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PRINCIPAL INVESTIGATOR: Dr. James William Harbour

RECIPIENT: University of Miami
Coral Gables, FL 33146

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6. AUTHOR(S)

J. William Harbour, MD
Mary Lou King, PhD
Jeffim Kuznetsov, PhD
Tristan Aguero, PhD

harbour@med.miami.edu

5d. PROJECT NUMBER

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5f. WORK UNIT NUMBER

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

University of Miami
1320 S Dixie Hwy STE 650
Coral Gables, FL 33146-2919

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our *objective* is to *identify therapeutic molecules that block cancer progression, metastasis and death in patients with loss of* BRCA1-associated protein molecular therapies that target BAP1 mutations could provide a powerful new strategy for treating these cancers. Unfortunately, there are several obstacles to developing such therapies. First, BAP1 is a tumor suppressor that is inactivated by mutations, such that targeted therapy would need to be directed against effectors of the BAP1 "pathway" that are deregulated by BAP1 loss. Second, the effectors of BAP1 that are relevant to cancer are not known. Third, BAP1 is difficult to study in cultured cells because the loss of BAP1 results in stem cell-like behavior; the cells proliferate slowly, become dedifferentiated and are difficult to propagate. These obstacles led us to shift from using *in vitro* to *in vivo* models for studying BAP1. One of the whole-animal models we have investigated is Xenopus laevis (African clawed frog), a widely used animal model for studying development. We found that targeted depletion of the Xenopus BAP1 protein (xBap1) causes a striking developmental phenotype failure to complete gastrulation at developmental stage 12. Our *rationale* is that *compounds that reverse or "rescue" the developmental phenotype caused by the loss of BAP1 in Xenopus laevis may also reverse the malignant phenotype caused by loss of BAP1 in human cancers*.

15. SUBJECT TERMS

Melanoma, mesothelioma, kidney cancer, Xenopus laevis, model organism, high-throughput drug screen, BAP1, morpholinos, neural crest, eye development

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1. INTRODUCTION

The BRCA1-associated protein 1 (BAP1) has recently received widespread attention as a tumor suppressor that is frequently mutated in some of the most lethal and treatment-resistant cancers, including melanoma, mesothelioma and kidney cancer. Molecular therapies that target BAP1 mutations could provide a powerful new strategy for treating these cancers. Our objective is to identify therapeutic molecules that block cancer progression, metastasis and death in patients with BAP1-mutant cancers by counteracting the cellular and molecular effects of BAP1 mutations in cancer cells. These ideas led us to shift from using in vitro to in vivo models for studying BAP1. One of the whole-animal models we have investigated is Xenopus laevis (African clawed frog), a widely used animal model for studying development. We found that targeted depletion of the Xenopus BAP1 protein (xBap1) causes a striking developmental phenotype - failure to complete gastrulation at developmental stage 12. This discovery confirms that BAP1 is a key developmental regulator, and it provides a tractable phenotype for high throughput drug screening. Our rationale is that compounds that reverse or "rescue" the developmental phenotype caused by the loss of BAP1 in X. laevis may also reverse the malignant phenotype caused by loss of BAP1 in human cancers.

2. KEYWORDS

Melanoma, mesothelioma, kidney cancer, Xenopus laevis, model organism, high-throughput drug screen, BAP1, morpholinos, neural crest, eye development

3. ACCOMPLISHMENTS

a. What were the major goals of the project?

Identify therapeutic compounds that rescue the BAP1-deficient phenotype in Xenopus laevis using in vivo high throughput compound screening.

b. What was accomplished under these goals?

We designed a gene-specific antisense oligonucleotide morpholino (MO-BAP1) to deplete xBAP1 protein levels when injected into *Xenopus laevis* embryos. MO-BAP1 works by binding to the xBAP1 mRNA and blocking protein synthesis. The control morpholino (MO-CTRL) consists of the same sequence as MO-BAP1 except that 5 nucleotides are mismatched with the wildtype sequence. Our data shows that MO inhibits protein expression in dose dependent matter (Fig 1). When up to 15 ng of MO-CTRL is injected into one cell at the two-cell stage of embryogenesis, the injected embryos develop similar to the uninjected embryos (Fig 1, green color). Whereas MO-BAP1 affected embryo development at doses as little at 2.5 ng per embryo (Fig 1, red color). MO-BAP1 at doses >7.5 ng per embryo led to an arrest in development at stage 12, indicating an early requirement for BAP1 in embryonic development (Fig 1, blue color).

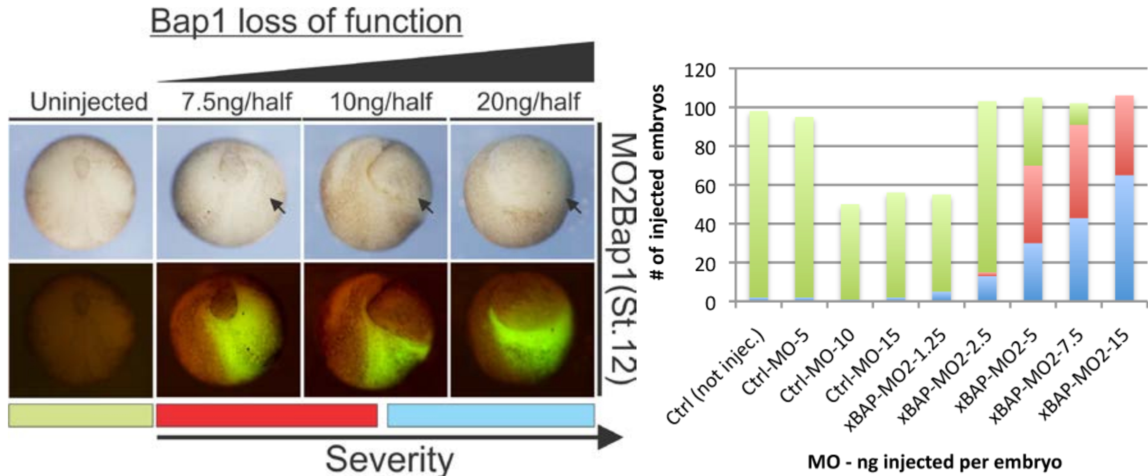


Fig 1. Dose-dependent effect of MO-BAP1 on embryo development. The absence of Bap1 function produce failures during gastrulation. Loss of function of Bap1 is achieved by using a specific morpholino oligonucleotide against Bap1 mRNA. To evaluate gastrulation abnormalities we define three levels of severity: LI, blastopore slightly open, LII, blastopore open with clear abnormal morphology, LIII complete failure of gastrulation.

We have found that Bap1 mRNA and protein present a distinct expression pattern throughout embryonic development. In particular, Bap1 protein is expressed in neural crest cells, the precursors of melanocytes. We have established a tractable phenotype by knocking-out Bap1 protein expression in *X. laevis* embryos at different developmental time points according to the desired effect we want to analyze. Our data indicate an early requirement for BAP1 during gastrulation in blastopore closure at stage 10-12 (**Fig. 2A**, arrow). Embryos with gross systemic defects that resulted from failed gastrulation at stage 12 due to a complete depletion of BAP1 (**Fig. 2C**, arrow) offer a more obvious phenotype that is easily distinguished by visual inspection in a high throughput format. Bap1-depleted embryos that complete gastrulation usually exhibit signs of incomplete blastopore closure, spina bifida and severe mesodermal foreshortening –traits easily detectable by visual inspection (**Fig. 2D**).

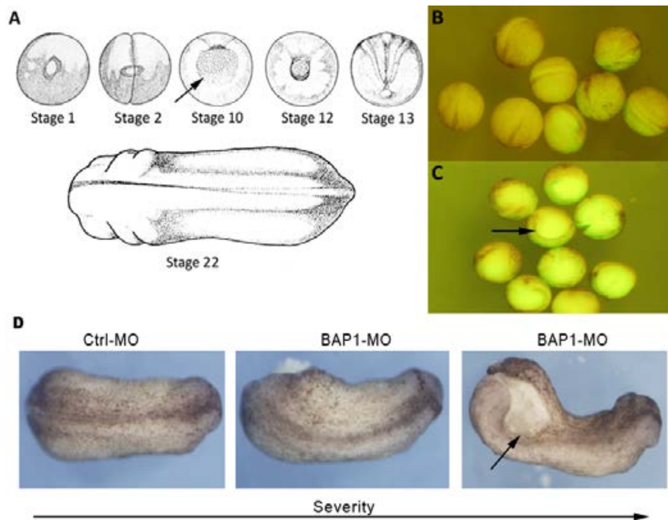


Figure 2. BAP1-depletion phenotype causes gastrulation defect in *X. laevis* embryos. (**A**) Relevant developmental stages for *Xenopus laevis* embryos used in this study. Embryos were injected at either stage 1 (single cell) or stage 2 (two cells). At stage 10, the blastopore (arrow) has not yet closed, whereas at stage 13 the blastopore has closed. **(B)** Embryos injected at stage 2 with 10 ng of MO-CTRL into one cell progressed through stage 12 and are shown here at stage 13 with normal morphology. **(C)** Embryos that were injected at stage 2 with 10 ng of BAP1-MO into one cell, at the same time point as the control embryos in B, show failure to close the blastopore (arrow) and to progress to stage 13. **(D)** Partial depletion of BAP1 with 7.5 ng of MO leads to incomplete gastrulation, characterized by spina bifida (arrow) and mesodermal foreshortening (curved axis).

Our initial results identify histone deacetylase inhibitors, such as SAHA, as a class of compounds that rescue embryos the arrested gastrulation phenotype associated with BAP1 depletion. Furthermore, the restorative effects of the HDAC inhibitor SAHA were dose dependent (**Fig.3**). As little as 0.5 μM of SAHA reduced the number of abnormal embryos by 50%, while 20 μM eliminated the BAP1-deficient phenotype altogether.

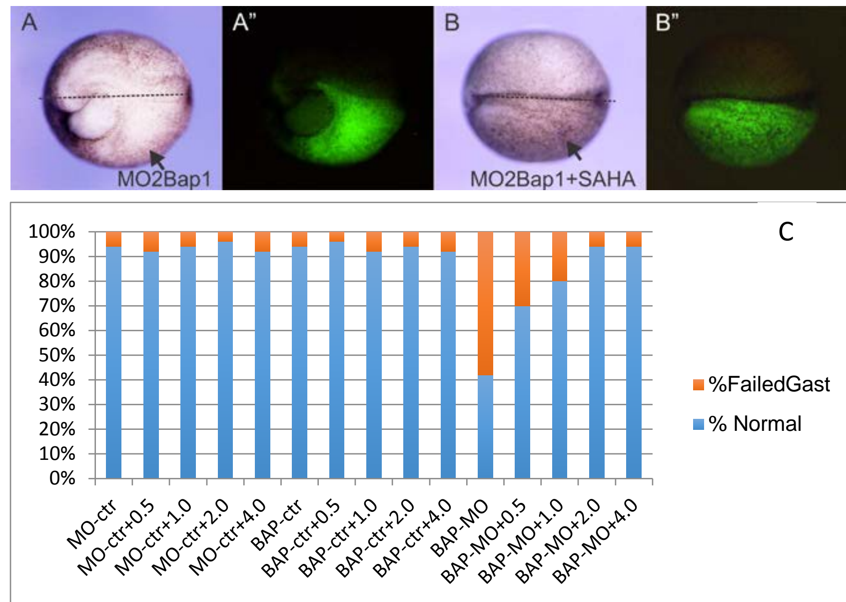


Figure 3. HDAC inhibitor SAHA reverses the gastrulation defects in BAP1-depleted Xenopus embryos. Embryos were injected at stage 2 (two cells) into one cell with 7.5 ng of either CTRL-MO or BAP1-MO (A). SAHA (final 5-20 μM) was added to the medium containing injected and uninjected embryos at stage 9, prior to gastrulation (B). The embryos were scored for gastrulation defects at stage 15. Failed gastrulation (orange) embryos include incomplete blastopore closure phenotypes (C).

To further evaluate the rescue of phenotype observed with SAHA we analyzed the expression of a tissue-specific marker normally affected in the absence of Bap1, the epidermis marker keratin (Fig.4). When Bap1 protein synthesis is blocked using a specific BAP1 morpholino, keratin expression is down-regulated (Fig.4 B). This phenotype is rescued by the co-expression of the Bap1 human ortholog (Fig.4 C), or when BAP1-defieicne embryos were treated with SAHA 20uM (Fig.4 D).

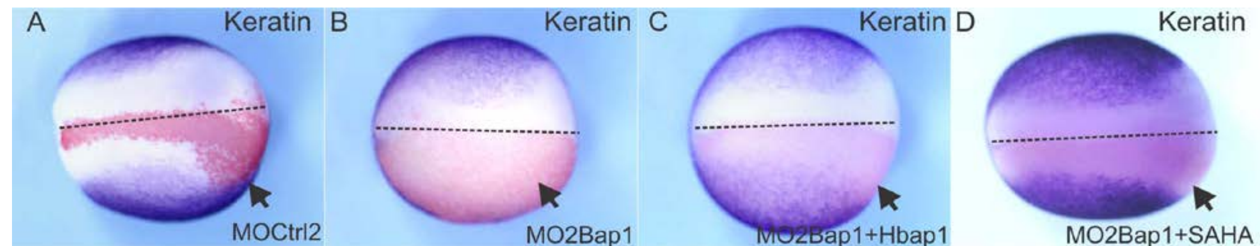
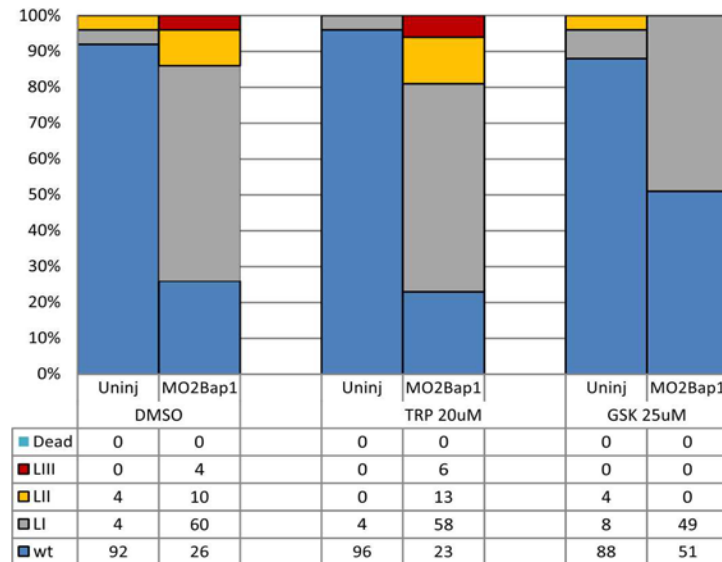


Figure 4. SAHA can restore expression pattern of the prospective epidermis marker keratin1. Embryos were injected at stage 2 cells into one cell with 7.5 ng of either MOCtrl or MOBap1 and 1ng of HBap1. (A) Control morpholino produce no changes in keratin expression. B) In the absence of xBap1 protein, expression of xKeratin is significantly reduced. (C) The co-expression of a functional human Bap1 protein rescues the expression of keratin in BAP1 morphants embryos. (D) SAHA rescues keratin expression pattern in 50% of BAP1 morphants.

To further investigate the association of BAP1 function and the polycomb repressor complexes 1 and 2 (PRC) we decided to use specific inhibitors of PRC1 and 2. We used PRC1 inhibitor TRP4165 that inhibits the protein RINGA1 in the complex (Ismail, McDonald et al. 2013). The result is the inhibition of histone H2A ubiquitination and the accumulation of ubiquitin at the DNA double-strand break sites. The PRC1-mediated histone ubiquitination plays an important role in aberrant gene silencing in human cancers therefore the rationale behind the use of this inhibitor was to block H2A ubiquitination in BAP1-depleted embryos and determine if blocking the opposite mechanism can restore the BAP1 depletion phenotype. First, we tested different concentrations of TRP4165 and determined the lowest concentration that does not affect uninjected embryos was 20uM. The results showed that this PRC1 inhibitor is not able to rescue the phenotype produce

for the absence of Bap1 activity (Fig.5). We cannot rule out a possible interaction between Bap1 and the PRC1 but we can infer that PRC 1 is acting in parallel or upstream of Bap1 function.

We used the PRC2 inhibitor GSK126 which inhibits EZH2 methyltransferase activity (Tien et al 2015 Development). First we tested the doses and found that 25uM was the lowest dose that produces no morphological changes in uninjected *X. laevis* embryos. We incubated Bap1MO-injected embryos with GSK126 from a pre-gastrulation to mid-neurulation stage. We observed that Bap1-depleted embryos presented a morphological improvement in treated embryos compared with morphants incubated in DMSO 25uM. The phenotypic recovery was approximately 50% (Fig.5). This results suggest the existence of a hierarchy between Bap1 and the PRC2 where PRC2 could be downstream in the Bap1 pathway.



	Uninj	MO2Bap1		Uninj	MO2Bap1		Uninj	MO2Bap1
	DMSO			TRP 20uM			GSK 25uM	
Dead	0	0		0	0		0	0
LIII	0	4		0	6		0	0
LII	4	10		0	13		4	0
LI	4	60		4	58		8	49
wt	92	26		96	23		88	51

Figure 5. The inhibition of the PRC2 complex partially recues the absence of Bap1 activity. Uninjected and MO2Bap1 injected embryos incubated in DMSO, TRP4165 or GSK126. A total number of 50 embryos were incubated in each condition. To evaluate gastrulation abnormalities we define three levels of severity: **LI**, blastopore slightly open, **LII**, blastopore open and abnormal shape, **LIII** complete failure of gastrulation. These results were obtained twice.

To further study the possible relationship of PRC1 and 2 and Bap1 function, we decided to evaluate the expression pattern of a tissue-specific marker normally affected in the absence of Bap1. We used a molecular marker of early neural crest development, FoxD3; the mesodermal master gene that is regulating gastrulation, brachyury (bra); and the epidermis marker keratin 1 (Fig.6). When Bap1 protein synthesis is blocked using a specific morpholino, early neural crest marker FoxD3, the mesodermal marker bra and the epidermal marker keratin are all down regulated (Fig.6 A and D). After incubation with the PRC1 inhibitor TRP4165 no changes were observed in morphants embryos (Fig6 B and E). Morphants embryos treated with the PRC2 inhibitor GSK126 presented a differential behavior, meanwhile foxD3 and Bra showed no changes, the epidermal marker keratin presented a restoration in the expression in about 40%. These results also suggest a selective relationship between PRC2 and Bap1 in the regulation of certain gene expression.

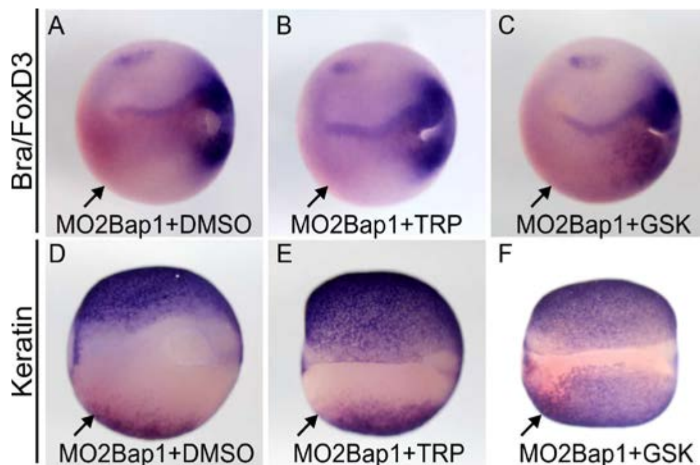


Figure 6. Inhibition of PRC2 selectively regulate gene expression. Embryos were injected at stage 2 cells into one cell with 7.5ng of MO2Bap1. Embryos were incubated in DMSO (A, D), TRP4165 20uM (B, E) or GSK126 25uM (C.F) from stage 10 (beginning of gastrulation) to stage 14 (mid-neurulation). In Bap1-depleted embryos expression of FoxD3, Bra and Keratin is down regulated. Embryos treated with PRC1 inhibitor present no changes in markers expression (B, E). PRC2 inhibitor GSK126 can partially rescue expression of keratin but failed in rescuing FoxD3 and Bra expression.

Our initial plan to breed sufficient numbers of immune-deficient NSG mice to be used in validating the effects of candidate chemical compounds discovered in Aim 2 was substantially scaled back, until complete evaluation of the LOPAC^{1280} compound library could be performed on Xenopus laevis in Aim 1. In August 2016 we began the breeding effort with a few young NSG breeding pairs from The Jackson Laboratory, with plans to expand the number of breeders using offspring. Although these mice should be good breeders in specific pathogen-free environments, as claimed by the manufacturer, our initial breeders and subsequent expanded breeders nurture only 50% of the offspring. It is likely that our in-house vivarium and husbandry procedure is unable to establish and maintain the ideal pathogen-free environment that would help produce greater offspring numbers. To date, our NSG colony has generated 59 viable offspring that has matured to breeding age. We will continue to maintain a small colony of NSG mice, and when appropriate, we will materially increase offspring production to provide significant numbers of subjects for our planned mouse xenograft experiments aimed to help validate any therapeutic effects of candidate compounds discovered in Aim 1.

c. **What opportunities for training and professional development has the project provided?**

Nothing to report.

d. **How were the results disseminated to communities of interest?**

Nothing to report.

e. **What do you plan to do during the next reporting period to accomplish the goals?**

A cell-based reporter gene luciferase assay will be used to identify top 100 promising compounds in the 1st round of screening. We will then use Xenopus embryos for the second round of screening with the top 100 compounds, identified in step 1, to further narrow down to about ~5-10 compounds, and these compounds will be prioritized for further evaluation in Aim 2 using a mouse xenograft model. In addition, to continue the breeding of NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ JAX® immunodeficient mouse strain, commonly referred to as NOD-SCID-Gamma or "NSG" mice, which we obtained from the Jackson Laboratory and maintain as a colony in our maximum barrier facility in the Gautier Building in preparation for Aim 2.

4. IMPACT

a. What was the impact on the development of the principal discipline(s) of the project?

Our previously published data showed that HDAC compounds, including SAHA, counteracts the abnormal ubiquitination of histone H2A in BAP1-depleted UM cells (Landreville, Agapova et al. 2012). Our data show that a histone deacetylase inhibitors (HDAC), such as SAHA, rescues embryos from the gastrulation defects that result from BAP1 depletion. Furthermore, the positive effects of SAHA were dose dependent (**Fig.3**). Our current findings are truly exciting and reinforce our hypothesis that *X. laevis* represents a suitable model for high-throughput chemical screens that will identify putative therapeutics to treat human diseases such as melanoma, mesothelioma, lung cancer, breast cancer, kidney cancer, gastric cancer, and other cancers harboring BAP1 mutations.

b. What was the impact on other disciplines?

Nothing to report.

c. What was the impact on technology transfer?

Nothing to report.

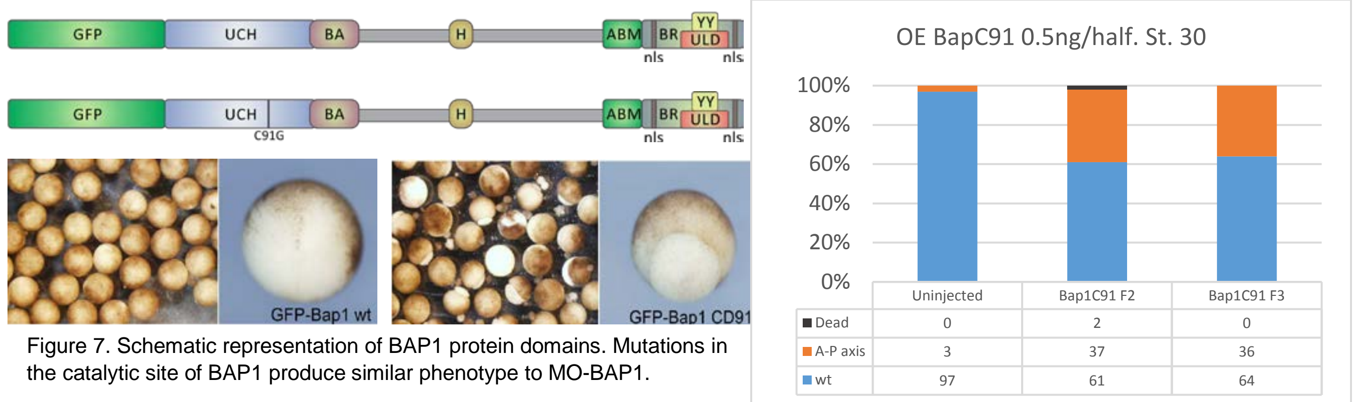
d. What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

a. Changes in approach and reasons for change

We originally designed a gene-specific antisense oligonucleotide morpholino (MO-BAP1) to deplete xBAP1 protein levels when injected into Xenopus laevis embryos. MO-BAP1 works by binding to the xBAP1 mRNA and blocking protein synthesis. The control morpholino (MO-CTRL) consists of the same sequence as MO-BAP1 except that 5 nucleotides are mismatched with the wildtype sequence. When injected into one cell at the two-cell stage of embryogenesis, MO-CTRL (15 ng) has no effect on embryonic development, whereas MO-BAP1 (15 ng) causes an arrest in development at stage 12, indicating an early requirement for BAP1 in embryonic development. We initially planned to screen compounds for the ability to rescue this “stage-12 arrest phenotype”. However, the technical limitations of injection apparatus and variations in frog genetic background led to higher than usual mortality in embryos injected with ctrl-MO, which negatively affected our ability to grow the injected embryos in 48-well plates. Additionally, many of the LOPAC compounds have a short half-life in the aqueous buffer in which morphants are incubated, and further masking any potential target compounds. To bypass this problem we designed a dominant negative mutant of xBAP1 by introducing a C91W mutation into the deubiquitinating catalytic site. Catalogue Of Somatic Mutations In Cancer (COSMIC) database showed that out of 108 disease-associated mutations identified 69 (~60%) are located in the catalytic domain of BAP1 (Fig7). (Bhattacharya, Hanpude et al. 2015)



	Uninjected	Bap1C91 F2	Bap1C91 F3
Dead	0	2	0
A-P axis	3	37	36
wt	97	61	64

Figure 7. Schematic representation of BAP1 protein domains. Mutations in the catalytic site of BAP1 produce similar phenotype to MO-BAP1.

It is well known that hot spot mutations of any gene or predisposition of genetic elements has severe consequences on structure and function of protein. Our preliminary data showed that catalytically dead dominant negative xBAP mutant (DN-xBAP1) produced similar phenotype to that of BAP1 morphans (Fig7). Furthermore, we fused the DN-xBAP1 protein to green fluorescent protein (GFP) tag to allow for greater stability of the recombinant protein in the injected embryos (Fig7). DN-xBAP1-GFP construct produces gastrulation failure in 80% of embryos, similarly to embryos injected with MO-BAP1. Our goal was to create a transgenic animal that can be induced with DOX to express DN-xBAP1-GFP in 100% of embryos, which could be then subjected to drug screening. This would allow us to avoid excessive mortality that results when embryos were injected at state 2 with either MO-BAP1 or mRNA for DN-xBAP1-GFP. However, when we analyzed the biomolecular markers associated with BAP1 depletion, we observed a discrepancy between embryos injected with MO-BAP1 and those expressing DN-xBAP1-GFP. This data suggests that loss of BAP1 expression likely causes a different signaling shift from that of mutant BAP1. This could be due to the ability of the mutant xBAP1 to bind to its binding partners (ASXL1 and HCF1), whereas in BAP1 morphans the BAP1 protein is absent and the binding partners are freed to alter other cellular signaling processes.

To bypass this problem we designed a cell-based assay for the initial high-throughput screen to further limit the pool of potential candidate compounds. The cell-based assay consists of the genetically engineered uveal melanoma cell line (92.1) that can be induced with DOX to decrease expression of endogenous BAP1. We have identified a gene that is consistently upregulated in response to BAP1 depletion (4-6 fold increase in expression). Based on these findings we have designed a reporter gene luciferase assay that allows for screening up to 300,000 pharmacological compounds in cost-effective way. A cell-based reporter gene luciferase assay will be used to identify top 100 promising compounds in the 1st round of screening. We will then use Xenopus embryos for the second round of screening with the top 100 compounds, identified in step 1, to further narrow down to about ~5-10 compounds, and these compounds will be prioritized for further evaluation in Aim 2 using a mouse xenograft model. Whereas mice would not be appropriate for the high throughput drug screening that will be performed in Aim 1, the mouse xenograft model is ideal for the validation experiments that will be performed in Aim 2. We will use the NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ JAX® immunodeficient mouse strain, commonly referred to as NOD-scid-gamma or "NSG" mice, which we obtained from the Jackson Laboratory and maintain as a colony in our maximum barrier facility in the Gautier Building. NSG mice are commonly used for cancer xenograft experiments since their profound immunodeficiency allows the growth of human cancer cells and cancer stem cells with significant fidelity to the original tumor.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

We initially planned to screen compounds for the ability to rescue this "stage-12 arrest phenotype". However, the technical limitations of injection apparatus and variations in frog genetic background led to higher than usual mortality in embryos injected with ctrl-MO, which negatively affected our ability to grow the injected embryos in 48-well plates. Additionally, many of the LOPAC compounds have a short half-life in the aqueous buffer in which morphants are incubated, and further masking any potential target compounds. A cell-based

reporter gene luciferase assay will be used to identify top 100 promising compounds in the 1st round of screening. We will then use Xenopus embryos for the second round of screening with the top 100 compounds, identified in step 1, to further narrow down to about ~5-10 compounds, and these compounds will be prioritized for further evaluation in Aim 2 using a mouse xenograft model.

c. **Changes that had a significant impact on expenditures**

The proposed changed would not increase expenditures. A cell based assay is far more cost-effective and allows for screening of up to 300,000 compounds as opposed to 1200 compounds originally suggested. The mammalian cell to frog embryo to mice pipeline will dramatically increase our chances of finding a compound that can reverse the malignant phenotype caused by the loss of BAP1 in human cancers.

d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

There were no significant deviations from the original approved protocols for the use or care vertebrate animals, biohazards, and/or select agents during the reporting period that would require new approvals.

6. PRODUCTS

a. **Publications, conference papers, and presentations**

i. **Journal publications.** Nothing to report.

ii. **Books or other non-periodical, one-time publications.** Nothing to report.

iii. **Other publications, conference papers, and presentations.** Nothing to report.

b. **Website(s) or other Internet site(s)**

Nothing to report.

c. **Technologies or techniques**

Nothing to report.

d. **Inventions, patent applications, and/or licenses**

Nothing to report.

e. Other Products Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. **What individuals have worked on the project?**

J. William Harbour, MD- no change
Mary Lou King, PhD- no change
Jeffim Kuznetsov, PhD- no change
Tristan Aguero, PhD- no change

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Completed Research Support for J. William Harbour
R01 CA161870 07/01/11 – 04/30/16
Genetics of Ocular Melanoma
Role: Co-PI

R01 CA125970 07/01/06 – 04/30/16
Molecular Predictive Testing in Ocular Melanoma

Role: PI
*Competing Renewal Submitted 07/05/2016 and Received a 1st Percentile Score

c. **What other organizations were involved as partners?**

Nothing to report.

1. **Organization Name:**
2. **Location of Organization:**
3. **Partner's contribution to the project**
    a. **Financial support;**
    b. **In-kind support**
    c. **Facilities**
    d. **Collaboration**
    e. **Personnel exchanges**
    f. **Other.**

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report.

References
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Bhattacharya, S., et al. (2015). "Cancer associated missense mutations in BAP1 catalytic domain induce amyloidogenic aggregation: A new insight in enzymatic inactivation." Sci Rep **5**: 18462.