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TITLE: Using Systems Genetics to Probe for Gene Interactions in Congenital Heart Disease

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: In this project we tried to understand the gene networks underlying congenital heart disease, specifically bicuspid aortic valve (BAV) and its relation to hypoplastic left heart syndrome (HLHS). Under this proposal we planned to use a two-pronged strategy to systematically identify cardiac gene networks: comprehensively identifying genetic interactors of cardiogenic genes NKX2-5/tinman and GATA4/pannier using the adult Drosophila (fruit fly) heart (aim 1), and iPSC-derived cardiac progenitors and cardiomyocytes from two families (parent/proband trios) with BAV+HLHS (aim 2). During year one and two, we identified ~163 regions in the fly genome that display either synthetic lethality or cardiac phenotypes in conjunction with tinman/pannier with about 80% completion of the genetic screen. These regions include several likely candidate genes such as Muscle-specific protein 300 (Msp300/Nesprin1), as well as many new potential loci with currently unknown role in heart development and function. Surprisingly, we found many loci that ameliorated the tin/pnr double-heterozygous phenotypes in transheterozygous condition. The next steps are to follow up with identification of the specific gene loci inside the candidate regions responsible for the interaction. For aim 2, we acquired the cell lines (iPSCs) necessary for conducting the proposed research and will process these cells for mass-screening using our collaborator's high-throughput assay.

15. SUBJECT TERMS
CHD, congenital heart disease, Drosophila, iPSC, genetics, systems biology, gene networks

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1. **INTRODUCTION:**

Our research is focused on the identification of genetic vulnerabilities that might lead to congenital heart defects (CHD) and could affect patient treatment and outcome prognosis. We hypothesize underlying gene networks of cardiac determinants (transcription factors, TFs) and their targets to be affected in cases of complex CHDs, such as hypoplastic left heart syndrome (HLHS). We proposed to build a genetic interaction map of cardiac TFs using the *Drosophila* heart model and analyze congenital heart disease networks in cells obtained from two families with CHD: bicuspid aortic valve (BAV) defects in a parent and child with HLHS.

2. **KEYWORDS:** CHD, congenital heart disease, Drosophila, iPSC, genetics, systems biology, gene networks

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Under Specific Aim 1, High-throughput screen for genetic interactors of cardiac determinants, we are using the model organism *Drosophila* to study the genetics of heart development and function, ultimately to understand human heart disease. This aim has the objective to systematically test the *Drosophila* genome for loci that interact with the cardiac transcription factors *tinman* and *pannier* (NKX2-5, GATA4/5/6, in humans). Under Specific Aim 2, High-throughput screen for genetic modifiers of BAV/HLHS, we sought to challenge cells (cardiac precursors (CPs) and cardiomyocytes (CMs)) with siRNA for candidate genes, alone and in combination with NKX2-5 and GATA4. Readouts are cardiac differentiation efficacy (for CPs) and CM-proliferation assay.

Specific Aim 1: High-throughput screen for genetic interactors of cardiac determinants	**Timeline**	**Site 1**	**% completion**
Genetic Screen using Drosophila deficiencies	Months		
Ordering of fly stocks	1-3	Dr. Vogler	100
Amplification of sensitized fly line	1-3	Dr. Vogler	100
Deficiency crosses (~470)	3-8	Dr. Vogler	100
Mounting and Filming	4-9	Dr. Vogler	100
Analysis of movies	9-10	Dr. Vogler	100
Prioritization of candidate genes from deficiency hits	10-11	Dr. Vogler	100
Identification of specific interactors within deficiencies			
Ordering of fly stocks	12-24	Dr. Vogler	10
Candidate gene crosses	12-23	Dr. Vogler	10
Mounting and Filming	12-23	Dr. Vogler	
Analysis of movies	12-23	Dr. Vogler	
Confirmatory experiments (qPCR, RNAseq)	12-23	Dr. Vogler	
Milestone(s) Achieved: identified specific interactors	24	Dr. Vogler	
Specific Aim 2: High-throughput screen for genetic modifiers of BAV/HLHS			
hiPSC-derived cardiac precursors and CMs			
Obtaining hiPSC from Mayo Clinic	1-2		100
Generation of cardiac precursors and banking	3-8	Drs. Vogler and Colas	
Generation of cardiomyocytes and banking	3-8	Drs. Vogler and Colas	
siRNA treatments, immunostaining of first gene sets	9-14	Dr. Colas	

Evaluation and repeat experiments	11-16	Dr. Colas	
Test candidates from Specific aim 1	17-23	Dr. Colas	
Milestone(s) Achieved: identified patient-specific pathways affected	24	Dr. Vogler	

What was accomplished under these goals?

In continuation of aim 1, we completed to probe the *Drosophila* genome for loci that are critical for heart development and function (aim 1) using a screen for deficiencies (deletions in the genome that remove a defined set of genes) to identify loci which, when placed *in trans* to a *tinman/pannier* double mutant condition, would alter the mild cardiac phenotype of *tin/pnr* alone (see previous technical report). We have crossed a total of 384 deficiency to flies that carry pnr^{VX6}, tin^{346} (loss-of-function alleles for *pannier* and *tinman*), covering all four chromosomes (12028 genes) and evaluated all deficiencies for phenotypes (Figure 1).

Among the deficiencies tested, we identified a total of 16 that caused synthetic lethality when placed *in trans* to pnr^{VX6}, tin^{346} (Figure 1; black bars). While not heart-specific, these loci contain one or more genes that strongly interact and now become haplo-insufficient when placed together with pnr^{VX6}, tin^{346} resulting in developmental lethality. As expected, these deficiencies included those that cover the *tin* and *pnr* loci, rendering these crosses as lethal due to non-complementation.

As previously reported, for all crosses, we aimed at collecting 20 female F1 flies per cross, to be analyzed along a control cross (pnr^{VX6}, tin^{346} x w^{1118}). Flies were mounted and imaged using our established pipeline and analyzed using our custom software. We then used several parameters describing heart structure (end-diastolic and systolic diameters (EDD, ESD)) and heart function, e.g., contractility (fractional shortening FS)), heart rate (HR), heart period (HP), rhythmicity (arrhythmia index and MAD indices), contraction time (systolic interval (SI)) and cardiac output (CO). If a deficiency crossed to pnr^{VX6}, tin^{346} showed deviation of one

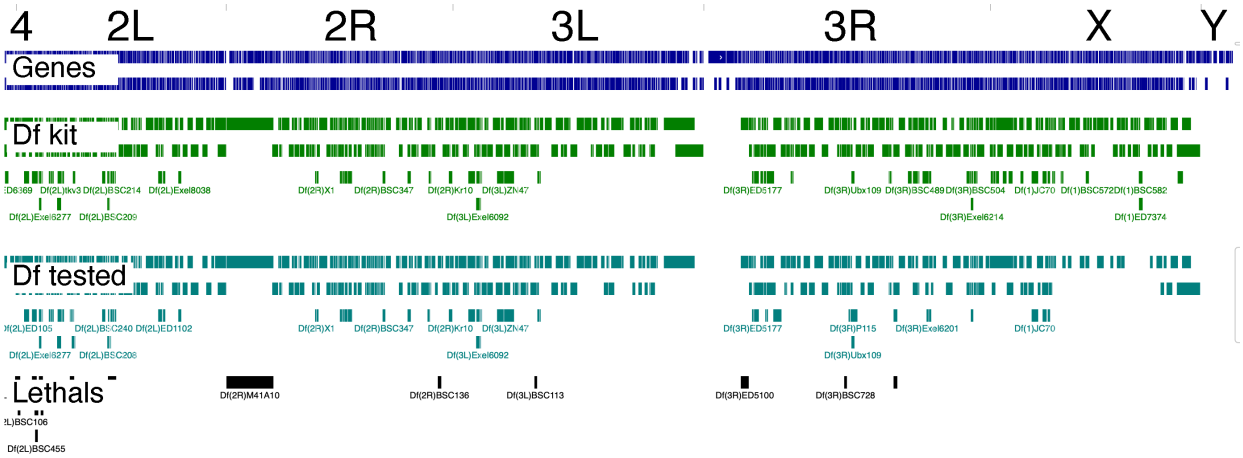

Figure 1. Map of the Drosophila genome and fly screen progress. Chromosomes and chromosome arms are shown on top. Progress on deficiency (Df) crosses is measured as Dfs tested (384) / all Dfs (BDSC Df kit). Dfs that cause synthetic lethality are shown in black.

or more heart parameters compared to the control cross it indicates a potential underlying genetic interaction of one or more loci inside the deficiency with pnr^{VX6}, tin^{346}. In contrast to synthetic lethality, were only the combined genotype is lethal whereas neither the deficiency nor pnr^{VX6}, tin^{346} alone are lethal, for all other phenotypes we need to consider that the phenotype is a result of an additive effect of the single genotypes.

Following data processing of high-speed recordings of all tested deficiencies we analyzed the data for statistical differences of each deficiency in any of the 32 cardiac parameters. To this aim we performed pairwise Wilcoxon tests of each deficiency against all control crosses. This approach ensured robust testing due to the conservative nature of the Wilcoxon ranked test compared to Student's t-test, as well as by pooling

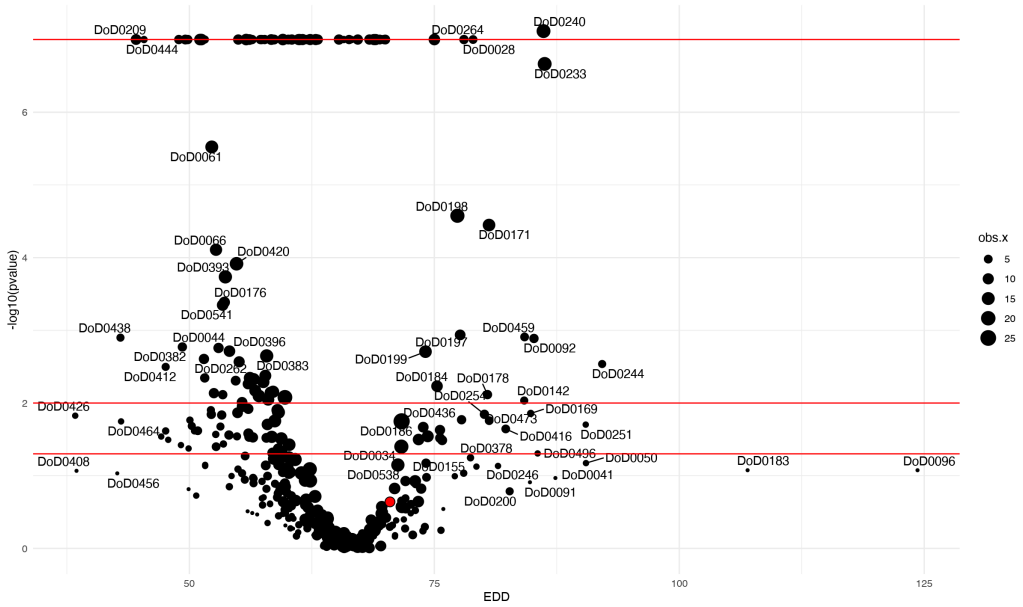

Figure 2. Volcano plot of all end-diastolic diameters (EDD) of all deficiencies tested. This screen identified genomic regions that altered heart size during relaxation (EDD) from baseline in *pnr*/*tin* double mutants. Interestingly, there are as many loci that further decrease heart size (synergism), as there are loci that increase heart size ('rescue'). Red lines indicate significance (p=0.05, p=0.01, in log-scale). Diameters are in micrometers.

all controls the baseline measurements are more accurately represented. All raw p-values were adjusted for multiple-testing.

297 deficiencies crossed to pnr^{VX6}, tin^{346} had viable and robust progeny (i.e., with enough heart recordings). Several crosses had sufficient progenies (> 10 flies), but defective hearts that could not be measured automatically due to structural defects. Among the 297 crosses, we identified 28 combinations with changes in EDD (Fig. 2), 19 in ESD, 5 in FS, 30 in SI, 25 in tt10r (90% of SI length), 63 in HP, 32 in MAD_HP, and 39 with changed cardiac output (CO). In total, 163 deficiencies showed a deviation from one or more of the baseline parameters (42%). For end-diastolic diameters, we find many deficiencies that caused an increase in diameter compared to *tin*, *pnr* double-heterozygotes that are constricted compared to wild type hearts indicating that loci exist that can reverse the cardiac defect found in *tin*, *pnr* double-heterozygotes and could serve as potential targets to ameliorate cardiac injury. Going forward we will focus on identifying those loci and

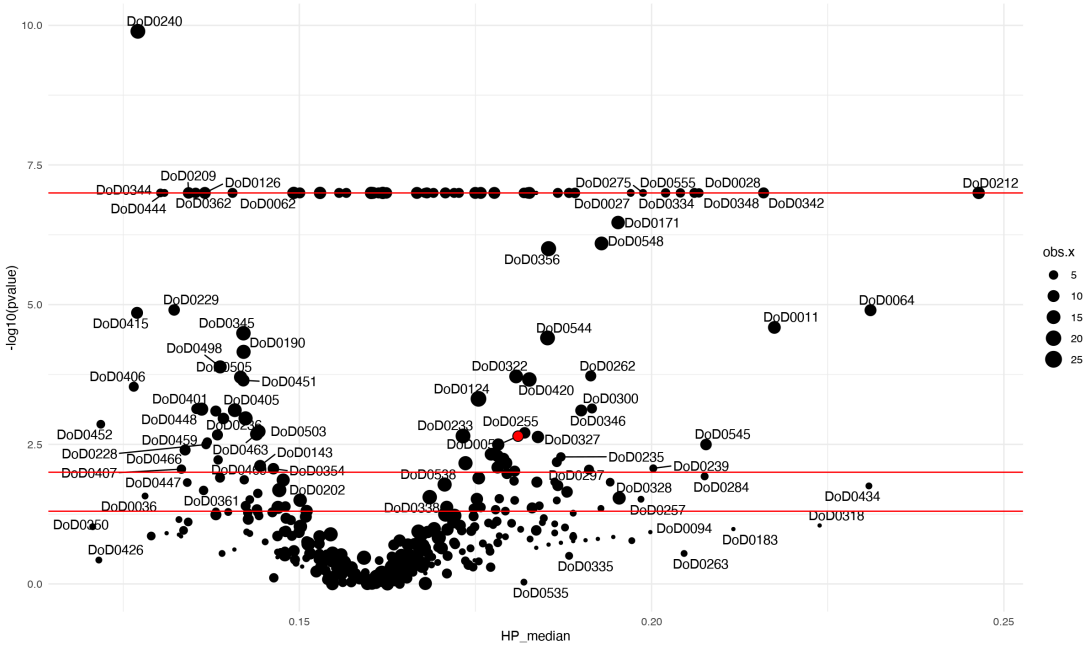

Figure 3. Volcano plot of deficiencies with reduced or prolonged heart periods (HP_median).

pathways that can rescue the *tin*, *pnr* deficiency. The most prominent phenotype with 63/163 deficiencies among all tested parameters was median heart-period (HP_median, Fig. 3), indicating that heart rate is most sensitive to genetic backgrounds compared to structural parameters (e.g., EDD, Fig. 2). Overall, this screen resulted in a large set of loci (n=163) that are likely genetically linked to the cardiac TFs *tin*/*pnr* in adult *Drosophila* hearts. Follow-up studies include testing each deficiency by itself to identify synergistic interactors versus genetic interactors, and prioritization of genetically interacting deficiencies to identify the interacting genes within each deficiency.

With respect to the analysis of iPSC-derived cells, we obtained clones for both families (7H and 158H) from our collaborators at Mayo Clinic, Rochester. We are still in the process of generating the cells necessary for the subsequent experiments (cardiac progenitors and cardiomyocytes).

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

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

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

Nothing to report (end of grant).

4. **IMPACT:**

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

Nothing to report**.**

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.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change
Nothing to Report**.**

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
Nothing to Report.

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.

6. **PRODUCTS:**

Publications, conference papers, and presentations

Journal publications. Nothing to Report

Books or other non-periodical, one-time publications. Nothing to Report

Other publications, conference papers, and presentations. Nothing to Report

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

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	Georg Vogler
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-8303-3531
Nearest person month worked:	5
Contribution to Project:	Dr. Vogler has designed the experiments, guided data acquisition and troubleshooting, and performed data analysis.
Funding Support:	N/A

Name:	Marco Tamayo
Project Role:	Lab Coordinator

Researcher Identifier (e.g. ORCID ID):	0000-0001-9891-0755
Nearest person month worked:	3
Contribution to Project:	Mr. Tamayo obtained and is maintaining all necessary fly stocks, performs fly husbandry, and data acquisition.
Funding Support:	N/A

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

CHANGES IN ACTIVE OTHER SUPPORT

Name of Individual: Vogler, Georg
Current Appointments: 09/2007 Research Assistant Professor

Other Support – Project/Proposal

CURRENT
Nothing to report.

CHANGES IN ACTIVE OTHER SUPPORT

Name of Individual: Colas, Alexandre
Current Appointments: 01/2008 Assistant Professor

Other Support – Project/Proposal

CURRENT
Nothing to report.

What other organizations were involved as partners?

1. **Organization Name:** Mayo Clinic

   Location of Organization: Rochester, MN

   Partner's contribution to the project:

   - **Other.** Mayo Clinic provided de-identified iPSCs from fibroblasts of donor tissues of two patient/parent trios (7H and 158H).

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to Report

9. **APPENDICES:** None