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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.

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REPLY TO
ATTENTION OF

September 11, 2019

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PC160590, Award Number W81XWH-17-1-0601 entitled, "Stathmin Phosphorylation as a Target for Blocking Metastasis in Prostate Cancer"

Principal Investigator Susan Kasper
University of Cincinnati
Cincinnati, OH

Dear Dr. Kasper:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, protocol PC160590 entitled, "Molecular Mechanisms of Cardiovascular Differentiation in Zebrafish," IACUC protocol number IACUC2019-0022, Protocol Principal Investigator Saulius Sumanas, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 06-SEP-2019 for the use of fish and will remain so until its modification, expiration or cancellation. This protocol was approved by the Cincinnati Children's Hospital Medical Center IACUC on 16-APR-2019.

Required Actions: When updates or changes occur, documentation of the following action or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)

- IACUC actions involving this protocol regarding
  a. any noncompliance;
  b. any deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  c. any suspension of this activity by the IACUC

- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program

- AAALAC, International status change

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Original Signed

Dawn C. Fitzhugh, VMD, MPH, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

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Dr. Angelica Schehr, Cincinnati Children's Hospital Medical Center
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Dr. Asif M. Rizwan, Congressionally Directed Medical Research Program (CDMRP)

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.

The major goals of the project are:

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	
Subtask 1: Generate the phospho-Stmn1 constructs using CRISR/Cas9 system Cell lines used: none.	1-5
Subtask 2: Generate cell lines using phospho-Stmn1 CRISR/Cas9 constructs Cell lines used: DU-145 [ATCC]	3-12
Milestone(s) Achieved: Production of stable cell lines expressing Stmn1serine substitutions.	12
Major Task 2: Characterization of Stmn1 phospho-mutants in cell lines	
Subtask 1: Analysis of Stmn1 phospho-mutants using cell culture assays Cell lines used: DU-145 [ATCC] and derivative DU-145/Stmn1 phospho-mutants + DU-145/shStmn1[made in our lab]	3-24
Milestone(s) Achieved: Evaluation of Stmn1 phospho-mutants	24
Specific Aim 2: Determine the impact of Stmn1 phosphorylation on metastasis in a zebrafish xenograft model in vivo	
Major Task 1: Analysis of Stmn1 phosphorylation on tumor formation and metastasis in vivo.	
Subtask 1: Submit documents for ACURO approvals	1-4
Milestone(s) Achieved: Obtain ACURO approval	4
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	1-4
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 the STMN1 phospho-mutant CRISPR/Cas9 constructs and cell lines
Subtask 1: Generate the phospho-STMN1 CRISR/Cas9 constructs

This task was completed in the previous report.

Subtask 2: Generate the cell lines stably expressing the STMN1 phospho-mutant constructs

Cell lines used: DU-145 [ATCC]

Previously: The CRISPR-generated DU-145 cell lines with the S16A, S16E, S25A, S25E, S38A, S38E, S63A, and S63E mutations (where S is serine, A is alanine, and E is glutamic acid) were initiated in the previous report.

Update: We are still in the process of characterizing the CRISPR lines. In addition, we have used combinations of guide RNAs to generate DU-145 cells where combinations of serines are substituted with A and/or E. This process is more labor-intensive since the Invitrogen CRISPR technology does not have a selection protocol, for example, a fluorescently-expressed protein which would allow sorting of fluorescent cells carrying the mutation, or a plasmid containing an antibiotic resistance gene, (e.g., puromycin), which would allow antibiotic selection of clones during cell culture. The outcome is that many more clones need to be sequenced to determine whether the mutations have been incorporated.

As an alternative strategy, we have used site directed mutagenesis (QuickChange Lightning Site-Directed mutagenesis kit -Agilent Technologies) and the pECFP-N1.STMN1 plasmid (purchased from Addgene, plasmid # 86783) containing the human STMN1 gene linked to a fusion protein sequence for ECFP to generate a series of plasmids containing STMN1 S-to-A and S-to-E mutations. The advantages of this approach are:

1) DU-145 cells expressing the mutant STMN1 protein can be selected by fluorescence sorting prior to cell line expansion and genomic sequencing to confirm the mutation(s), and

2) These plasmids can be transfected into our DU-145/shSTMN1 cell line which does not express the endogenous STMN1 protein, and therefore DU-145/shSTMN1 cells will only express mutant protein.

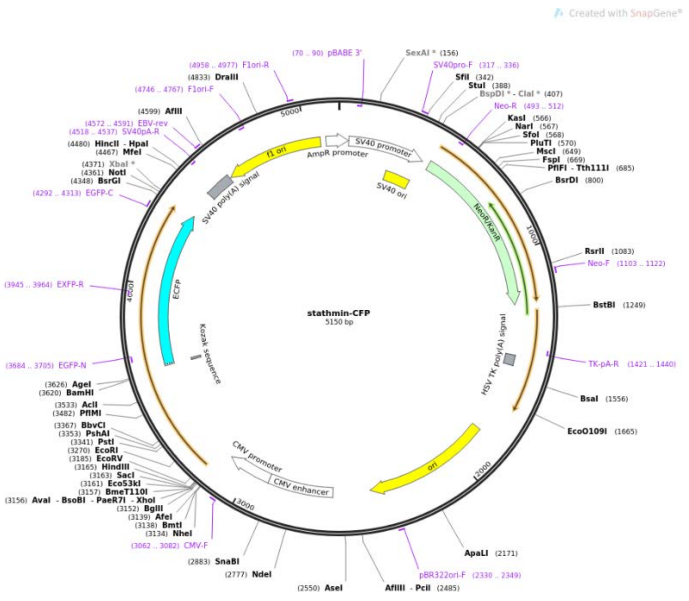


Figure 1. pECFP-N1.STMN1 plasmid to generate S-to-A and S-to-E mutations in the STMN1 gene using site-directed mutagenesis.

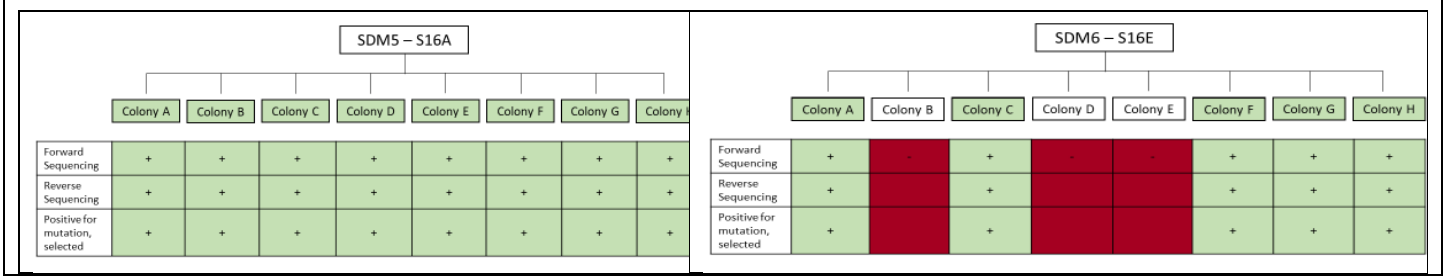
We first confirmed that the pECFP-N1.STMN1 plasmid contained the STMN1 gene by selecting single bacterial colonies and isolating and sequencing the plasmids. The resulting STMN1 sequence was confirmed by comparing it to the published STMN1 sequence, the verified STMN1 sequence expressed in our in-house DU-145 prostate cancer lines, and by standard plasmid restriction digest analysis.

Next, primers were designed (as outlined in Table 1) and purchased from Integrated DNA Technologies (IDT). Site-directed mutagenesis using the Quickchange Lightning kit was conducted as per the manufacturer's protocol. Briefly, reactions contained 5 uL reaction buffer, 100 ng of pECFP.N1.STMN1 plasmid DNA template, 125 ng of the forward and reverse primers for each mutation, 1 uL dNTP, 1.5 uL Quicksolution, and brought to 49 uL in ddH_20. One (1) uL of Quickchange Lightning enzyme was added to each reaction and amplified by PCR.

Table 1. Forward and reverse primers designed to generate mutant STMN1 gene in pECFP.N1.STMN1

Serine	Code	AA positions	S to A	Primers for replacing S with A	Primer length
16	TCA	**46,** 47, 48	**G**CA	R- 5'-aaagcctggcctgcggcacgcttctcc-3' F -5'-ggagaagcgtgccgcaggccaggcttt-3'	27 bp
25	AGC	**73, 74,** 75	**GC**C	5'-agattcttttgaccgaggggcgagaatcagctcaaaagcc-3' 5'-ggcttttgagctgattctcgcccctcggtcaaaagaatct-3'	40 bp
38	TCC	**112,** 113, 114	**G**CC	5'-ctgttccagaattccccccttgcccctccaaagaag-3' 5'-cttctttggaggggcaaggggggaattctggaacag-3'	35 bp
63	TCC	**187,** 188, 189	**G**CC	5'-gacctcagcttcatgggccttgcgtctttcttctg-3' 5'-cagaagaaagacgcaaggcccatgaagctgaggtc-3'	35 bp
Serine	**Code**	**AA positions**	**S to E**	**Primers for replacing S with E**	**Primer length**
16	TCA	**46,** 47, 48	**GA**A	5'-ctcaaaagcctggccttcggcacgcttctccagt-3' 5'-actggagaagcgtgccgaaggccaggcttttgag-3'	34 bp
25	AGC	**73, 74,** 75	**GAG**	5'-aacagattcttttgaccgaggctcgagaatcagctcaaaagcctg-3' 5'-caggcttttgagctgattctcgagcctcggtcaaaagaatctgtt-3'	45 bp
38	TCC	**112,** 113, 114	**GAA**	5'-aaagatccttcttctttggaggttcaagggggaattctggaacagattc-3' 5'-gaatctgttccagaattccccccttgaacctccaaagaagaaggatcttt-3'	49 bp
63	TCC	**187,** 188, 189	**GAA**	5'-cttcaagacctcagcttcatgttccttgcgtctttcttctgcagc-3' 5'-gctgcagaagaaagacgcaaggaacatgaagctgaggtcttgaag-3'	45 bp

Once amplified, the PCR product was treated with Dpn I restriction enzyme for 6 minutes to digest the parent plasmid and leave the mutated plasmid. The digested product was then transformed into XL10-Gold ultracompetent cells and grown in SOC broth for 1 hour at 37◦C. 10 – 50 uL of each transformed culture was then plated onto agar plates containing kanamycin for 16 hours. After 16 hours, 8 single colonies per mutation were selected and grown up in LB broth containing kanamycin for 16 hours and plasmid DNA was isolated from 1 mL bacterial suspension using Qiagen plasmid purification kit. Plasmid DNA was quantified using Nanodrop, and sequencing reactions to confirm the mutation were conducted using 600 ng of plasmid and either the forward or reverse pECFP.N1.STMN1 sequencing primer. The sense and antisense strands of each plasmid were sequenced by Sanger sequencing at the Cincinnati Children's Hospital Medical Center (CCHMC) Sequencing Core; and plasmids confirmed for carrying the mutation on both strands were selected, expanded in bacteria to generate glycerol stocks, and isolated in sufficient quantity to conduct the transfection assays. A summary of the characterization of the mutant clones is presented in Fig. 2.



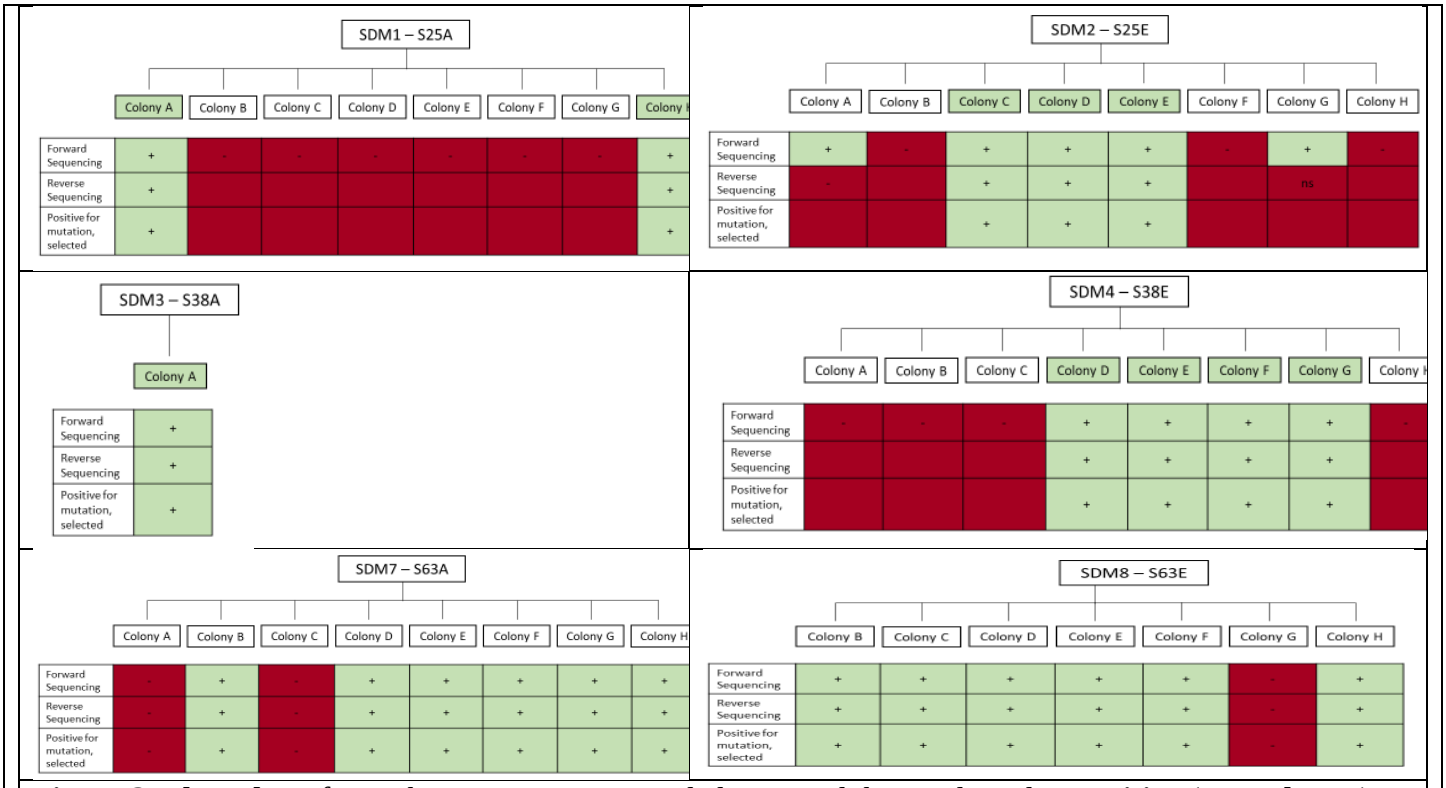


Figure 2. Flow chart for each mutation, sequenced clones, and those selected as **positive (green boxes)**. Top row, S16A, S16E; Second row, S25A, S25E; Third row, S38A, S38E; and bottom row, S63A, S36E.

In summary, we now have all of the single STMN1 mutations (S16A, S16E, S25A, S25E, S38A, S38E, S63A, and S63E) inserted into the human STMN1 sequence within the pECFP-N1.STMN1 expression vector. This vector will be transected into DU-145/shSTMN1 cells to determine the mechanisms by which STMN1 phosphorylation regulates PCa cancer cell proliferation and metastatic potential. Of particular advantage of this approach over CRISPR is the generation of the STMN/CFP fusion protein which will allow us to identify the cells expressing the mutant protein.

In addition, we designed double-stranded combinatorial oligonucleotides to determine the activity of one serine-of-interest and the impact that the remaining three serines had on that serine-of-interest. This was accomplished by substituting the serine-of-interest with an A while the other 3 serines were substituted with an E, and *vice versa* where the serine-of-interest was substituted with an E while the other 3 serines were substituted with an A. The oligonucleotides sequences (represented by the sense strand) are as follows:

S16A/S(25,38,63)E:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGCAGGCCAGGCTTTTGAGCTGATTCTC
GAACCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGAACCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGAACATGAAGC|TGAG
GTCTTG

S16E/S(25,38,63)A:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGAAGGCCAGGCTTTTGAGCTGATTCTC
GCCCCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGCCCCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGCCCATGAAGC|TGAG
GTCTTG

S25A/S(16,38,63)E:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGAAGGCCAGGCTTTTGAGCTGATTCTC
GAACCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGAACCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGAACATGAAGC|TGAG

GTCTTG

S25E/S(16,38,63)A:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGCAGGCCAGGCTTTTGAGCTGATTCTC
GAGCCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGCCCCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGCCCATGAAGC|TGAG
GTCTTG

S38A/(16,25,63)E:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGAAGGCCAGGCTTTTGAGCTGATTCTC
GAACCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGCCCCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGAACATGAAGC|TGAG
GTCTTG

S38E/S(16,25,63)A:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGCAGGCCAGGCTTTTGAGCTGATTCTC
GAGCCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGAACCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGCCCATGAAGCT|GAG
GTCTTG

S63A/S(16,25,38)E:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGAAGGCCAGGCTTTTGAGCTGATTCTC
GAACCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGAACCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGCCCATGAAGC|TGAG
GTCTTG

S63E/S(16,25,38)A:
CTTCTGAT|ATCCAGGTGAAAGAACTGGAGAAGCGTGCCGCAGGCCAGGCTTTTGAGCTGATTCTC
GAGCCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTGCCCCTCCAAAGAAGAAGGATCTTTCC
CTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGGAACATGAAGCT|GAG
GTCTTG

These double-stranded oligonucleotides are being subcloned into the corresponding region of the human STMN1 gene in the pECFP.N1.STMN1 plasmid through a straight-forward substitution of the wild type sequence with the mutant sequence using standard molecular biology methodology (plasmid cloning by restriction enzyme digest). The mutations will be confirmed via Sanger sequencing and expressed in DU-145/shSTMN1 cells. If one (or more) of the resulting mutant STMN1 proteins demonstrate a response in modulating PCa cancer cell proliferation and/or metastatic potential, we will generate additional permutations of S-to-A and S-to-E to determine their effects on the activity of that mutant STMN1 protein.

In summary, we have generated all the mutants required to investigate the mechanisms by which the individual serines in STMN1 modulate metastasis.

Major Task 2: Determine which STMN1 phospho-serine(s) regulates proliferation and/or metastatic phenotype

Subtask 1: Cell culture experiments to analyze cell proliferation, migration and invasion

Cell lines used: DU-145/STMN1 phospho-mutant, DU-145 and DU-145/shSTMN1

Previously: We used inhibitors that were selective for the pathways that regulate STMN1 phosphorylation on each of these sites

Table 2. 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

(summarized in Table 2). The CAMKII Inhibitor KN93 (for S16) greatly decreased DU-145 proliferation while the inactive analog KN92 did not alter cell proliferation (except for the highest two concentrations which were deemed toxic, as determined by the Trypan Blue viability assay). In addition, KN93 treatment did not affect DU-145/shSTMN1 cell proliferation, which is not surprising since these cells did not express STMN1, thus confirming that inhibition of proliferation was STMN1-regulated. In addition, both the cell cycle regulator p34cdc2 inhibitor, Roscovitine (for S38) and the Protein kinase A (PKA) inhibitor H89 (for S63) decreased cell proliferation, implying that they may modulate CAMKII/S16 activity. This will need to be confirmed in subsequent experiments.

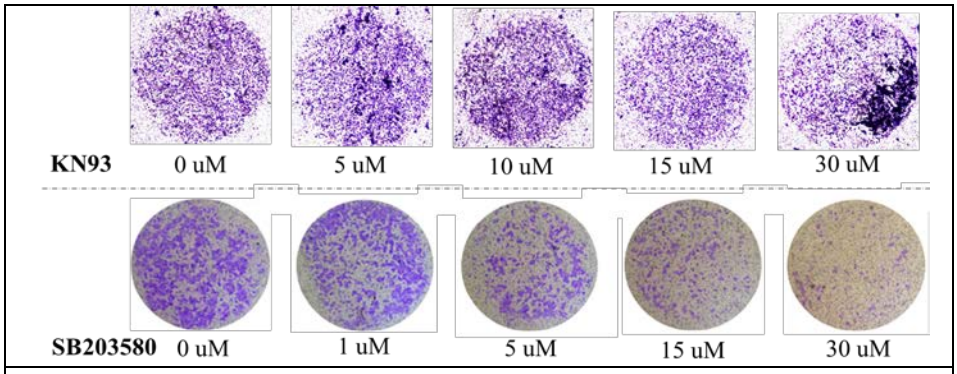


Fig. 5. DU-145 migration assay. Top panel, KN92 treatment. **Lower panel**., SB203580 treatment.

Update: We tested the CAMKII activator, oleic acid, and determined that proliferation of wild type (wt) DU-145 cells expressing normal STMN1 protein levels increased in response to oleic acid treatment in a dose-dependent manner. In contrast, preliminary experiments using oleic acid treatment implied that oleic acid did not regulate DU-145/shSTMN1 cell proliferation, as seen by a constant rate of proliferation under all doses tested.

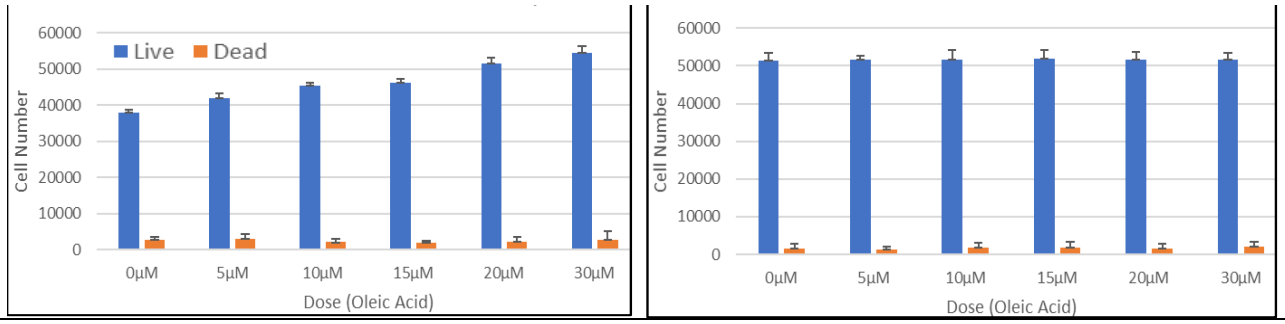


Fig. 3. Oleic Acid increased wt DU-145, but not DU-145/shSTMN1 cell proliferation. Left panel. wt DU-145 cell line. **Right panel.** DU-145/shSTMN1 cell line.

We have also initiated analysis of the effects of the p38/MAPK inhibitor SB203580 (for S25) on DU-145 cell proliferation. Treatment with SB203580 (for S25) did not dramatically increase, or decrease, cell proliferation, except at the highest dose used, suggesting that *S25 may not be directly involved in regulating proliferation*. The high dose was not toxic using the Trypan Blue viability assay and will be confirmed using western blot analysis with antibodies that evaluate apoptosis, e.g., cleaved caspase 3.

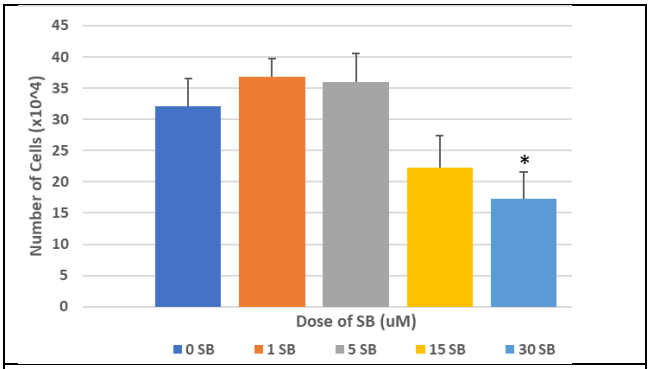


Fig. 4. DU-145 proliferation assay. SB203580 treatment did not greatly modulate cell proliferation, except at the highest dose used. * p = 0.02

To determine whether CAMKII and/or p38/MAPK promoted a metastatic phenotype, DU-145 cell migration was analyzed using the Neuroprobe assay as described previously (Williams et al., 2017. Inhibition of Stathmin1 Accelerates the Metastatic Process. Cancer Res. 72:5407-17). As shown in Fig. 5, KN93-mediated inhibition

of CAMKII signaling did not modulate DU-145 cell migration at all concentrations tested while SB203580-mediated inhibition of p38/MAPK signaling decreased DU-145 cell migration in a dose-dependent manner.

In summary, these observations indicate that CAMKII primarily regulates DU-145 cell proliferation while it has little effect on cell migration, a process that facilitates metastasis. These data support the premise that the CaMKII pathway is a rational target for inhibiting PCa cell proliferation without activating a metastatic phenotype. We are currently performing western blot analyses to determine which cell cycle regulators promote DU-145/shSTMN1 cell proliferation where STMN1 is not expressed and spindle formation is compromised, as compared to wt DU-145 cell proliferation under conditions where STMN1 regulates normal spindle formation. In addition, invasion and western blot assays are being done as described in the Williams *et al*. 2012 reference to confirm that CAMKII signaling does not induce a metastatic phenotype, as determined by the inability to induce MMP2 and MMP9 expression or promote cell invasion (as measured by the ability of DU-145 cells to digest and invade through Matrigel).

In contrast, p38/MAPK signaling primarily appears to regulate cell migration and metastatic potential while having little effect on DU-145 cell proliferation. These data begin to separate out the differential functions of the individual serines and infer that if a drug inhibited S16 activity alone, it would selectively block PCa proliferation; however if the drug inadvertently activated the p38/MAPK pathway, a side-effect of the treatment would be the emergence of metastasis. We are currently performing invasion and western blot assays to confirm that p38/MAPK signaling induces metastasis, as seen by induction of MMP2 and MMP9 expression and promotion of cell invasion.

Major Task 3: RNA-seq analysis to develop profiles that distinguish between cell proliferation and metastatic potential for prediction of PCa progression and/or metastasis

Subtask 1: RNA-seq, pathway analysis, and biological validation of key genes. The later date of this task is to ensure that the CRISP/Cas9 cell lines are well-characterized and optimal cell lines are selected for the RNA-seq analysis.

Cell lines used: DU-145/STMN1 phospho-mutant, DU-145 and DU-145/shSTMN1cell lines

This task will be addressed in the coming year.

Major Task 4: Analysis of small molecule inhibitors with/without androgen deprivation to inhibit PCa cell growth

Subtask 1: Test small molecule inhibitors to individual phospho-serines with/without androgen deprivation in cell culture

Cell lines used: DU-145, DU-145/AR (made in our laboratory), LNCaP [ATCC], LAPC4 [provided by Dr. Charles Sawyers]

This task will be addressed in the coming year.

Milestone(s) Achieved:

- *We have generated the CRISPR lines. These are still being selected.*

- *We have generated pECFP-N1.STMN1 expression vectors for all of the single STMN1 mutations (S16A, S16E, S25A, S25E, S38A, S38E, S63A, and S63E). Of particular advantage is the STMN/CFP fusion protein will allow us to identify the cell lines expressing the mutant protein. The mutant STMN proteins will be expressed in DU145/shSTMN1 cells to determine their effects on PCa cell proliferation and metastatic potential.*

- *Double-stranded combinatorial oligonucleotides have been made and are being subcloned into the pECFP-N1.STMN1 expression vector. These combinatorial mutant proteins will be expressed in DU145/shSTMN1 cells to investigate how the phosphorylation-mediated activity of one serine-of-interest is modulated by the*

remaining three serines.

- Our data indicate that the CaMKII pathway is a key regulator of PCa cell growth, but not metastasis. In contrast, the p38/MAPK pathway is a key regulator of metastatic potential, but not PCa cell proliferation.

Specific Aim 2: Determine the impact of STMN1 phosphorylation on metastasis in a zebrafish xenograft model in vivo

Major Task 1: Delineate the actions of STMN1 phosphorylation in mediating tumor formation and metastasis in vivo.

Subtask 1: Culture and provide cell lines, determine optimal drug doses, and perform xenograft experiments in vivo.

Cell lines used: DU-145 and DU-145/shSTMN1

Update: Cell lines are being cultured for each experiment. We are in the process of determining optimal drug doses.

We have initiated the zebrafish assays. The first task was to generate the *casper/flk:mCherry* zebrafish line carrying the Tg(mpv17a9/*mitfaw2ix*) mutation (*Casper*) and the *kdrl:mCherry* gene which was expressed in all endothelial cells. The resulting line was a transparent zebrafish line that lacked pigment in its skin and scales, and had a brilliantly red tagged vasculature which was due to the selective expression of fluorescent red mCherry protein in endothelial cells. To accomplish this, the *Tg(kdrl:mCherry)* zebrafish line was crossbred with the *casper* zebrafish line (purchased from Zebrafish International Resource Center). Embryos were selected for *kdrl:mCherry* red fluorescence, and grown to sexual maturity at 3 months of age. *kdrl:mCherry* positive adults were then back-crossed with the *casper* zebrafish line and embryos positive for krdl:mCherry red fluorescence but lacking pigmentation were selected. These embryos were grown to sexual maturity which took another 3 months. The *casper/flk:mCherry* line is housed and maintained in the aquatic facilities at Cincinnati Children's Hospital Medical Center (CCHMC) and embryos for each experiment are generated through in-crossed breeding. Embryos are incubated at 28°C prior to injection and 32°C post-injection.

The first assays were performed to optimize the injections and determine the average tumor and metastasis numbers. The wt DU-145 cell line was used for these experiments since they will serve as controls for all DU-145-derived cell lines. Briefly, cells were stained with the green fluorescent living dye, CMFDA (5-chloromethylfluorescein diacetate) according to the manufacturer's protocol. The advantages of this dye are: i), it is taken up by cells and passed to daughter cells through cell division, ii) the green fluorescent cells remain bright green through the length of the experiment and iii), metastasis can be tracked through imaging the zebrafish embryos. In addition, many initial technical replicate studies were conducted to optimize the injection procedure. After the injection procedure became routine, we performed three optimization experiments, and the preliminary results and experimental details are summarized in Table 3.

Table 3. Summary of preliminary results and experimental details from three optimization zebrafish experiments

	Preliminary Experiment 1	**Preliminary Experiment 2**	**Preliminary Experiment 3**
Cell line	wt DU-145	wt DU-145	wt DU-145
Passage number	191	11	82
Cell density (cells/mL)	8.6x10^6	8.25x10^6	2.3x10^7
Zebrafish line	Casper/flkmCherry	Casper/flkmCherry	Casper/flkmCherry
Injection conditions			
pressure balance	-1.5	-1.5	-1.5
pulse	50 ms	50 ms	50 ms
inject pressure	5-10 PSI	5-10 PSI	5-10 PSI

injection time	50-100 ms	50-100 ms	50-100 ms
needle pull	pull 9	pull 9	pull 9
Injection Data			
Injected zebrafish	40	37	50
Positive tumors	34	37	25
Metastasizing tumors 24 hrs	1	NA	2
Metastasizing tumors 48 hrs	0	2	4
Metastasizing tumors 72 hrs	1	2	0
Total percent metastasis	6	11	24

These data indicated that wt DU-145 cells metastasized in 14% of injected zebrafish. Representative images detailing tumor formation and the tracking of metastatic lesions are shown in Fig. 6 and 7. The low metastatic rate is not surprising since DU-145 cells exhibit a moderate metastatic rate as compared to the highly metastatic PCa cell line, PC3].

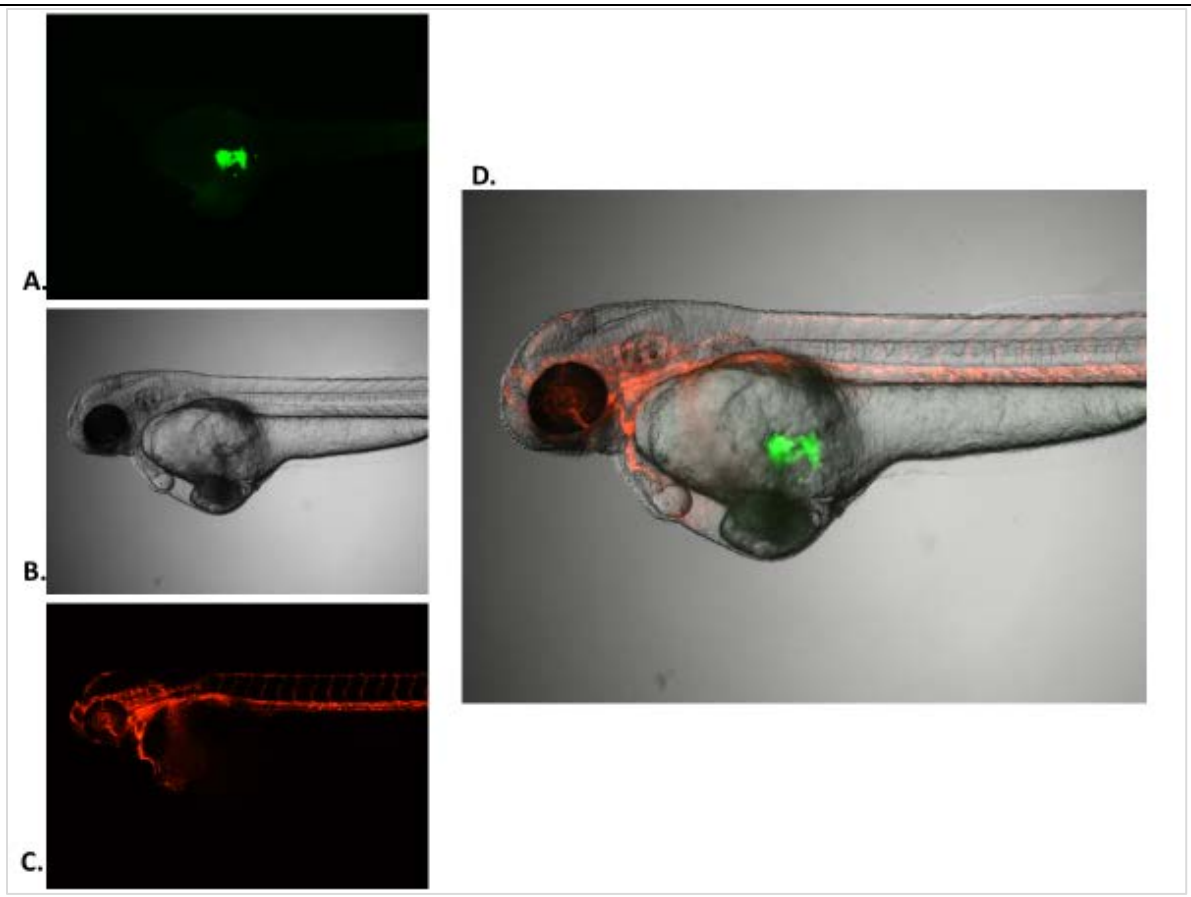


Fig. 6. Representative image of zebrafish xenograft assay. **A**. GFP filter of CMFDA-stained DU-145 cells into the perivitelline space in 48-hour post fertilization *Casper/flk:mCherry* zebrafish. **B**. Bright field filter of same embryo. **C**. Texas red filter of same embryo for imaging vasculature. **D**. Composite Z stack image generated by overlaying A, B, and C.

Analysis of the metastatic lesions showed that most lesions were detected in the tail region. However, a few lesions were detected in the brain (as labeled in Fig. 7). Since the DU-145 cell line was initially established from a PCa lesion that had metastasized to the brain, our data suggests that these PCa cells could still metastasize to this site.

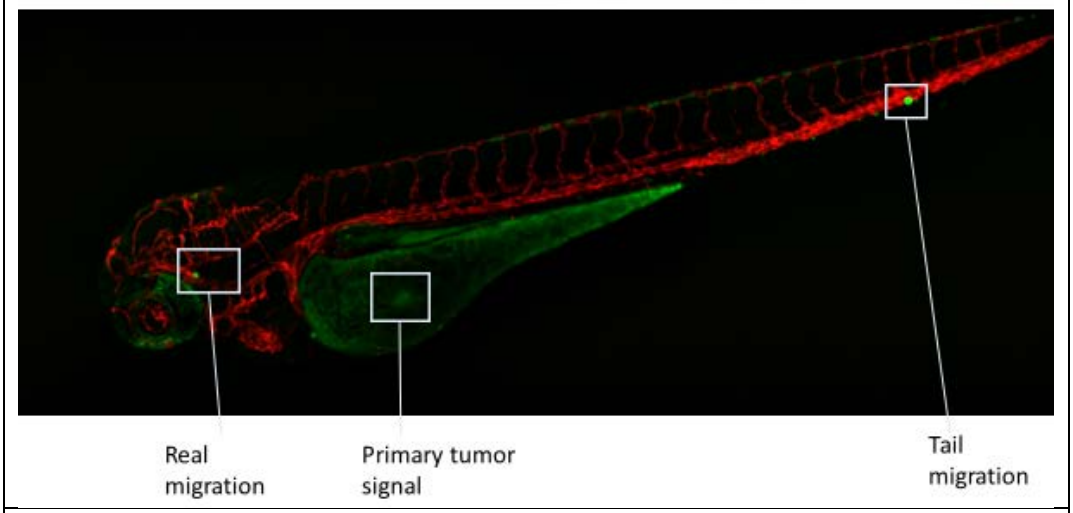


Fig. 7. Representative image of zebrafish xenograft assay containing primary tumor migration to brain and tail (as marked in image). Image taken using confocal microscopy.

The *Casper/flk:mCherry* zebrafish will be used to determine the ability of DU-15 cells expressing pECFP-N1.STMN1 expression vectors for the single STMN1 mutations (S16A, S16E, S25A, S25E, S38A, S38E, S63A, and S63E) to form tumors and metastasize. An advantage is that these cells express the cyan blue fluorescent protein which will be highly visible within mCherry red-tagged endothelial cells. In addition, zebrafish will be injected with wt DU-145 cells stained with CMFDA and treated with small molecule inhibitors, e.g., SB203580, to determine their effects on tumor formation and metastasis *in vivo*.

Major Task 2: Determine the actions of PCa cells carrying STMN1 S/E and S/A substitutions in mediating tumor formation and metastasis
Subtask 1: Culture and provide STMN1 phospho-mutant cell lines, test mutations for their activity to promote/inhibit tumor formation and metastasis in vivo
Cell lines used: DU-145/STMN1 phospho-mutant, DU-145 and DU-145/shSTMN1cell lines
This task will be addressed in the coming year.

Major Task 3: Pre-clinical analysis of small molecule inhibitors with/without antiandrogen treatment to inhibit tumor formation and metastasis in vivo
Subtask 1: Small molecule inhibitors to individual phospho-serines will be tested with/without androgen deprivation.
Cell lines used: DU-145, DU-145/AR
This task will be addressed in the coming year.

Milestone(s) Achieved:
- *We have established the casper/flk:mCherry zebrafish line.*
- *The injection protocols have been optimized.*
- *The pECFP-N1.STMN1 expression vectors for the single STMN1 mutations (S16A, S16E, S25A, S25E, S38A, S38E, S63A, and S63E) have been generated and characterized and are ready for analysis in the zebrafish xenograft assay.*

Specific Aim 3: Determine the clinical relevance of STMN1 phosphorylation in human prostate cancer progression.
Major Task 1: human PCa TMA analysis
Subtask 1: Preparing TMAs (required when using antibodies to phosphorylated proteins), probing TMAs with phospho-serine specific antibodies

Previously: The TMA for total STMN1 protein expression was previously completed
Update: The remaining four TMAs have been processed using commercial antibodies specific for the four phosphorylated serines (S16, S25, S38, and S63).

Subtask 2: Scoring of TMAs and analysis of the data

This task will be addressed in the coming year. We are now in the process of scoring the results. Since the TMAs represent the range of prostate cancer progression - from benign to metastatic cancer – this analysis will determine the major phospho-form(s) expressed during the different stages of prostate cancer progression. in addition, since these specimens have been molecularly characterized with clinical and pathological information available, 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, this data will allow an in-depth analysis of the correlation of Stmn1 phosphorylation with these variables.

Milestone(s) Achieved:
- *Processing of the human PCa TMAs is complete.*
- *Evaluation of the primary phospho-serines expressed in benign, low-grade, high grade, and metastatic PCa and correlation of STMN1 phosphorylation with disease progression, metastasis and antiandrogen treatment are in process.*

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 PhD student on this project. The PI has also accepted Alison Pecquet as a PhD student on this project. The PI has provided group and one-on-one training on all aspects of this project. There are group lab meetings every Monday morning to discuss topics of common interest, e.g., cell biology, molecular biology, normal prostate development, mechanisms of metastasis, lab protocols, and new technologies. We also hold one-on-one- meetings every Thursday to discuss the previous week's work in detail, to analyze and interpret data, and to plan the next week's research. The students also attend several seminar series, including the Wednesday Department of Environmental Health seminar series and the Cancer Cell Biology seminar series. Other training activities included presenting their 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 posters at the annual UC Graduate Student Research Forum.

We had hired Dr. Kirill Fedorov as a postdoctoral fellow. However once he began his research project, he determined that the work was not within his scope of interest and therefore resigned. This research is now being conducted by Alison Pecquet.

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.

Both students presented posters at the UC Graduate Student Research Forum.

Paul Deford also presented a poster an the Annual Meeting of the Society for Basic Urologic Research (SBUR) which was held Nov 08 - 11, 2018, in Palm Springs, CA. National and international scientists involved on urologic research, including prostate cancer research, attend the SBUR Annual Meetings. Thus, Paul had opportunity 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. Continue characterizing the CRISPR lines

b. Determining the effects of single and combinatorial pECFP.N1.STMN1 mutants generated by site-directed mutagenesis

c. Working on the proliferation, migration and invasion assays,

d. Confirming the data generated by the CRISPR and pECFP.N1.STMN1 mutants using small molecule inhibitors that selectively inhibit the individual serines

e. Performing the xenograft assays using the new mutant STMN1 cell lines

f. Analyzing the data generated the completed 5 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).

We have developed a series of unique expression vectors that generate specific STMN1 mutant proteins. These vectors would be made available to the research community upon publication of our work.

Our data indicate that the CaMKII pathway is a key regulator of PCa cell growth, but not metastasis. In contrast, the p38/MAPK pathway is a key regulator of metastatic potential, but not PCa cell proliferation.

These observations begin to separate out the differential functions of the individual serines in Stathmin; and this knowledge could be used in future to develop treatment strategies that selectively kill PCa cells and limit/do not cause unwanted side-effects. For example, the observations suggest that a drug that only inhibits the CaMKII pathway (and S16 activity) would basically block PCa proliferation and decrease tumor burden; and show decreased ability of activating other processes that could lead to metastasis. However the p38/MAPK pathway that activates S25 is well-known to promote metastasis. Therefore, if a drug inadvertently activated S25, a side-effect of the treatment could be the emergence of metastatic disease.

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 licenses**

  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.*

We have developed a series of unique expression vectors that generate specific STMN1 mutant proteins. These vectors would be made available to the research community upon publication of our work.

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. In addition, his work focuses on the mechanisms of S16 (in context of the other serines) in regulating PCa cell proliferation, but not metastasis.

Name: Alison Pecquet
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 12
Contribution to Project: Ms. Pecquet has generated the series of unique expression vectors that generate specific STMN1 mutant proteins in cultured cells. In addition, her work focuses on the role of S25 (in context of the other serines) in regulating metastasis, but not cell proliferation. She has receiving training in zebrafish biology and is performing the xenograft assays.

Name: Kirill Fedorov, PhD
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 4
Contribution to Project: Dr. Fedorov was recruited to work on aspects of the project including, CRISPR technology, cell culture, analysis of small molecule inhibitors, and zebrafish biology. However once he began his research project, he determined that the work was not within his scope of interest and therefore he resigned on January 1, 2019. This research has been seamlessly taken over by Alison Pecquet as a PhD project. Therefore, the project has not suffer any interruption in the performing of the work.

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.

CCHMC Institutional Animal Care and Use Committee

The following protocol was approved by the IACUC on 4/16/2019.

ID	PI	Submission Type
IACUC2019-0022	Saulius Sumanas	IACUC Protocol

Annual Review Date: 4/16/2020

Renewal Date: 4/16/2022

All cage labels must reflect the above protocol ID within 5 - 7 business days.

Cincinnati Children's Hospital Medical Center
Office of Research Compliance and Regulatory Affairs
3333 Burnet Avenue, Cincinnati, Ohio 45229-3026
1-513-636-8039 | orcra@cchmc.org

ACURO Animal Use Appendix for Research Involving Animals
FULL VERSION

*****NOTE: ACURO will ONLY review protocols that have been approved by your IACUC. *****
****Animal work MAY NOT be initiated until the awardee receives ACURO approval. ****
Animal work initiated without ACURO approval is noncompliant and may not be funded.

Institutions using DOD funds to support the use of animals in research, development, testing and evaluation (RDT&E) projects must provide electronic copies of the following documents to the US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Animal Care and Use Review Office (ACURO) for review and approval prior to initiation:

A. a copy of the **IACUC-approved institutional protocol(s)** and documentation of initial IACUC approval- Note: If the protocol expires within the next 60 days, please wait to submit until your protocol is renewed.

B. a copy of all existing IACUC-approved protocol amendments or modifications and documentation of IACUC approval (**future modifications or amendments** must be reviewed and approved by ACURO **PRIOR** to implementation)

C. completed corresponding ACURO Animal Use Appendix for each IACUC-approved protocol.

D. a copy of the research site's most recent facility inspection report from the USDA (This requirement also applies to all subcontractors using animals in support of DoD-funded projects or programs)

Specific information requested in the following animal use Appendix is derived from requirements in the Animal Welfare Regulations (AWRs), the *Guide for the Care and Use of Laboratory Animals*, and other applicable Federal and DOD regulations. The DOD policies and requirements for the use of animals in DoD-supported RDT&E are described in DoD Instruction 3216.01, *Use of Animals in DOD Programs*, dated September 13, 2010 and Army Regulation 40-33, *The Care and Use of Laboratory Animals in DOD Programs*, dated February 16, 2005. These requirements differ from those of other funding agencies. Use of the Appendix is intended to meet the requirements of these documents.

Questions concerning animal use and review should be directed to the USAMRMC ACURO:

Phone: 301-619-6694
Email: Usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil

~~~~~~

Each section of this Appendix must be completed. To assist you in completing this appendix, instructions and explanations are provided as *hidden, red and italicized text*. To view the instructions and/or examples for each section, select the "**Show/Hide ¶**" button on your tool bar (the button itself appears as the ¶ symbol). To print the hidden text, select "Print Hidden Text" in the print options section. It is important that you carefully **read the instructions** for each paragraph to ensure you provide a comprehensive response. Begin typing responses after the colon (":") for each section to ensure your typing is not within the hidden text.

Submit electronic copies of the appendix only; please **do not** submit printed copies to ACURO.
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Refer to the cover page for directions on viewing the hidden text in this document.
WHEN COMPLETING THIS APPENDIX, INCLUDE ONLY ANIMALS, EXPERIMENTS AND PROCEDURES THAT ARE FUNDED BY THIS GRANT/CONTRACT

1. DOD Funding of Protocol

[X] Yes [ ] No The attached IACUC-approved protocol contains experiments that are not funded by this DOD award. If all experiments within the protocol are funded, STOP! You must use the **abbreviated version** of the Appendix.

2. Administrative Data:

DOD Award PI Name:	Susan Kasper
DOD Award PI Email:	kaspersn@ucmail.uc.edu
DOD Award PI Phone:	**513-558-2126**
DOD Funding Source:	
DOD Grant/Award/Proposal Number:	**W81XWH-17-1-0601**
Institution Where Animal Studies are Conducted:	**Cincinnati Children's Hospital Medical Center**
IACUC Protocol PI Name	**Saulius Sumanas**
IACUC Protocol PI Email	**saulius.sumanas@cchmc.org**
IACUC Protocol PI Phone	**513-803-0435**
IACUC Protocol Title	**Molecular Mechanisms of Cardiovascular Differentiation In Zebrafish**
IACUC Protocol Number	**IACUC2019-0022**
Attending Veterinarian Name	**Sai Tummala**
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Institutional Grants Manager Name	**Heather Kinsman**
Grants Manager Email	**Heather.Kinsman@cchmc.org**
Grants Manager Phone	**513-803-6713**

3. Brief Objective Summary:

Stathmin (Stmn1) is an oncoprotein overexpressed in many cancers, including prostate cancer. Stmn1 phosphorylation regulates cancer cell migration and metastasis. We will use zebrafish xenograft model to determine the impact of Stmn1 phosphorylation on metastasis

4. Total Number of Animals Used (by Species) and USDA Pain/Distress Category- Note: All animals proposed for use must be accounted for in this section, including species not currently regulated by the USDA.

USDA Pain/Distress Category Definitions:

- **Column B*:*** Animals being bred and animals being held for use but not yet used for such purposes.
- **Column C:** Animals that will experience no more than slight or momentary pain or distress
- **Column D:** Animals that will potentially experience accompanying pain or distress and for which appropriate anesthetic, analgesic, or tranquilizing drugs **WILL** be used.
- **Column E:** Animals that will potentially experience accompanying pain or distress and for which appropriate anesthetic, analgesic, or tranquilizing drugs **WILL NOT** be used.

SPECIES	HIGHEST USDA PAIN/DISTRESS CATEGORY (B, C, D, E*) See table below for definitions	TOTAL NUMBER
Danio rerio (zebrafish) adults	B	100
Zebrafish embryos / larvae	C	5000

5. Statistical/Other Basis for Number of Animals Used:
We estimate that 100 adults will be needed to generate 5,000 embryos in the period of 2 years for the study.

6. Column E only- If any animals are listed in USDA Column E (Unalleviated Pain or Distress), the PI must provide a scientifically valid justification for withholding pain relieving medication:

7. Consideration of Alternatives to Painful Procedures (Columns D or E only). If there are no animals listed in USDA Column D or E, mark this section "N/A." If **any animals are listed in USDA Column D or E**, the PI must perform this literature search. **You MUST provide a narrative summary of the results of the literature search for alternatives to painful procedures.** The Animal Welfare Act regulations specifically state that the P.I. must provide a narrative description of the methods and sources that he/she used to determine that alternatives to the painful or distressful procedure(s), including those in which pain or distress is alleviated, were not available: **DOD regulations require this for all animals undergoing painful procedures including species not covered by the Animal Welfare Act.**
N/A

8. Rationale for Using Animals:
In vivo model is needed to study metastasis.

9. Animal Model Rationale:
Zebrafish is a lower vertebrate alternative to mouse model. Its tramsparent embryos and fast develop makes it a superior model for metastasis studies.

10. Animal Procurement:

☐ Yes ☒ No Does the protocol involve Animal Welfare Act-regulated species?

☐ Yes ☐ No If so, are the animals obtained legally from suppliers licensed by the USDA?

If the supplier claims exemption from USDA licensing, provide confirmation from the research site's IACUC that the exemption criteria have been met.

[ ] Yes [X] No Are wildlife species are used? If so, provide IACUC assurance that animals have been obtained legally and provide copies of all applicable capture and use permits.

11. Experimental Design: DU-145 prostate cancer derived wild-type and Stmn1 knockdown cell lines will be labeled with the green fluorescent dye CMFDA and engrafted into wild-type or vascular endothelial reporter fli1:GFP zebrafish embryos at 2 days-post-fertilization. Embryos will be generated by mating adults raised in the in-house facility. Tumor engrafted and control zebrafish embryos will be incubated in varied concentrations of small inhibitors of pathways that regulate Stathmin phosphorylation (including KN-93, KN-92, SB203580, roscovitine and H 89 dihydrochloride compounds). Subsequently embryos will be anesthetized in 0.25% Tricaine and imaged by fluorescent microscopy at 3 - 10 days post fertilization to analyze tumor formation and metastasis. Similar analysis will be also performed using SU-145 cells that carry different mutations in Stmn1. All tumor cell work will be performed in BSL2 facility under IBC approved protocols. At the end of experiment, tumor injected embryos will be euthanized using 1% Tricaine and disposed as biohazardous waste.

12. Specific Procedures / Technical Methods: In the subparagraphs listed below, provide a complete description of all procedures the animals will experience

a. Animal Observations and Health Status Assessment Criteria: Adults are examined daily by veterinarians. Any individuals that appear sick (thin, bleeding, obvious infections or abnormal behavior) will be removed and euthanized. Embryos and larvae will be examined daily. Embryos with severe morphological abnormalities will be removed and euthanized.

b. Anesthesia/Analgesia/Chemical Restraint and /or Non-pharmaceutical Methods of Relieving Pain or Distress:

[X]	Yes	[ ]	No	Anesthetics/Analgesics/Tranquilizers will be used to relieve pain or distress.
[ ]	Yes	[X]	No	Non-pharmaceutical methods will be used to relieve pain or distress.
[ ]	Yes	[X]	No	Drugs will be used for chemical restraint.

If "Yes" to any of the above,

i. Describe methods or strategies planned to effectively relieve pain and/or distress:
Embryos will be anesthetized in 0.25% Tricaine solution during tumor cell injection and imaging analysis

ii. Intraprocedural Observations: list the observational or monitoring criteria used to assess depth of anesthesia while the procedure is being performed and/or to determine if animals are experiencing pain or distress and require additional anesthetics, analgesics, tranquilizers or non-pharmaceutical pain relief:
Embryo/larvae movement will be monitored

c. Paralytic Agents (Note: the use of paralytic agents without anesthesia is <u>prohibited</u>):

[ ] Yes [X] No Paralytic agents will be used during this protocol.

If "Yes," describe the following:

i. Rationale for using paralytic agents:

ii. Paralytic Agent Protocol (e.g., drug, dose, frequency of injection, etc.):

iii. Monitoring methods to ensure adequate depth of anesthesia while animal is under influence of paralytic agents:

d. Surgery:

[ ] Yes [X] No Surgical procedures are performed on live animals during this protocol.

If "Yes," describe the following:

i. Pre-operative Considerations and Animal Preparation:

ii. Surgical Procedures:

iii. Immediate and Long-Term Post-operative Monitoring/Observations/Treatment:

e. Multiple Major Survival Surgeries (performed on the same animal):

[ ] Yes [X] No Multiple major survival surgeries will be performed on the same animal.

If "Yes," provide a scientifically valid justification: Be sure to include time between surgeries.

f. Biosamples:

[ ] Yes [X] No Biosamples are collected from animals during this protocol.

If "Yes," state the frequency, volume, harvest site, and collection method for each sample type collected ante-mortem (before the animal is euthanized)

g. Adjuvants:

[ ] Yes [X] No Adjuvants are used in animals during this protocol.

If "Yes," list any adjuvants used and the plan for their use:

h. Genotyping/DNA Analysis:

[ ] Yes [X] No Genotyping/DNA analysis will be performed on animals during this protocol.

If "Yes," describe any methods used for genotyping or other DNA analysis:

i. Agents Administered to Animals: In this section, list all substances, compounds, or special diets administered to animals by any method.

Agent	Dose/Volume	Route of Administration
KN-93	1-20 ug/ml	Embryo incubation in a solution
KN-92	1-20 ug/ml	Embryo incubation in a solution
SB203580	1-20 ug/ml	Embryo incubation in a solution
Roscovitine	1-20 ug/ml	Embryo incubation in a solution
H 89 dihydrochloride	1-20 ug/ml	Embryo incubation in a solution

j. Prolonged Restraint:

[ ] Yes [X] No Animals will undergo prolonged restraint (as defined by institutional IACUC policies or the Animal Welfare Act regulations) during this protocol.

If "Yes," justify and describe restraint and duration in detail:

k. Behavioral Studies or Behavioral Modification Techniques:

[ ] Yes [C] No Animals will be involved in behavioral studies or undergo behavioral modification during this protocol.

:

l. Other Procedures (e.g., electrocardiograms, radiology or other imaging procedures, tissue perfusions, stress induction, etc.):

13. Endpoints for Animals:

a. **Study Endpoint**: State the projected study endpoint for each animal, animal group, or experiment (e.g., survival until the specified time point and then euthanasia, recovery, death without early euthanasia)

Larvae will be euthanized at 1-2 weeks of age after observations are complete.

b. **Humane Early Endpoints**: Define specific, and objective criteria that will be used to determine early removal (for example, percentage of weight loss, tumor size, number of abdominal taps, abdominal distension, loss of locomotion, significant lowering of body temperature, decreased food or water consumption, decreased activity, etc.).

General tissue necrosis or grossly abnormal larvae development

c. **Death as an Endpoint**: Specifically address and scientifically justify any proposal in which animals are allowed to die as a result of the experimental procedures without the benefits of treatment or early euthanasia.

14. Euthanasia:
Euthanasia performed using incubation in 1% Tricaine for 5 minutes. Death will be confirmed by transferring sample larvae into fish water and examining them for the absence of any visible movement, lack of touch response and absence of blood circulation.

15. Literature Search for Unnecessary Duplication: This search is required for all animal use proposals.

a. Source(s) Searched :

Research Portfolio Online Reporting Tool Expenditures and Results (RePORTER)
http://projectreporter.nih.gov/reporter.cfm

http://www.ncbi.nlm.nih.gov/pubmed

http://awic.nal.usda.gov/alternatives

www.google.com

b. Date(s) Search of Search : 9/5/2019

c. Years Covered by Search : all years searchable

d. Keywords Used and/or Search Strategy Please note that this is not a search for alternatives and alternatives terms should not be used here. :

Stathmin AND metastasis, zebrafish AND cancer xenograft model, zebrafish AND metastasis

Results of Search 4 funded projects:

PAX2 LOSS IN FALLOPIAN TUBE LESIONS AND STRATEGIES FOR RESTORATION IN SEROUS CANCER
SPATIALLY-DELINEATED SYSTEM-LEVEL ANALYSES AND CONTROL OF CYTOSKELETAL REGULATION
CONNEXIN HEMICHANNELS IN SUPPRESSION OF BREAST CANCER BONE METASTASIS

None of the projects relate to Stathmin role in metastasis in vivo.

STATHMIN PHOSPHORYLATION AS A TARGET FOR BLOCKING PROSTATE CANCER METASTASIS

16. Qualifications:

STUDY PERSONNEL QUALIFICATIONS/TRAINING

Protocol activity or procedure (e.g., tail vein injections, euthanasia)	Name of person performing activity	Qualifications or experience of person performing activity in the proposed species (e.g., research technician; 2 yrs experience with intracranial surgical procedure; performed IP injections on 100s of mice)	**Specific** training in this activity or procedure (e.g., rodent handling class; trained to do surgical procedure by PI; aseptic surgical techniques training; rabbit intubation)
Tumor cell microinjections and imaging	Saulius Sumanas	Associate Professor; 18 years of experience	Previously performed the procedure

****The following questions refer to the Research Site and Protocol PI.****

Information and/or documents required in these questions should be obtained from the research site's IACUC or veterinary staff. Documents may be provided directly to ACURO by the Protocol PI or institution staffs.

17. Institutional Accreditation / Assurances:

a. Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) Accreditation (do **NOT** provide AAALAC correspondence)**:**

[X] Yes [ ] No Animal work is being performed at an AAALAC International-accredited facility.

b. Public Health Service Animal Welfare Assurance Statement:

[X] Yes [ ] No Animal work is being performed at a PHS-assured facility.

c. Non-accredited, Unassured Facilities: If neither above apply to the facility where animal work is being performed, submit a statement signed by the Institutional Official that states the care and use of animals will be conducted in accordance with the National Research Council's 2011 *Guide for the Care and Use of Laboratory Animals* and applicable Federal and DoD regulations.

18. Continuing Review:

[X] Yes [ ] No Does your IACUC conduct continuing review of all animal use protocols, no less than once per year, in accordance with the Animal Welfare Act Regulations and

Department of Defense requirements? All animal use protocols funded by the DOD, even in part, must be reviewed by the IACUC no less than annually, including those that do not utilize USDA regulated species.

19. Institution's Veterinary Care: Provide a brief description of the veterinary care plan at the research site.

a. Describe routine care of animals by vivarium staff

b. Describe weekend, holiday, and emergency care of animals by vivarium staff.

c. Identify whether the attending veterinarian is on staff full-time or by contract. If contracted, address frequency of visits to the facility.
Veterinarians feed and inspect zebrafish twice daily (once per weekends and holidays). Any sick animals are removed and euthanized. Veterinarians are employed full-time. On-call staff is available for emergency care.

Protocol Principal Investigator Assurances:

The law specifically requires several written assurances from the P.I. Please read and sign the assurances as indicated (this page may be photocopied and signed).

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Painful Procedures: I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals.

B. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC and the U.S. Army Medical Research and Materiel Command **Animal Care and Use Review Office** (ACURO) prior to its implementation.

C. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

D. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

E. Training: I verify that the personnel performing the animal procedures/manipulations/observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to implement animal use alternatives where feasible, and conduct humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Saulius Sumanas

(Protocol Principal Investigator Name)

5/17/19

(Protocol Principal Investigator Signature and Date)