation and acts as a gatekeeper to inhibit a cascade leading to metastatic PCa*. To address this hypothesis, *Specific Aim 1* will determine the function of Stmn1 S16 and the inter-relationship between S16, S25, S38 and/or S63 phosphorylation in regulating cell proliferation and a malignant phenotype; *Specific Aim 2* will determine the impact of Stmn1 phosphorylation on metastasis using a zebrafish xenograft model *in vivo* to track tumor formation, cell migration and metastasis; and *Specific Aim 3* will determine the clinical relevance of Stmn1 phosphorylation in human prostate cancer progression using commercial antibodies to the 4 phosphorylated serines in Stmn1 to analyze human Tissue Microarrays representing the range of prostate cancer progression - from benign to metastatic cancer. This approach will identify the major Stmn1 phospho-forms expressed during the different stages of prostate cancer progression and determine whether a specific isoform could serve as a biomarker for prostate cancer progression.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Stathmin, Stmn1, Phosphorylation, Ca2+/calmodulin-dependent kinase II, CaMKII, metastasis, prostate, epithelial mesenchymal transition, EMT, human prostate TMA

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

For Year, 1, the major goals of the project were:

Specific Aim 1: Elucidate the mechanisms by which Stmn1 phosphorylation regulates PCa cell growth and metastatic potential.	**Months**

Major Task 1: Generate Stmn1 phospho-mutant CRISPR/Cas9 constructs and cell lines	
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Milestone(s) Achieved: Obtain ACURO approval	4
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Milestone(s) Achieved: Obtain ACURO approval	4
Subtask 2: Preparation and analysis of human PCa TMAs	4-15

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 1: Elucidate the mechanisms by which Stmn1 phosphorylation regulates PCa cell growth and metastatic potential.

Major Task 1: Generate Stmn1 phospho-mutant CRISPR/Cas9 constructs and cell lines

Subtask 1: Generate the phospho-Stmn1 constructs using CRISR/Cas9 system

Overview of the CRISR/Cas9 system:

Figure 1 provides an overview of the CRISPR/Cas9 technology that was used to generate single amino acid (AA) substitution mutation DU-145 cell lines. Specific guide RNA's (gRNAs) were generated to allow the Cas9 protein to precisely target the Stmn1 sequences. This generates double stranded nicks into which the single strand Oligo (ssOligos) is inserted. Once this occurs, a complementary strand is then produced, thus creating a single AA substitution mutation. The steps and corresponding sequences taken for each of these components is outlined below.

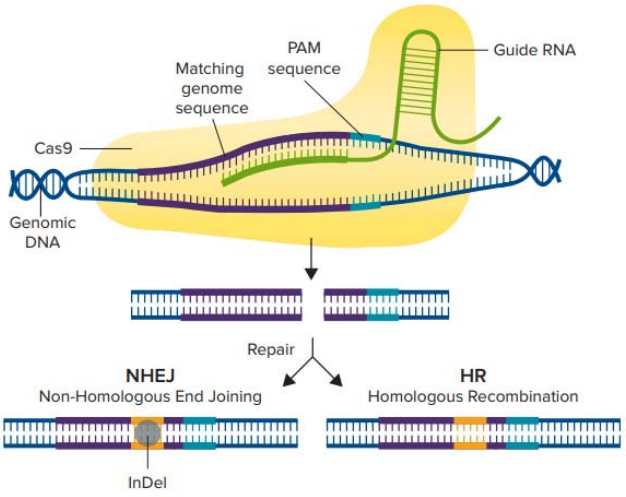

Fig.1. Overview of the CRISR/Cas9 system.

Step 1: Prior to designing the guide RNAs to be used in the CRISPR experiments, we sequenced the Stmn1 gene found in the DU145 cell line to confirm that it was wild type (i.e., to ensure that it did not contain any mutations). DU-145 cells were cultured under standard conditions (MEM/EBSS medium supplemented with 10% FBS), RNA was isolated, and cDNA was prepared using standard reverse transcription. Primers to the 5' and 3' ends of the gene were designed, and the gene product was amplified by PCR and sequenced at the Cincinnati Children's Hospital Medical Center DNA sequencing Core. This analysis confirmed that DU-145 cell express wild type Stmn1.

Step 2: Next, we designed guides (ssOligos) to replace all four serines with either an alanine (A) or glutamic acid residue (E), resulting in 8 specific targeted substitutions. The substitution mutation strategy is outlined below. The locations of the amino acids to be substituted are highlighted in green. The substitutions that have be made are highlighted in yellow.

Table 1. Serine substitutions to alanine or glutamic acid.

Serine location	Substitution
Alanine Substitution	
S16 - TCA	A16 - GCA
S25 - AGC	A25 - GCA
S38 - TCC	A38 - GCC
S63 - TCC	A63 - GCC
Glutamic Acid Substitution	
S16 - TCA	E16 - GAA
S25 - AGC	E25 - GAA
S38 - TCC	E38 - GAA
S63 - TCC	E63 - GAA

Design and Synthesis of IVT gRNA

We identified the genomic sequence for **wild type** Stmn1 (NCBI Reference Sequence: NC_000001.11), mapped out all exon and intron junctions as well as the precise locations for S16, S25, S38, and S63 on the genomic DNA. This information was provided to Thermo Fisher Scientific who then designed the gRNAs. Sequences and quality parameters were as follows:

Table 2. Design and Synthesis of IVT gRNA.

in vitro transcribed gRNA	gRNA Sequence (5'-3')	Concentration (µg/µL)	Total Yield (µg)	A260/280
S16E	GAACTGGAGAAGCGTGCCTC	2.01	201.42	2.26
S16A	GAACTGGAGAAGCGTGCCTC	2.06	206.49	2.25
S63A	AGACCTCAGCTTCATGGGAC	2.03	203.19	2.35
S25E	TTGAGCTGATTCTCAGCCCT	0.72	71.8	2.26
S25A	TTGAGCTGATTCTCAGCCCT	0.73	73.28	2.24
S38A	CCTTCTTCTTTGGAGGGGAA	1.47	147.25	2.36
S38E	CCTTCTTCTTTGGAGGGGAA	1.62	161.85	2.36
S63E	TTTCCAGTCCCATGAAGCTG	0.47	46.71	2.31

Step 4**: Design and Synthesis of crRNA (CRISPR RNAs)**
In conjunction with Thermo Fisher Scientific, the crRNA were designed by Invitrogen. Sequences and quality parameters were as follows:

Table 3. Design and Synthesis of IVT crRNA.

Serine location	Sequence
Alanine Substitution	
S16A	G*A*A*CUGGAGAAGCGUGCCUC + modified linker
S25A	U*U*G*AGCUGAUUCUCAGCCCU + modified linker
S38A	C*C*UUCUUCUUUGGAGGGGAA + modified linker
S63A	A*G*A*CCUCAGCUUCAUGGGAC + modified linker
Glutamic Acid Substitution	
S16E	G*A*A*CUGGAGAAGCGUGCCUC + modified linker
S25E	U*U*G*AGCUGAUUCUCAGCCCU + modified linker
S38E	C*C*U*UCUUCUUUGGAGGGGAA + modified linker
S63E	U*U*U*CCAGUCCCAUGAAGCUG + modified linker

Step 5: Design and Synthesis of ssOligos (single stranded Oligos)
CRISPR Oligo Sequences were designed by Invitrogen and are as follows:

S16A:
TEETTTTCTGAATTATAAATATAATCAATTCTAGATATCCAGGTGAAAGAACTGGAGAAGCGTGC
CGCAGGCCAGGCTTTTGAGCTGATTCTCAGCCOZC

S16E:
TEETTTTCTGAATTATAAATATAATCAATTCTAGATATCCAGGTGAAAGAACTGGAGAAGCGTGC
CGAAGGCCAGGCTTTTGAGCTGATTCTCAGCCOZC

S25A:
TFEATATCCAGGTGAAAGAACTGGAGAAGCGTGCCTCAGGCCAGGCTTTTGAGCTGATTCTCGC
ACCTCGGTCAAAAGAATCTGTTCCAGAATTCCCOOT

S25E:
TFEATATCCAGGTGAAAGAACTGGAGAAGCGTGCCTCAGGCCAGGCTTTTGAGCTGATTCTCGA
ACCTCGGTCAAAAGAATCTGTTCCAGAATTCCCOOT

S38A:
TZZCTTCTGCAGCTTCTAATTTCTTCTGAATTTCCTCCAGGGAAAGATCCTTCTTCTTTGGAGGGG
CAAGGGGGAATTCTGGAACAGATTCTTTTGAOOG

S38E:
TZZCTTCTGCAGCTTCTAATTTCTTCTGAATTTCCTCCAGGGAAAGATCCTTCTTCTTTGGAGGTT

CAAGGGGGAATTCTGGAACAGATTCTTTTGAOOG

S63A:
TOZGAAGCACTTCTTTCTCGTGCTCTCGTTTCTCAGCCAGCTGCTTCAAGACCTCAGCTTCATGG
GCCTGGAAAAAAAAGTTTAATAGGCTAGGCACZOT

S63E:
AOFCCCAGCCTGAATACATTTTAGAGTGCCTAGCCTATTAAACTTTTTTTTCCAGGAACATGAAG
CTGAGGTCTTGAAGCAGCTGGCTGAGAAACGAEFG

Note that the codes E, F, J, L, O, P, Q, and Z are for phosphorothioate bonds that indicate wobble. The letters have the following meanings:
F - Phosphorothioate-A
O - Phosphorothioate-C
E - Phosphorothioate-G
Z - Phosphorothioate-T

Milestone(s) Achieved: Production of stable cell lines expressing Stmn1serine substitutions

Subtask 2: Generate cell lines using phospho-Stmn1 CRISR/Cas9 constructs

We used the DU-145 cell line (ATCC® HTB-81™) to create cell lines in which each Stmn1 serine (S16, S25, S38, and S63) was substituted by a bi-allelic knock-in of alanine (A) to mimic dephosphorylation. These new lines have been termed S16A, S25A, S38A, and S63A. In a similar manner, each serine was substituted by a bi-allelic knock-in of glutamic acid (E) to mimic phosphorylation. These new lines have been termed S16E, S25E, S38E, and S63E. The steps to create the new substitution mutation cell lines were as follows.

Step 1: To begin generating the CRISPR lines, three 10 mm plates of DU-145 cells (~80% confluent) were trypsinized, washed three times with phosphate buffered saline (PBS), and resuspended in PBS. Cells were counted to determine the total number to use for subsequent experiments discussed below.

Step 2: Cas9 System and Electroporation
Approach 1. We selected the Invitrogen™ TrueCut™ Cas9 Protein V2 system. **In the first approach**, sgRNAs were used, where the crRNA and tracrRNA (transactivating CRISPR RNA) were engineered as a fusion sequence called single guide RNA (sgRNA). sgRNAs and ssOligos were diluted to stock solutions (100 µM concentrations in TE buffer). Stock solutions were stored at -20◦C. The sgRNAs and the CAS9 enzyme were mixed in Resuspension Buffer (R buffer) for 15 minutes at room temperature to allow the RNP (RiboNuclear Protein) to form. The RNP and ssOligos were then mixed with a calculated volume of DU-145 cells (resuspended in R buffer) so that the resulting cell concentration in each well of a 12 well plate was $1x10^5$ cells/mL. A master mix was created for each guide assuming four replicate wells per guide (Table 4).

Table 4. Total amount of master mix for each CRISPR reaction for each S substitution for approach 1.

Approach 1				
	Cas9 Protein	**sgRNA**	**ssOligo**	**R Buffer**
Serine to alanine substitution				
S16A	1 µL	0.48 µL	0.4 µL	18 µL
S25A	1 µL	1.32 µL	0.4 µL	17.2 µL
S38A	1 µL	0.92 µL	0.4 µL	17.6 µL
S63A	1 µL	0.48 µL	0.4 µL	18 µL
Serine to glutamate substitution				
S16E	1 µL	0.48 µL	0.4 µL	18.12 µL

S25E	1 µL	1.3 µL	0.4 µL	17.3 µL
S38E	1 µL	0.6 µL	0.4 µL	18 µL
S63E	1 µL	2 µL	0.4 µL	16.6 µL
Serine to glutamate substitution mixtures				
S16E	0.25 µL	0.12 µL	0.1 µL	17.75 µL
S25E	0.25 µL	0.32 µL	0.1 µL	
S38E	0.25 µL	0.15 µL	0.1 µL	
S63E	0.25 µL	0.25 µL	0.1 µL	
Serine to alanine substitution mixtures				
S16A	0.25 µL	0.12 µL	0.1 µL	17.8 µL
S25A	0.25 µL	0.33 µL	0.1 µL	
S38A	0.25 µL	0.23 µL	0.1 µL	
S63A	0.25 µL	0.12 µL	0.1 µL	
Serine to alanine and serine to glutamate substitution mixtures				
S16E	0.25 µL	0.12 µL	0.1 µL	15.55 µL
S25E	0.25 µL	0.32 µL	0.1 µL	
S38E	0.25 µL	0.15 µL	0.1 µL	
S63E	0.25 µL	0.25 µL	0.1 µL	
S16A	0.25 µL	0.12 µL	0.1 µL	
S25A	0.25 µL	0.33 µL	0.1 µL	
S38A	0.25 µL	0.23 µL	0.1 µL	
S63A	0.25 µL	0.12 µL	0.1 µL	

Briefly, DU-145 cells were diluted to a working concentration of 5 x 10^6 cells/mL after centrifugation and resuspension. Cells were rinsed with PBS (without Ca^{2+} or Mg^{2+}) and resuspended in the appropriate volume of R buffer. Cells resuspended in R buffer were added to each reaction master mix to bring the total volume to 40 µL (10 µL per reaction, with 4 replicates each) (Table 4). 500 µL of antibiotic-free media were placed into each well of the 24-well plates and pre-warmed. For electroporation, 10 µL of master mix (containing Cas9, gRNA or sgRNA, and ssOligo; or pEGFP) was drawn into the Neon pipette tip, placed into the Neon pipette station containing 3 mL of electrolytic buffer. The electroporation procedure took place under aseptic technique using a biological hood. Each sample was electroporated according to the manufacturers protocol for DU-145 cells (pulse voltage: 1260 v; pulse width: 20 ms; pulse number: 2). After electroporation, contents of the tip were immediately transferred into a well (of the 24-well culture plate) containing pre-warmed, antibiotic-free medium. Following electroporation, the culture plates were incubated at 37◦C with 5% CO_2. After 24 hours, media was changed and replaced with antibiotic-containing media.

Viable cells were obtained in all wells and cultured for a number of days. We purchased sterile plates for these cultures; however, unbeknownst to us, one sleeve of plates was not sterile. Therefore, the cell cultures became contaminated despite our best efforts to save them. Only two lines, S16A and S38A, were rescued. We therefore had to buy new gRNAs and repeat the experiment.

Approach 2. During the time we used the first approach, Invitrogen had modified their CRISPR strategy. Therefore, we used this **second approach** to repeat the assay. The tracrRNA and crRNA (gRNA) were ordered separately and annealed following the manufacturer protocol using a standard thermocycler. The Cas9 Protein V2 and gRNA were incubated in R buffer at room temperature for 15 minutes to allow the RNP to form. The RNP and ssOligos were then mixed with the appropriate volume of DU-145 cells resuspended in R buffer so that the resulting concentration in a 12 well plate was roughly 1x10^5 cells/mL. A master mix was created for each guide assuming four replicate wells per guide (Table 5).

Table 5. Total amount of master mix for each CRISPR reaction for each S substitution for approach 2.

Approach 2				
	Cas9 Protein	**gRNA (cr:tracr)**	**ssOligo**	**R Buffer**
Serine to alanine substitution				
S16A	1 µL	1.5 µL	4 µL	13.5 µL
S25A	1 µL	1.5 µL	4 µL	13.5 µL
S38A	1 µL	1.5 µL	4 µL	13.5 µL
S63A	1 µL	1.5 µL	4 µL	13.5 µL
Serine to glutamate substitution				
S16E	1 µL	1.5 µL	4 µL	13.5 µL
S25E	1 µL	1.5 µL	4 µL	13.5 µL
S38E	1 µL	1.5 µL	4 µL	13.5 µL
S63E	1 µL	1.5 µL	4 µL	13.5 µL

Viable cells were obtained in all wells and the new cell lines are described in Major Task 2.

Positive control: One treatment included electroporation using a GFP-expressing plasmid (pEGFP) so that quality of the transfection could be assessed visually on a fluorescent microscope; concentration of pEGFP per reaction was roughly 1 µg.

***Milestone(s) Achieved**: Production of stable cell lines expressing Stmn1serine substitutions.*

Major Task 2: Characterization of Stmn1 phospho-mutants in cell lines
Subtask 1: Analysis of Stmn1 phospho-mutants using cell culture assays

Outcome of Approach 1.
a. positive control. The positive control, i.e., cells transfected with pEGFP expressed the green fluorescent protein, demonstrating that the electroporation via the Neon method was successful.

b. CRISPR lines. Viable cells were obtained in all wells and cultured for a number of days. After 48 hours, we determined that cell viability was low (approximately 10%) as compared to the manufacturers estimate of 80% viability using their protocol (described above). However, the remaining cells were viable and began to proliferate. We had purchased sterile culture plates for these experiments; however unbeknownst to us, one sleeve of 5 culture plates was not sterile. Therefore, the cell cultures became contaminated despite our best efforts to save them. Since we had only used 3 plates, we took one plate from this sleeve and another plate from a different sleeve of 5 plates selected from the box at random and plated sterile medium in each as a test control. Only the plate from the sleeve of plates used in the CRISPR experiment was contaminated. Therefore, only two lines, S16A and S38A, were rescued. We purchased new gRNAs and repeated the CRISPR experiment.

Outcome of Approach 2. All plates were tested prior to use by adding sterile medium to a few wells and incubating the plates overnight to ensure that the medium remained sterile. Again, viable cells were obtained in all wells and cell viability was low (approximately 10%) as compared to the manufacturers estimate of 80%. The outcome is that we have eight new cell lines, S16A, S25A, S38A, and S63A as well as S16E, S25E, S38E, and S63E.

These lines are being cultured and as soon as the wells become confluent, we will begin the protocol for single cell selection. All single-cell selected clones will be sequenced to confirm the substitution mutations. The effects of the substitution mutations on cell proliferation, migration, and metastasis will be initiated once the substitution mutations have been confirmed.

Additional experiments. To confirm the effects resulting from the substitution mutations, we will use inhibitors that are selective for the pathways that regulate Stmn1 phosphorylation on each of these sites (summarized in Table 6). A number of these small molecule inhibitors could selectively inhibit (or promote) the phosphorylation of S16, S25, S38, and S63. We have begun the experiments to determine their effects on PCa proliferation. Briefly, the protocol for the following experiments was as follows. Cells are plated in 24-well plates at 20,000 cells/mL and incubated overnight at 37°C and 5% CO_2 to allow cell attachment. The next morning, medium is replaced with medium containing increasing concentrations of drugs (as shown in the figures), and cells are incubated for 3 days, after which they are trypsinized, and counted using the Trypan Blue viability test.

Table 6. Summary of pathway inhibitors and activators.

Pathway	Abbreviation	Serine	inhibitor	Activator
Ca2+/calmodulin-dependent protein kinase II	CamKII	S16	KN-93 - inhibitor KN-92 - an inactive derivative of KN-93 to serve as control	Oleic Acid
Mitogen-activated protein kinase signaling	MAPK	S25	SB203580	Anisomycin
$p34^{cdc2}$ (cell cycle regulator)	$p34^{cdc2}$	S38	roscovitine	Not known
Protein Kinase A signaling	PKA	S63	H 89 dihydrochloride	8-Bromo-cAMP

a. CAMKII Inhibitor KN93 (for S16) – Increasing doses (0μM – 30 μM) of KN93 and the KN93 inactive isomer, KN92, were analyzed. Fig.2 demonstrates that treating DU-145 cells with increasing concentrations of the small molecule inhibitor drug KN-93 greatly decreased DU-145 proliferation. In contrast, KN92 did not alter cell proliferation except at the highest doses. KN92 solubility is low and requires DMSO for solubility. Therefore, DMSO only was added in the same volumes as that used for DMSO+drug. This demonstrated that the decrease in cell number was due to DMSO toxicity at higher levels (data not shown).

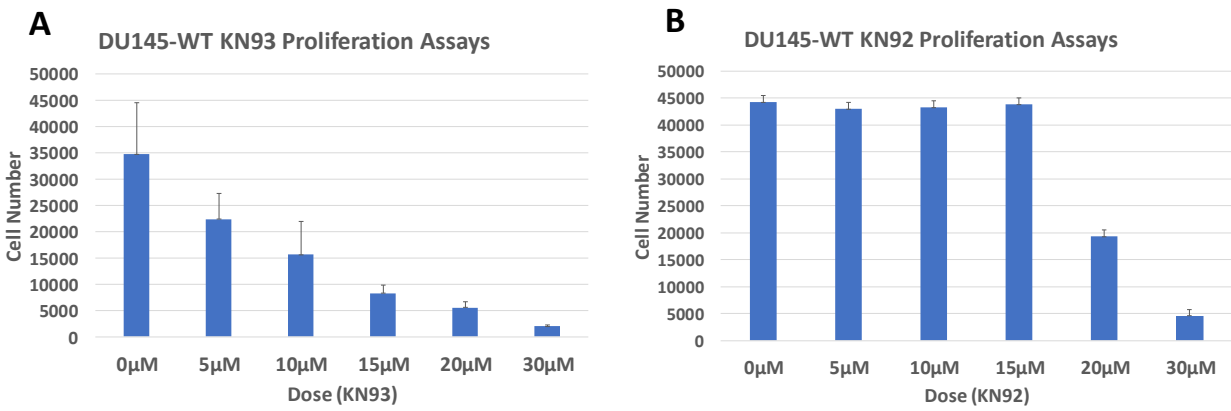

Fig.2. Treatment of DU-145 with the CAMKII Inhibitor KN93 (A) and the KN93 inactive isomer, KN92 (B).

b. p38/MAPK inhibitor SB203580 (for S25). As seen in Fig.3, increasing concentrations of SB203580 did not affect cell proliferation, suggesting that S25 is not directly involved in regulating cell proliferation in DU145-WT cells.

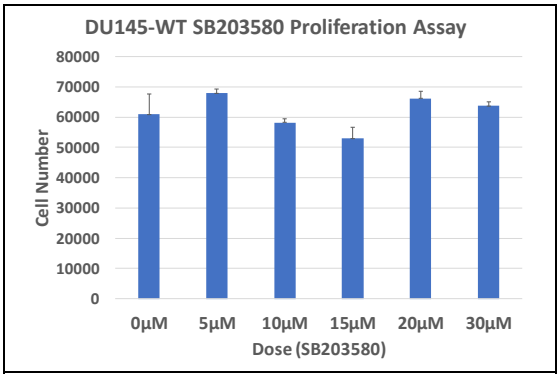

Fig.3. Treatment of DU-145 with the p38/MAPK inhibitor SB203580

c. The cell cycle regulator $p34^{cdc2}$ inhibitor, Roscovitine (for S63). Cyclin-dependent kinase 1 (Cdk1) also known as $p34^{cdc2}$ is encoded by the CDC2 gene. Cdk1 forms complexes that phosphorylate a variety of target substrates and phosphorylation of these proteins leads to cell cycle progression. Roscovitine inhibits this activity. Therefore, treating DU-145 cells with increasing concentrations of Roscovitine decreased cell proliferation in a dose-dependent manner (Fig.4).

d. Protein kinase A (PKA) inhibitor H89 (for S63). PKA is also known as cAMP-dependent protein kinase. Treating DU-145 cells with increasing concentrations of H89 decreased cell proliferation in a dose-dependent manner (Fig.5).

In summary, the phosphorylation status of S25, S38 and S63 in Stmn1 appears to regulate cell proliferation of DU145-WT cells while S25 does not appear to be involved in this process. The cell lines under development will complement this data set by having increased phospho-specificity and direct effect of Sthmn1 modulation on cell proliferation and other cellular properties.

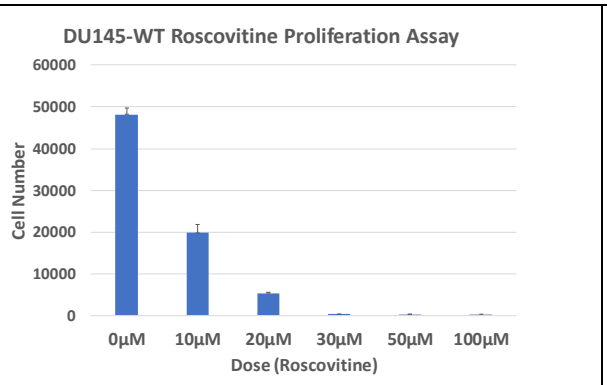

Fig.4. Treatment of DU-145 cells with the cell cycle regulator p34^{cdc2} inhibitor, Roscovitine

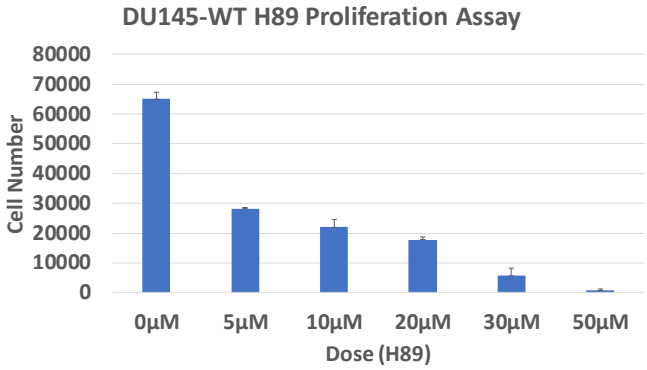

Fig.5. Treatment of DU-145 cells with the PKA inhibitor H89.

We have begun to analyze the inhibitors in the DU-145-shStmn1 cell line which does not express to confirm that the activity is specific to the phosphorylation status of Stmn1. This work will continue into the next year.

The first proliferation assay testing KN93 activity in DU-145-shStmn1 cells Proliferation determined that KN93 was inactive over all concentrations tested (Fig.6). This was not surprising since the cells did not express Stmn1 and confirmed that inhibition of proliferation was Stmn1-regulated.

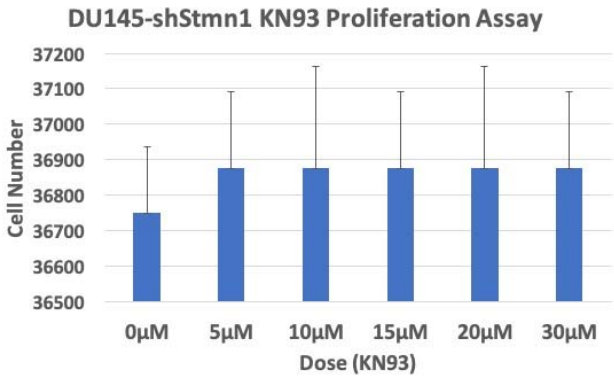

Fig.6. Treatment of DU-145-shStmn1 with the CAMKII Inhibitor KN93.

Specific Aim 2: Determine the impact of Stmn1 phosphorylation on metastasis in a zebrafish xenograft model in vivo

Subtask 1: Submit documents for ACURO approvals

Milestone(s) Achieved: Our ACURO protocol has been approved (letter attached)

Specific Aim 3. Determine the clinical relevance of Stmn1 phosphorylation in human prostate cancer progression.

Major Task 1: Characterization of Stmn1 phosphorylation in human PCa TMAs

Subtask 1: Submit documents for ACURO approvals

Milestone(s) Achieved: Our ACURO protocol has been approved (letter attached)

Subtask 2: Preparation and analysis of human PCa TMAs
The following tasks have been achieved in Year 1:

a. Commercial antibodies to the 4 phosphorylated serines in Stmn1 as well as to total Stmn1 and an IgG control were purchased.

b. Optimal conditions for staining with anti-total Stmn1 antibody were determined

c. We prepared a primary PCa TMA containing 2 tissue cores of tumor with matched benign prostate from approximately 127 patients and a metastasis TMA containing 2 tissue cores of up to 4 different metastasis sites per patient from approximately 45 patients. These specimens have been molecularly characterized with clinical and pathological information available which will allow an in-depth analysis of the correlation of Stmn1 phosphorylation with numerous variables including paired benign and tumor comparison, Gleason score, proliferation score, PSA levels, time to recurrence, heterogeneity within various metastatic sites in an individual, osseous versus soft lesions, and the most recent abiraterone/enzalutamide treatment.

d. The TMA was successfully processed.

e. The TMA is in the process of being sent to the Pathologist at UC for analysis. Since Dr. Masineni is no longer at UC, this work will be done by Dr. Jiang Wang.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Paul Deford is a graduate student on this project. The PI has provided one-on-one training on all aspects of this project. Paul has taken opportunity to learn from senior PhD student in other labs on how to perform Western blot analysis. In addition, Paul attends several seminar series, including the Wednesday Department of Environmental Health seminar series and the Cancer Cell Biology seminar series. Other training activities included presenting his project at laboratory meetings, at the Seminar Series for students in the Division of Genetics and Molecular Toxicology, at the ImunoTox seminar series, and as a poster at the UC Graduate Student Research Forum on Thursday, October 25, 2018.

Kirill Fedorov is a postdoctoral fellow who just started on the project. He is currently being trained in all the technologies used in the Laboratory and he is starting to perform cell culture-based experiments. He will continue to receive on-on-one training from the PI and attend the same seminar series indicated above.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Paul Deford presented a poster on CRISPR technology and generating cell lines at the UC Graduate Student Research Forum on Thursday, October 25, 2018. This provided opportunity for his to share his knowledge on Stathmin in cancer progression and how to generate cell lines using a CRISP-based substitution mutation strategy.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will continue following the Statement of Work in performing the next experiments including:

a. Generating singe cell selected cell lines for the substitution mutations

b. Completing the proliferation assays

c. Working on the migration and invasion assays,

d. Taking the training to work with zebrafish

e. Initiating the zebrafish assays in Aim 2.

f. Staining the remaining TMAs with phospho-antibodies to serines 16, 25,38 and 63.

4. IMPACT: *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to*:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable*:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page

numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to Report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

- **Website(s) or other Internet site(s)**

  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**

  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

- **Inventions, patent applications, and/or license**s

  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name: Susan Kasper, PhD
Project Role:
Researcher Identifier (e.g. ORCID ID): Dr. Kasper will apply for an ORCID ID.
Nearest person month worked: 3.6

Contribution to Project: Dr. Kasper was responsible for the experimental design of the research plan as well as the overall direction, administration and oversight of this research project.

Name: Paul Deford
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 12
Contribution to Project: Mr. Deford has performed work in the area of CRISPR technology, cell culture, and analysis of small molecule inhibitors.

Name: Kirill Fedorov, PhD
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Dr. Fedorov has just been recruited and is receiving training in the areas of CRISPR technology, cell culture, analysis of small molecule inhibitors, and zebrafish biology.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);

- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other.

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Not applicable.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Attached are 3 files indicating ACURPO approval of our protocols.