AWARD NUMBER: W81XWH-15-1-0111

TITLE: Drosophila as a Screening Platform for Novel Lung Cancer Therapeutics

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REPORT DATE: November 2017

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
November 2017	Final	1 Sep 2015 - 31 Aug 2017

4. TITLE AND SUBTITLE
Drosophila as a Screening Platform for Novel Lung Cancer Therapeutics

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0111

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Icahn School of Medicine at Mount Sinai
New York, NY 10029

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The research in this grant is designed to explore the effects of genetic and genomic complexity in lung cancer progression and response to therapeutics. Using Drosophila, we are establishing a set of 'personalized fly lines', each which represents a separate patient. In this Progress Report, I discuss our initial studies in building these lines. In addition, I discuss our results with screening a library of 1200 FDA approved drugs. We provide evidence that a two drug cocktail, trametinib plus fluvastatin, synergize to improve efficacy and minimize toxicity in fly and human cell line models.

15. SUBJECT TERMS

None listed

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

The research in this grant was designed to explore the effects of genetic and genomic complexity in lung cancer progression and response to therapeutics. Using Drosophila, we are completing a set of 'personalized fly lines', each which represents a separate patient. In this Final Technical Progress Report, I discuss our completion of these lines and the first drug experiments. In addition, I discuss our results using one line with screening a library of 1200 FDA approved drugs. We provide evidence that a two drug cocktail, trametinib plus fluvastatin, synergize to improve efficacy and minimize toxicity in fly and human cell line models. We are currently executing similar studies with a new set of patient-matched fly lines, and initial results are consistent with the view that patient-specific mutations can strongly alter drug response.

KEYWORDS:

Drosophila, lung adenocarcinoma, trametinib, fluvastatin

Final Technical Progress Report for LCRP grant W81XWH-15-1-0111

Drosophila as a Screening Platform for Novel Lung Cancer Therapeutics

Accomplishments

What were the major goals of the project?

This proposal uses Drosophila to explore how tumor complexity—common plus rare variants—affect tumor progression. To address this issue, I proposed to build 11 fly models, each modeling a different patient or commonly used cell line. These fly lines will be used to explore tumor complexity including response to drugs.

Two Specific Aims were proposed:

Specific Aim 1: Assess functional relevance of rare variants. Using sequencing data from patients and from cell lines, we designed 11 'avatars' designed to mimic patients' mutation load. Using EGFR alone as a commonly reported 'base' model, our goal was to compare it to more genetically complex avatars for differences in tumor progression by quantitating eight phenotypic and biochemical assays. This has been completed.

Specific Aim 2: Examine drug response in Drosophila lung cancer models. As a step towards understanding the role of rare variants in drug response, I proposed to screen each avatar with a broad set of 1200 FDA approved drugs plus combinations (drug cocktails), comparing the base EGFR model with the more genetically complex fly lung cancer models. This is ongoing, but the initial results indicate that increasing genetic complexity—including patient-specific alterations—can have strong effects on drug response.

What was accomplished under these goals?

Specific Aim #1. I proposed to have my laboratory develop a set of 'base' (genetically simple) and 'complex (more extensively multigenic) fly models to match specific patients. Since writing the Proposal, the TCGA data has been updated. To reflect this updated information, we re-analyzed EGFR-class patients (EGFR, ERBB2, ERBB4) to include copy number variation and to capture newly included patients. We took advantage of advanced analysis now available within the Sloan-Kettering 'CBioportal' which assessed each variant to determine its likely impact on protein function. Table 1 shows the updated analysis, translated to specific fly lines.

	patient#	*oncogenes* *tumor suppressors*
1	*base*	*EGFR*
2	*E5b*	*EGFR* *P53*
3	*4080/2708b*	*EGFR* *P53 PTEN*
4	*2722b*	*EGFR HTL* *P53*
5	*2787*	*EGFR* *P53 CDK1 KUG/FAT*
6	*2708*	*EGFR SCRO MYC FKH* *P53 PTEN*
7	*3789*	*EGFR CDK4 RAC1* *P53*
8	*2698*	*EGFR CycD CycE* *P53 AGO VAP MSH6*
9	*4593*	*EGFR CycD CycE* *P53 AGO LAR*
10	*1081*	*EGFR HTL* *P53 PTEN*
11	*2722*	*EGFR HTL* *P53 AGO*

Table 1. Fly avatars.

The assembling of the transgenic lines proved challenging. While not all of the difficulties are clear, two main technical difficulties slowed progress. First, we used a series of 'hairpin' inverted repeat constructs to knock down tumor suppressor function through RNA degradation. Several of these inverted repeats proved (i) difficult to grow in bacteria without deletions and (ii) difficult to PCR for adding 'sticky ends' due to the paired repeats structure. We eventually solved these difficulties by changing transformation bacteria and by changing the order of the inverted repeats. Injection of the constructs also proved challenging, as the majority led to lethality in injected embryos (Bestgene, *personal communication*). This can occur if the plasmid directs transient expression of the (otherwise silent) transgenes in the early stage embryo. Despite these challenges, we were finally able to successfully build all 11 models, in part by using two transformation vectors to split the gene load. We then completed the 11 models by recombining transformation vectors by standard recombination:

EGFR

EGFR-P53

EGFR-P53-PTEN

EGFR-P53-CDK1-FAT

EGFR-P53-AGO-BAP-MSH6

EGFR-P53-HTL

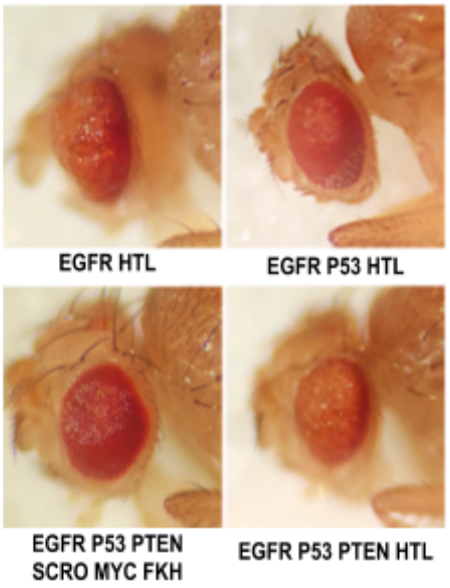
EGFR-P53-CDK4-RAC1

EGFR-P53-AGO-LAR

EGFR-P53-AGO-HTL

EGFR-P53-PTEN-HTL

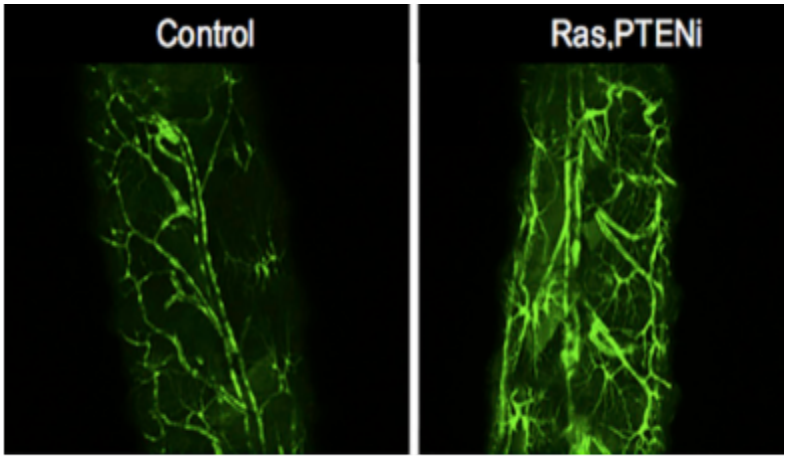
EGFR-P53-PTEN-SCRO-MYC-FKH



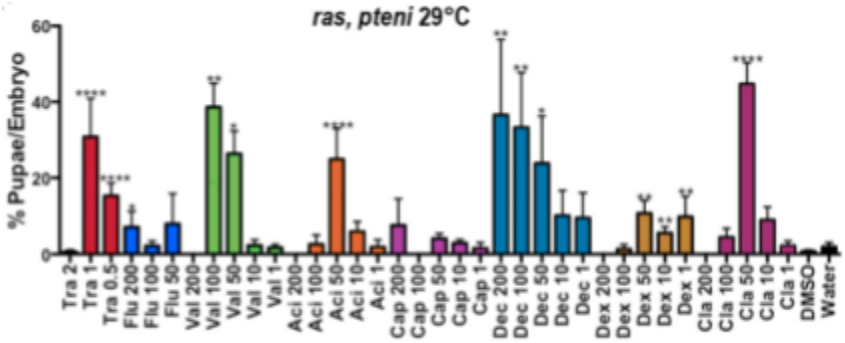
1. Complex Drosophila models. HTL= fly FGFR ortholog, SCRO= NK-like transcription factor, FKH= forkhead class transcription factor.

Expressing the transgenes broadly with a tubulin driver led to lethality the 8 lines tested. To compare transformation 'expressivity', we expressed each transgene using the eye-specific GMR promoter. Figure 1 shows four examples with differing levels of severity in the eye phenotype. In general, animals with P53 showed a weaker phenotype; note for example that EGFR-P53-HTL has a stronger phenotype than EGFR-HTL. We have previously observed this effect of P53 (*e.g.*, Bangi et al, 2016) and find that, despite reduced levels of transformation, the blocking of senescence by loss of P53 will eventually lead to more extensive long-term tumorigenesis. We also found that expression of the FGFR Heartless (HTL, Figure 1) leads to the strongest transformation when paired with EGFR. Overall, the 11 lines lie along a spectrum of transformation intensity.

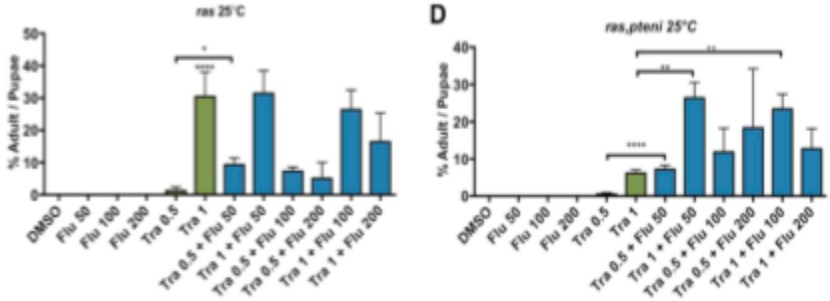
Specific Aim #2: In this Aim, I proposed to screen a library of 1200 FDA-approved drugs for the ability to 'rescue' our fly lung cancer lines. While assembling the personalized fly models, we embarked on this screen against a 'base' Ras model and a Ras/PTEN model (Levine and Cagan, 2016). As described in the original proposal, transgenes were targeted to the developing fly trachea using the *btl-GAL4* driver, yielding *btl>Ras* and *btl>Ras/PTEN flies.* As shown in Figure 2, the result was enlargement of trachea including tracheal branches (*from* Levine and Cagan, 2016). We then established and calibrated a 'rescue from lethality' assay.



2. *btl>Ras/PTEN* animals displayed enlarged trachea.



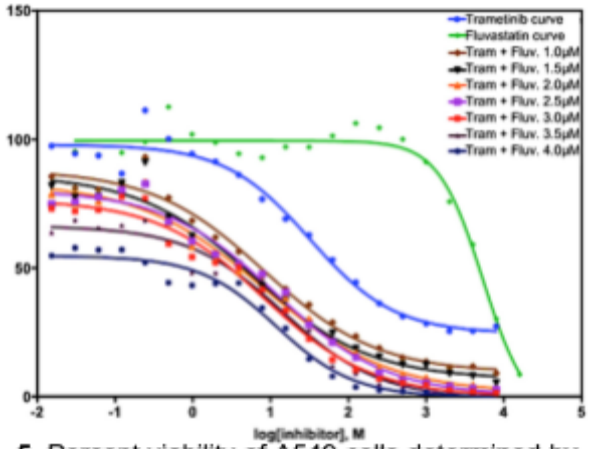
3. Eight positive 'hits' rescued *btl>Ras/PTEN* animals to pupariation. Drug concentrations are mM. Aci, aciclovir; Cap, capecitabine; Cla, cladribine; Dec, decitabine; Dex, dexrazoxane; Flu, fluvastatin; Tra, trametinib; Val, valaciclovir. (*p%0.05, ** p%0.01, *** p % 0.01, and **** p % 0.0001). From *Levine and Cagan, 2016.*



4. 50 μM fluvastatin + 0.5 mM trametinib showed better rescue to adulthood than 0.5 mM trametinib alone (p=0.05). From Levine and Cagan, 2016.

Screening the full FDA library yielded eight 'hits', shown in Figure 3. These hits included chemotherapy-related drugs as well as two targeted drugs, trametinib and fluvastatin. Trametinib is an inhibitor of MEK, a downstream target of EGFR and Ras. Fluvastatin is a statin-class drug that inhibits HMG-CoA reductase. Our data indicated that at least part of fluvastatin's activity is through its ability to suppress the cholesterol modification required to target the Ras protein to the surface.

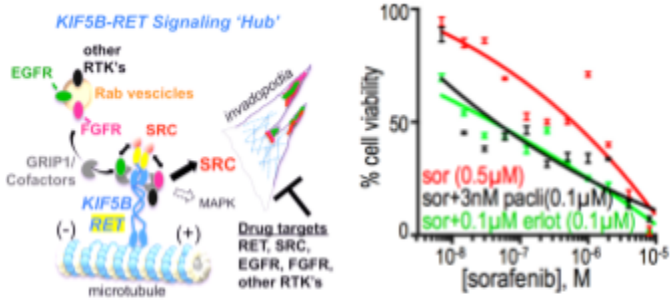
A key hypothesis of the Grant is that drug combinations may prove more useful than single targeted therapies. This proved to be the case in some instances. In particular, trametinib plus fluvastastin proved better able to rescue *btl>Ras/PTEN* flies to adulthood, as shown in Figure 4. The synergistic rescue was observed for *btl>Ras/PTEN* but not for *btl>Ras* alone, emphasizing the changing response to drugs as tumors increase in complexity.



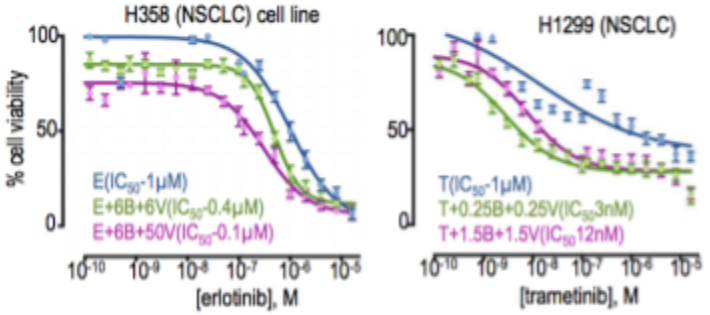
5. Percent viability of A549 cells determined by MTT assay plotted on a logarithmic molar dose curve scale. Based on Chou-Talalay CI index, 1-3 μM fluvastatin synergistically lowered the IC50 of trametinib. From Levine and Cagan, 2016.

We were able to validate this drug combination in an A549 lung cancer cell line (Figure 5). As we develop the EGFR-based fly avatars, we will be testing these initial hits to determine how useful they are in suppressing the transformation phenotypes observed in the fly avatar lines.

To further explore the concept of polypharmacology (multi-targeting) in lung cancer, we modeled lung cancer patients with the KIF5B-RET fusion, the most common fusion in lung cancer and an especially aggressive disease subtype. To model the disease, we developed both transgenic fly and transgenic human cell line KIF5B-RET transgenic models. In KIF5B-RET patients, the N-terminus of the kinesin-associated protein KIF5B is fused to the intracellular domain of the RET receptor tyrosine kinase. We recently published a paper (Das and Cagan, Cell Reports 2017) demonstrating how this fusion activates a complex set of signaling pathways including RET, EGFR, FGFR, and SRC (Figure 5). To address this cancer network complexity required drug cocktails, for example (i) sorafenib plus (ii)



6. *Left:* The KIF5B/RET fusion activates multiple pathways including EGFR and FGFR through un unusual transport mechanism. *Right:* The result is an aggressive, metastatic tumor that responds best to drug combinations. From Das and Cagan, 2017.



7. In two separate NSCLC cell lines, erlotinib (E) or trametinib (T) synergized with a cocktail of bortezomib (B) plus vorinostat (V). From Das et al, 2018.

erlotinib or paclitaxel (Figure 6). We have recently expanded this point, showing a similar requirement for multi-targeting in H1299 NSCLC cells (Figure 7; Das et al, 2018).

Finally, a key part of the proposal was to explore the importance of genetic complexity, including 'passengers' using our novel set of genetically complex fly lines from Aim #1. In preliminary experiments, we have treated four of the lines with the MEK inhibitor Trametinib (100 μM). Notably, while EGFR alone responded strongly to trametinib as we previously published (Levine and Cagan, 2016), EGFR PTEN P53 HTL showed no detectable rescue when the transgenes were expressed in the eye. Together, these results are consistent with the view that one emergent property of genetically complex models is drug resistance. We have demonstrated a small number of 2-3 drug cocktails that can handle greater genetic complexity. Whether any of these cocktails would prove effective across a broad cross-section of lung cancer patients is unknown.

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

Through weekly meetings and journal clubs, this Project provided scientific mentorship. In addition, I meet with each member of the Project one-on-one every other week to provide more extensive mentorship on both the science and regarding careers decisions.

How were the results disseminated to communities of interest?

I have given approximately 15 external, invited talks in the past year to discuss our approach using screening of multigenic cancer models. In addition, we recently published papers reporting our initial characterization and drug screening of our Drosophila lung cancer models and cell line work:

Levine B and Cagan R. (2016). Drosophila Lung Cancer Models Identify Trametinib Plus A Statin as a Candidate Therapeutic. Cell Reports, doi: 10.1016/j.celrep.2015.12.105.

Das T, Cagan R. KIF5B-RET Oncoprotein Signals through a Multi-kinase Signaling Hub (2017). Cell Rep. 5;20(10):2368-2383. PMC5590648

Das T, Esernio J, and Cagan R (2018). Restraining Network Response to targeted Cancer Therapies Improves Efficacy and Reduces Cellular Resistance. Canc Res doi: 10.1158/0008-5472.CAN-17-2001

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

While this is a Final Report, I anticipate that we will complete drug screening to match drugs/drug cocktails to specific genotypes.

Impact

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

Cancer remains a key challenge to our healthcare system. Our initial results emphasize the utility of screening in an unbiased fashion, the challenge of tumor genetic complexity to drug response, and the potential utility of drug cocktails in addressing tumor complexity.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change
No significant changes were made in the approach or scope. Technical difficulties were resolved by standard approaches.

Actual or anticipated problems or delays and actions or plans to resolve them
We faced some challenges in building the highly complex transgenes for knockdown. After some technology development, we settled on a 'stitching' method in which PCR products were assembled into a single knockdown construct. This delayed completion of the multigenic models towards the end of the funding period. As discussed above, after additional challenges relating to the complexity of the constructs, all constructs have been successfully generated as stable lines.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

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.

PRODUCTS

One journal publication:

Levine B and Cagan R. (2016). Drosophila Lung Cancer Models Identify Trametinib Plus A Statin as a Candidate Therapeutic. Cell Reports, doi: 10.1016/j.celrep.2015.12.105.

Das T, Cagan R. KIF5B-RET Oncoprotein Signals through a Multi-kinase Signaling Hub (2017). Cell Rep. 5;20(10):2368-2383. PMC5590648

Das T, Esernio J, and Cagan R (2018). Restraining Network Response to targeted Cancer Therapies Improves Efficacy and Reduces Cellular Resistance. Canc Res doi: 10.1158/0008-5472.CAN-17-2001

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- ***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."***

What individuals have worked on the project?
Ross Cagan, no change

Name:	*Ross Cagan*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.4*
Contribution to Project:	*Dr. Cagan provided overall project guidance.*
Funding Support:	*NA*

Name:	*Masahiro Sonoshita*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8*
Contribution to Project:	*Dr. Sonoshita has taken the lead in the project, working with Dr. Das to develop the transgenic animals.*
Funding Support:	*NA*

Name:	*Alexander Teague*
Project Role:	*Senior Associate Researcher*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*8*
Contribution to Project:	*Mr. Teague has provided overall technical support including animal care.*
Funding Support:	*NA*

Name:	*Tirtha Das*
Project Role:	*Associate scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Das worked with Dr. Sonoshita to develop the required transgenic animals.*
Funding Support:	*NA*

Name:	*Erdem Bangi*
Project Role:	*Senior scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Bangi provided the initial genomics analysis of lung cancer patients.*
Funding Support:	*NA*

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

The following were listed as “Active” but have now ended:

NIH R01 EY11495
Programmed cell death in the *Drosophila* eye
Role: PI. Expired 7-31-15

NIH R01 CA170495
A Drosophila Model Linking Diet-induced Obesity and Cancer (PQ1)
Role: PI. Expired 6-30-16.

ACS RSGM-11-018-01CDD
A Novel Class of Therapeutic Kinase Inhibitors for Treatment of Men2
Role: PI. Expired 12-31-15

The following grants are currently active:

U54OD020353-01 (Cagan) 08/01/2015-06/30/2020 3.6 calendar
NIH

A New Disease Platform Leveraging Complex Drosophila and Mammalian Models
Builds a therapeutic platform, Drosophila-to-mammals
Role: PI

R01HL071207 (Gelb) 08/01/02-01/31/18 0.7 calendar
Molecular basis of Noonan syndrome and related disorder
Discover additional RASopathy genes and to seek therapies that reverse the hypertrophic cardiomyopathy associated with these disorders.
Role: Investigator

What other organizations were involved as partners?
Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

APPENDICES

N/A