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TITLE: **Molecular Mechanisms of Transcription Factor Dosage in Heart Development and Congenital Heart Disease**

PRINCIPAL INVESTIGATOR: Benoit Bruneau, Ph.D.

CONTRACTING ORGANIZATION: The J. David Gladstone Institutes

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Benoit Bruneau, Ph.D.

E-Mail: benoit.bruneau@gladstone.ucsf.edu

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The J. David Gladstone
Institutes
1650 Owens Street
San Francisco, CA 94158-2261

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14. ABSTRACT
We have discovered that we can indeed understand molecular mechanisms of Congenital Heart Disease using cultured pluripotent stem cells when differentiated to cardiac cells. This requires the study of thousands of single cells rather than the whole population. These results will help us understand what type of cell is most affected upon loss of the congenital heart disease gene TBX5. Our findings in mice are also pointing a specific cell type within the developing heart that is most relevant to congenital heart disease.

15. SUBJECT TERMS
heart, gene expression, congenital heart disease, human

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Introduction.

Congenital heart defects (CHDs) are the most prevalent and serious birth defects, occurring in over 1% of live births, and higher still when stillbirths and spontaneous abortions are considered. Major subsets of congenital heart defects are defective septation of the atria or ventricles, and conduction system defects, which often co-exist within an individual. The molecular and cellular basis of congenital heart defects remains poorly understood, and is necessary to develop new diagnostic and therapeutic modalities. The genetic basis of human CHD is largely dominant mutations in gene regulators, including transcription factors and chromatin-modifying factors. How the altered dosage of a transcriptional regulator translates to altered genomic function is not known, nor is it known how these altered gene regulatory networks then disrupt heart development resulting in CHDs. TBX5 is a member of the T-box family of transcription factors, haploinsufficiency of which causes heart defects associated with Holt-Oram syndrome (HOS). We have developed an induced pluripotent stem (iPS) cell model of HOS, and in parallel we have studied a new mouse model of TBX5-dependent CHD. Current data using single cell RNAseq indicates that we can elucidate gene regulatory networks sensitive to TBX5 dosage in both models. We hypothesized that in the developing heart, TBX5 dosage in specific cell populations drives gene regulatory networks. that control finely regulated cellular behaviors, with consequences for cardiac development. We will test this hypothesis by elucidating the genomic dysregulation that results from TBX5 haploinsufficiency in a human iPSC model of CHD, and the genetic and cellular defects in the cells that define the interventricular septum boundaries in a mouse model of TBX5 haploinsufficiency in vivo.

Keywords.

Heart, congenital heart disease, gene regulation, stem cells, mouse models

Accomplishments.

What were the major goals of the project?

Specific Aim 1: Define the consequences of TBX5 haploinsufficiency in mouse and human models of CHD.
Major Task 1: Clonal reduction of Tbx5 function.
Major Task 2: Clonal reduction of Tbx5 function and relationship to Mef2cAHF lineage
Major Task 3: Gene expression changes at the compartment boundary upon loss of tbx5

Specific Aim 2: Identify the mechanisms for TBX5 haploinsufficiency.
Major Task 4: Obtain material for ChIP-exo
Major Task 5: ChIP-exo for Tbx5, Nkx2-5, Gata4
Major Task 6: Obtain material for ChIP-seq for histone marks, in Tbx5 allelic series.
Major Task 7: ChIP-seq for histone marks in Tbx5 allelic series.
Major Task 8: Gene expression changes in human iPS cells
Major Task 9: TBX5 ChIP-exo in human iPS cells

Specific Aim 3: Modulation of TBX5 haploinsufficiency by a novel genetic interactions with Mef2c.
Major Task 10: Imaging of heart defects in Tbx5/Mef2c mutant mice.
Major Task 11: Gene expression program regulated by Tbx5 and Mef2c dosage relationships.

What was accomplished under these goals?

1) major activities;

Over the course of the project we examined the function of TBX5 in mouse and human stem cell models of congenital heart disease, as outlined in the Major Tasks listed above.

We have generated single cell RNAseq data using the 10X Genomics DropSeq technique for over 50,000 human iPS cell-derived cardiomyocytes. We deployed several computational approaches to measure statistically significant change in gene expression.

We also completed the generation of single cell RNAseq analysis for mouse embryonic hearts with a clonal reduction of Tbx5 function, and did so in the context of the relationship to Mef2cAHF lineages. We have completed and are analyzing these data, along with the expression program that is altered at the compartment boundary.

Regarding the epigenomic basis of TBX5 haploinsufficiency, we deployed ChIP-seq and ATAC-seq to examine histone modifications and chromatin accessibility, respectively. We completed the ATAC-seq on the entire time course and allelic series. This massive undertaking has yielded terabytes of data that are still undergoing computational analysis. In parallel, we completed ChIP-seq or have the material for ChIPseq for several histone modification and cardiac transcription factors. We also compiled new data from our collaborators on in vivo transcription factor occupancy for all the factors that we were interested in, with others as well. This allowed a computational assessment of co-occupancy of TBX5 with a broad cadre of partner factors.

We imaged extensively several samples of *Tbx5*/*Mef2c* mutant mice and their respective controls, using histology and now deploying 3D light sheet microscopy. We have obtained some of the samples required for RNAseq analysis.

2) specific objectives;

1. Define the consequences of TBX5 haploinsufficiency in mouse and human models of CHD. Using new mouse models of disease, we wished to elucidate the importance of TBX5 dosage in maintaining a compartment boundary that is important for ventricular septation, deploying single-cell RNAseq to identify stochastic vs rheostatic gene regulation. These in vivo mouse studies were complemented with a human induced pluripotent stem cell model of CHD, to compare gene expression changes in the context of TBX5 haploinsufficiency.
2. Identify the mechanisms for TBX5 haploinsufficiency. We aimed to determine if reduced TBX5 dosage leads to an overall reduction in occupancy, reduced occupancy at discrete loci, or an absence of binding at a few loci. We also wished to evaluate the epigenomic consequences of reduced TBX5 dosage, by examining enhancer RNAs, open chromatin by ATAC-seq, and histone modifications by ChIP-seq.
3. Modulation of TBX5 haploinsufficiency by a novel genetic interactions with Mef2c. We discovered a novel genetic interaction between *Tbx5* and *Mef2c*, in which reduced dosage of both leads to specific and reproducible muscular ventricular septal defects. We wished to deploy the lineage marking and RNAseq approaches used in Aim 1 to explore the basis of this genetic interaction.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

1. Define the consequences of TBX5 haploinsufficiency in mouse and human models of CHD
An allelic series of *TBX5* was derived by CRISPR-Cas9 editing of a well-characterized iPS cell line, WTc11. WT (parental, $TBX5^{+/+}$), Control (has seen editing machinery but has not been edited at *TBX5*, $TBX5^{+/+}$), heterozygous ($TBX5^{+/-}$), or homozygous ($TBX5^{-/-}$) cells can differentiate into cardiomyocytes (Fig 1A-E), although $TBX5^{-/-}$ cells have a delayed onset of beating and differentiate less efficiently. Electrophysiological analyses show that TBX5 alters action potential characteristics and calcium handling properties in a dose-dependent manner (Fig 1F-J). Alterations are detectable in $TBX5^{+/-}$ cells, indicating that in vitro-differentiated iPS cells can reveal TBX5 dosage-related cellular phenotypes relevant to human disease. We have generated preliminary single-cell RNAseq data using the 10X Genomics DropSeq technique for >50,000 human iPS cell-derived cardiomyocytes. The data provide a highly quantitative view of gene expression in individual cells (Fig 2A). We find a progressive increase in the complexity of the cell population during iPSC differentiation, concomitant with increased definition of cell types that have altered dosage of TBX5. We used the trajectory inference software package URD (named after the Norse god of fate) to detect distinct developmental paths for the $TBX5^{-/-}$ line (Fig 2B,C), including a delay in *NKX2-5* activation (Fig 2C). We focused on cardiomyocytes at day 23, and examined pairs of clusters for altered gene expression between $TBX5^{+/+}$ and $TBX5^{+/-}$ cells. Fig 3 shows the quantitative analysis, which thanks to the profiling of thousands of cells offers strong statistical power, and a measure of gene expression levels in addition to the number of cells expressing a particular gene. We find genes with altered expression in many or all examined clusters, while most cluster-cluster comparisons reveal unique signatures. Within these groups are CHD-associated genes, and genes involved in contraction and electrophysiological parameters, providing potential mechanisms for the cellular defects. Using fluorescent in situ hybridization (RNAScope), we validated the sensitivity of one such transcript, *NPPA*, to

TBX5 dose (Fig 3E), which aligned with previous findings in mouse models. Our preliminary results show that we can learn important disease-related aspects of altered gene expression in our *TBX5* allelic series.

To discover the GRNs modulated by TBX5 dosage, we employed an approach completely independent and orthogonal to differential gene expression, using transcript correlations across single cells to infer networks. This method builds networks and quantitatively assigns nodes of centrality within the GRN, which can be compared across conditions. This analysis (Fig 4) revealed networks with altered centrality in the *TBX5*$^{+/-}$ cardiomyocytes for genes known to be involved in CHD and heart development. Genes such as *MEF2C* have reduced centrality in the *TBX5* gene regulatory network, but do not have altered expression. TBX5 and MEF2C are part of the trio

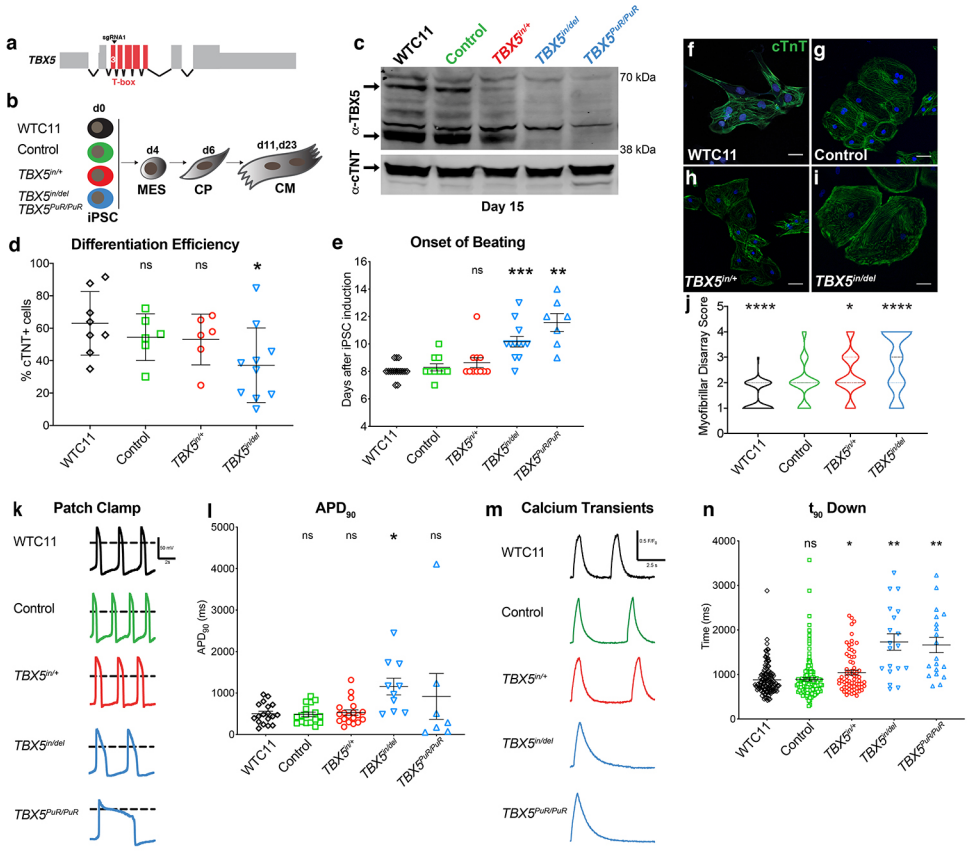

Fig 1: *TBX5* allelic series. a. Gene diagram. b. experimental design. c. Western blot across allelic series. d. Differentiation efficiency. e. Onset of beating. f-j: defective sarcomere structure. k. Action potentials. l. quantitation of APD90. m. Calcium transients. n. Quantitation of t90 down.

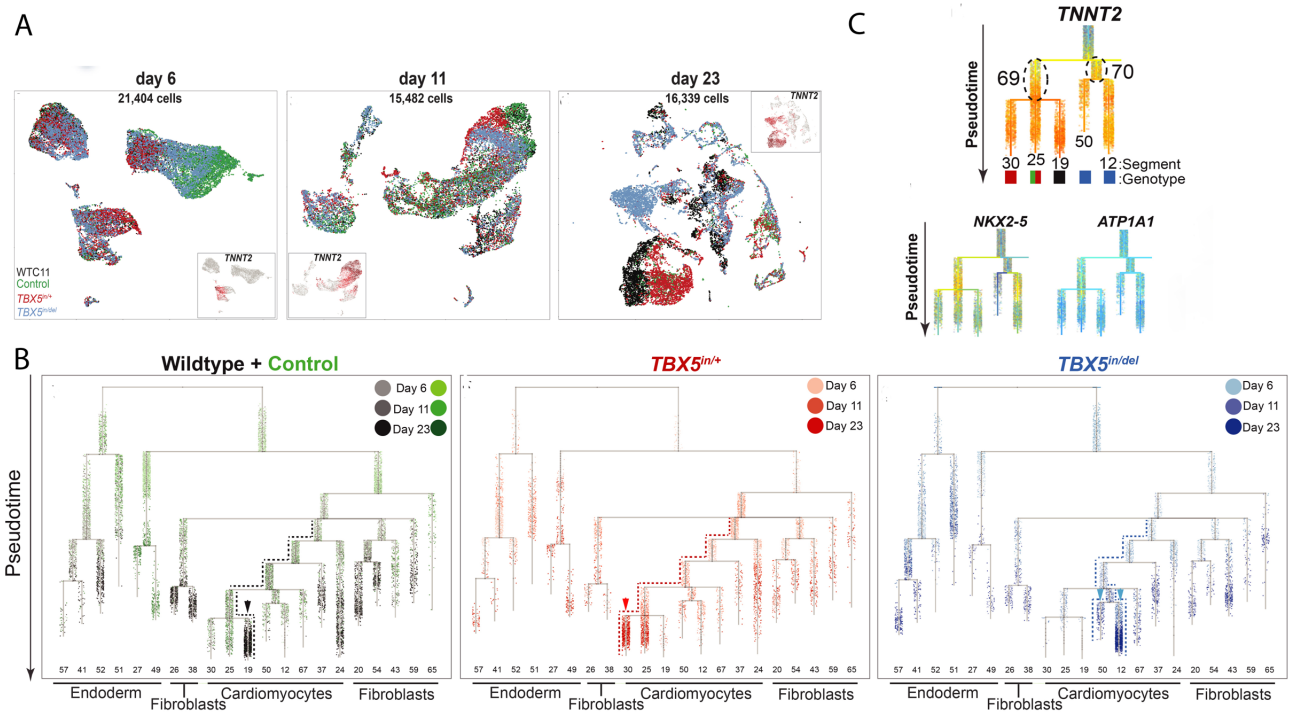

Fig 2: TBX5-dependent fate trajectories. A. Overall preliminary scRNAseq. B. Overall trajectories encompassing all genotypes. Inferred pseudotime is from top to bottom. Note the enrichment of cells from one genotype at certain branch points (arrowheads). C. Delayed or absent activation *NKX2-5* activation in *TBX5*$^{-/-}$ cells.

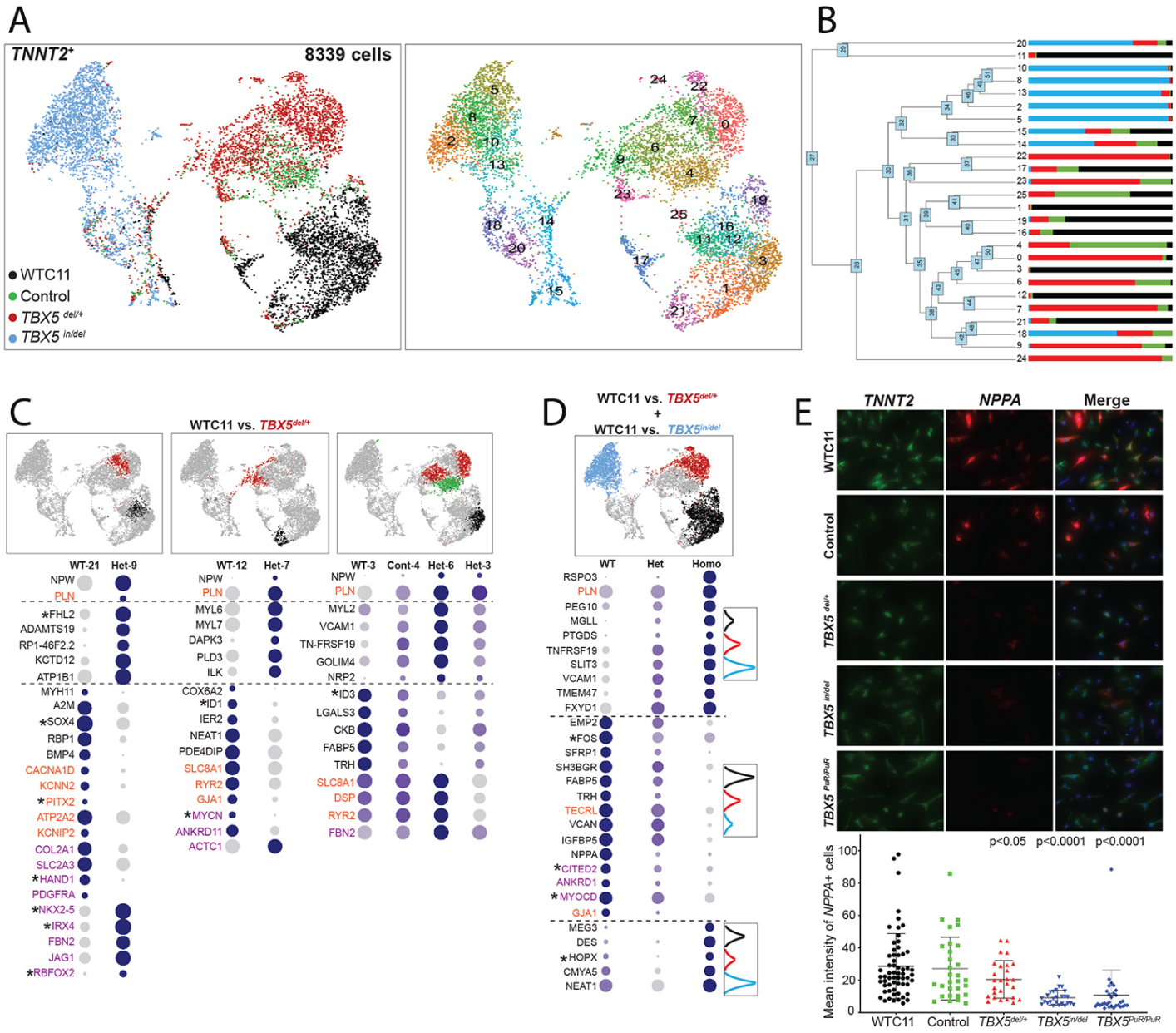

Fig 3: Quantitative analysis of single cell analysis across the *TBX5* allelic series. A. Cardiomyocyte clusters, genotype-labeled (left) and all clusters (right). B. "Family tree" of all clusters, with proportion of each genotype indicated. C. Three pair-wise cluster comparisons, chosen based on their relatedness in B. Genes labeled orange are electrophysiology-related, those in purple are mutated in CHD, asterisks indicate TFs. Size of bubble is number of cells, while intensity of color is level of expression. D. Dose-dependence of gene expression across the *TBX5* allelic series. E. Validation of dose-dependent expression of *NPPA* (red, center) by RNAScope, quantitated in graph below.

of cardiac reprogramming factors, and have been shown to physically interact and coactivate a reporter construct, making this node in the TBX5-dependent gene regulatory network particularly compelling. These observations validate this approach for the discovery of GRNs that are affected by TBX5 dosage.

All these results were published in Kathiriya et al, *Developmental Cell* 2020.

In the mouse model, we performed high-resolution light sheet imaging to assess the relationship of the IVS boundary and Tbx5-dependent CHDs in the mouse. We determined that mice in which one allele of *Tbx5* is deleted by $Tbx5^{CreERT2/+}$ have ventricular septation defects. We used $Tbx5^{CreERT2/+}$;*Mef2cAHF-DreERT2*;$ROSA^{Ai66/Ai6}$ and $Tbx5^{CreERT2/flox}$;*Mef2cAHF-DreERT2*;$ROSA^{Ai66/Ai6}$ mice to induce *Tbx5* loss of function, while tracing the cells that specifically label the left side of the interventricular septum (Fig 5).

Tbx5$^{CreERT2/+}$ is a hypomorphic allele, so the deletion of the other floxed allele of *Tbx5* creates a loss of TBX5 that is slightly greater than in *Tbx5*$^{del/+}$ mice. In this model, the *Tbx5* lineage is labeled by ZsGreen from the Ai6 reporter, while the intersectional lineage expresses tdTomato from the Ai166 reporter, as shown in Fig 6. Sections revealed that reduced TBX5 function leads to ventricular septation defects, and a loss of the sharp boundary between TBX5 lineage-labeled cells and the adjacent lineage-negative cells. Preliminary single cell RNAseq data from our *Tbx5* loss of function model collected at E13.5 show that we can identify the various cell types in the developing heart, and the specifically labeled populations by the presence of either the ZsGreen (*Tbx5* lineage) or the tdTomato (intersectional lineage) reporters (Fig 6). We further have been able to measure quantitative changes in gene expression that point to potentially important cellular pathways that are affected in the Tbx5 mutant cells. For example, the TF *Id2*, previously shown to be downstream of *Tbx5*, has reduced expression in several clusters, and in the tdTomato intersectional lineage.The upregulation of the cell adhesion/repulsion genes *Slit2* and *Netrin1* (*Ntn1*) provides compelling potential cellular mechanisms via their increased expression in the affected cells. The power of single-cell RNAseq is clearly manifest *in vivo* as it was for our human *in vitro* experiments, as comparison of different clusters yields different sets of altered transcripts, and by gating on the tdTomato+ (intersectional lineage) cells, we can directly interrogate changes in expression in this unique population without sorting and with the ability to simultaneously interrogate all other cells. Our preliminary results clearly show that with single-cell RNAseq, we will be able to gain clear insights into stochastic vs. rheostatic gene regulation determined by reduced TBX5 dosage. We are pursuing with RNAscope the expression of the potential patterning molecules, and will also integrate single-cell ATACseq to define altered regulatory elements. This part of the project is being finalized into a manuscript.

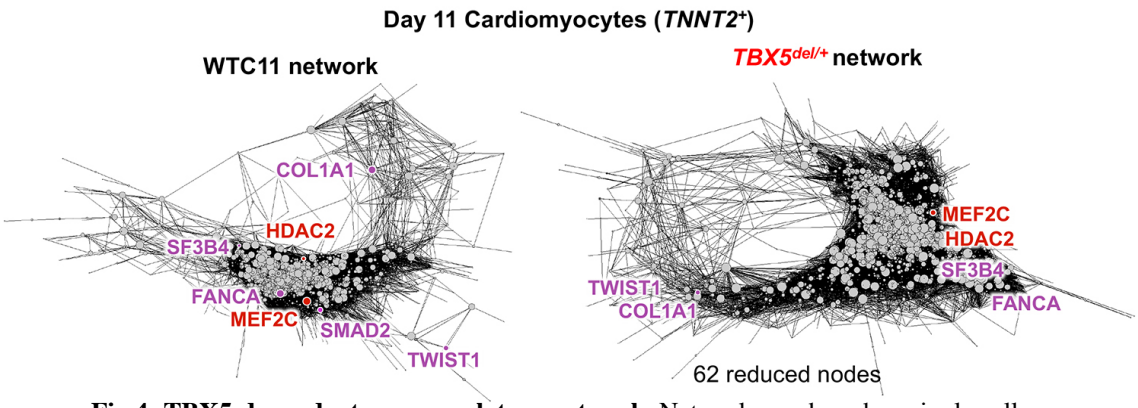

Fig 4: TBX5-dependent gene regulatory network. Networks are based on single cell RNAseq data. Shown are networks for d11 cardiomyocytes for WT (left) and *TBX5*$^{del/+}$ (right). CHD-related genes are noted in purple, and heart development genes in red. Note the absence of *SMAD2* and the reduced centrality of *MEF2c* (smaller circle) in the *TBX5*$^{del/+}$ network.

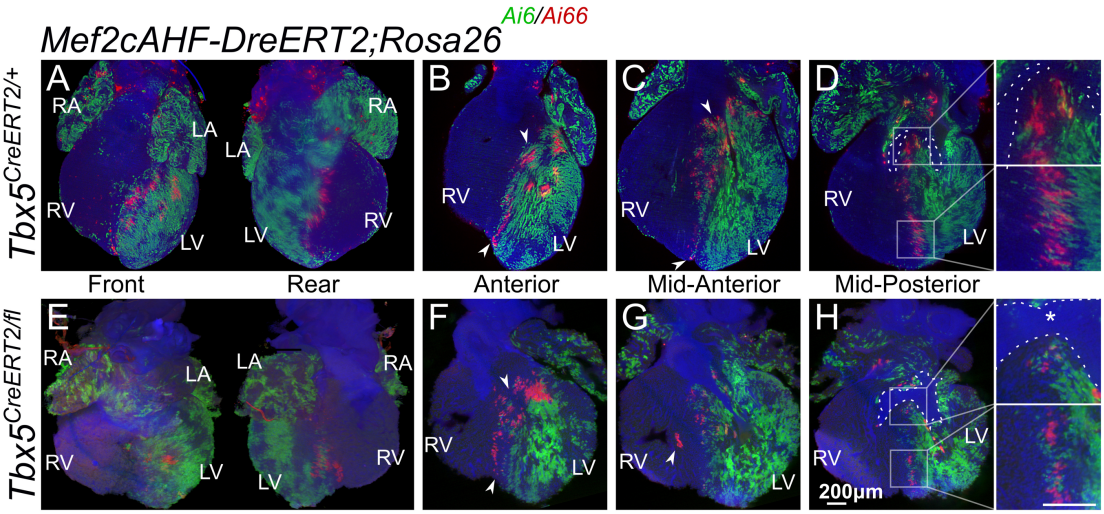

Fig 5: Mislocalization of double-positive *Mef2cAHF*+/*Tbx5*+ intersect lineage cells is associated with ventricular septal defect in *Tbx5* mutant hearts. Dams were injected with tamoxifen at E6.5 and embryonic hearts were collected at E14.5. DAPI is labeled in blue, *Tbx5*+ lineage is labeled in green, and double-positive *Mef2cAHF*+/*Tbx5*+ intersect lineage is labeled in red. In Tbx5 mutants, intersect cells do not uniformly align with the center of the IVS (arrowheads in F and G, compare with arrowheads in B and C). In mid-chamber and posterior positions, apical intersect cells appear grossly normal (lower insets in H vs. D); however, they are almost completely absent from the basal septum (upper insets in H vs. D), where a large ventricular septal defect (asterisk) is present.

In our current analysis of the single cell RNAseq from mouse embryonic hearts with a clonal reduction of Tbx5 function, in the context of the relationship to Mef2cAHF lineages, we have identified several transcription factors and signaling molecules that we will experimentally test as potential downstream mediators of TBX5 function in the context of ventricular septation.

2. Identify the mechanisms for TBX5 haploinsufficiency.
We also generated ATAC-seq data in the TBX5 allelic series and find qualitatively altered regions of accessibility near genes that are downregulated when TBX5 is reduced or absent (Fig 7). We are in the process of combining this with other histone modifications that we are still gathering data on.

3. Modulation of TBX5 haploinsufficiency by a novel genetic interactions with Mef2c.
We also identified a novel genetic interaction between *Tbx5* and *Mef2c*. This was predicted from the human iPS cell-derived *TBX5*-sensitive gene regulatory network, in which *MEF2C* was identified as a node of centrality disrupted in *TBX5*$^{del/+}$ iPSC-derived cardiomyocytes (Fig 4). The hearts of mice singly heterozygous for *Tbx5* (*Tbx5*^{CreERT2}) or a null allele of *Mef2c* are intact (Fig 8A, B), while the hearts of *Tbx5*^{CreERT2};*Mef2c*$^{+/-}$ mice have clear muscular VSDs or subaortic membranous VSD (Fig 8C-E), revealing a powerful and highly sensitive genetic interaction. This is remarkable as this is rarely seen in mice, and yet is a very common CHD in humans. Using newly available in vivo ChIP-seq data we were able to make a statistically robust correlation between TBX5 and MEF2C genomic occupancy. These results were published in Kathiriya et al 2020. We are continuing to examine this genetic interaction with single cell and spatial transcriptomics.

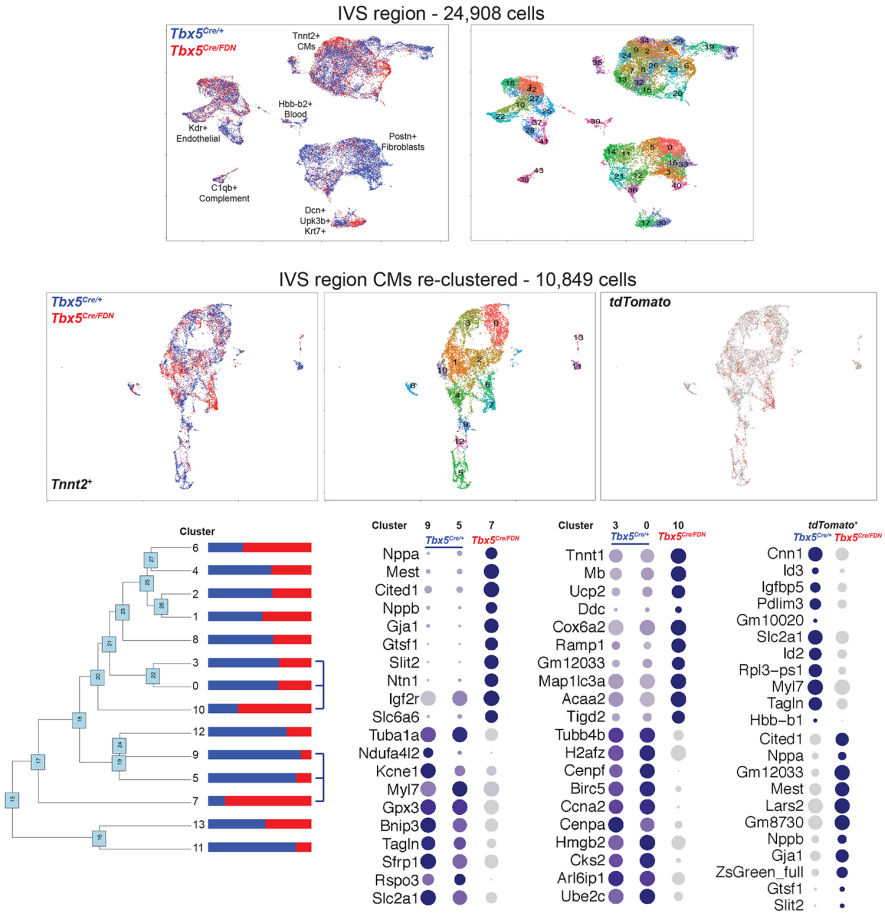

Fig 6: Single cell RNAseq from E13.5 interventricular septum. A: all cells are shown in UMAP labeled by genotype (left) or by individual cluster (right). B: Subset of Tnnt2+ cardiomyocytes, reclustered. Left is colored by genotype, middle are all clusters, right highlights tdTomato+ (intersectional lineage) cells. C: correlation tree of the 13 clusters in B. D: top 10 increased or decreased transcripts in *Tbx5*$^{CreERT2/fdn}$ vs *Tbx5*$^{CreERT2/+}$, for three sets of cluster comparisons. Size of bubble indicates number of cells expressing, while color intensity is transcript level.

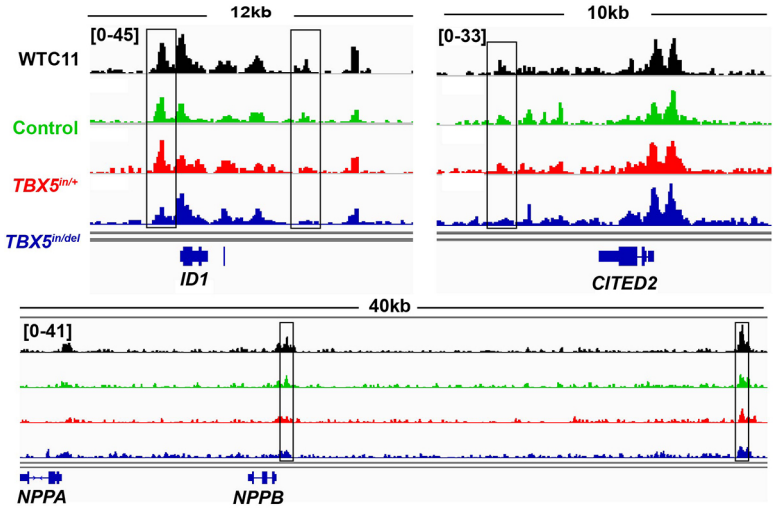

Fig 7: ATAC-seq in the TBX5 allelic series. ATAC-seq tracks are shown for *ID1*, *CITED2*, and *NPPA*/*NPPB*. Regions with reduced accessibility are boxed.

4) other achievements
In the course of the analysis of the data described above, we designed a new computational tool called Cell Layers, which permits the supervised subclustering of single cell RNAseq data in Sankey plots. This convenient approach was presented in a bioRxiv preprint (Blair et al 2020).

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.

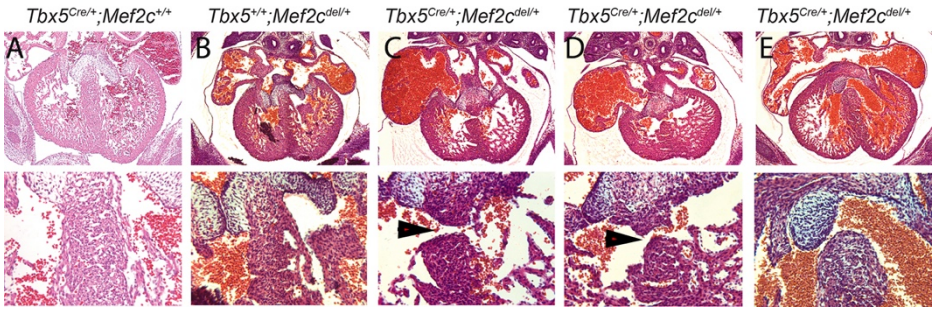

Fig 8: Genetic interaction between *Tbx5* and *Mef2c*. Genotypes are indicated above each sample. Magnified views of the interventricular septa are shown in the bottom row. Note muscular VSDs (arrowheads in C,D), subaortic membranous VSD (E) and dilated blood-filled atria in the $Mef2c^{+/-};Tbx5^{CreERT2/+}$ embryos.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
The impact of the project was to discover the molecular and genetic underpinning of congenital heart disease. The findings have broad ramifications for fundamental concepts in cardiac biology, and also for the potential management of human congenital heart disease and, importantly, its associated co-morbidities. The findings also provide exciting testable hypotheses related to the genetic etiology of congenital heart disease. Finally, the project has for the first time pinpointed the specific cell types that are involved in septation defects, which after several decades of investigation now allow us to probe their importance further.

What was the impact on other disciplines?
In general the project has impacts on cardiac electrophysiology, and to gene regulation in general. Many diseases are caused by transcription factor dosage reduction, and this work now informs the potential mechanisms underlying many of these types of disease. Our work also has general and broad impact on the use of human iPS cell models combined with single cell RNAseq and innovative computational approaches to interrogate these data.

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
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
We only had delays in the ChIP-exo experiments due to the shutdown of Santa Cruz Biotechnologies, our antibody provider. We have since identified other suitable antibodies and will complete this aim.

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:

Publications, conference papers, and presentations

***Journal publications*.**

"Modeling Human TBX5 Haploinsufficiency Predicts Regulatory Networks for Congenital Heart Disease"
Kathiriya IS, Rao KS, Iacono G, Devine WP, Blair AP, Hota SK, Lai MH, Garay BI, Thomas R, Gong HZ, Wasson LK, Goyal P, Sukonnik T, Hu KM, Akgun GA, Bernard LD, Akerberg BN, Gu F, Li K, Speir ML, Haeussler M, Pu WT, Stuart JM, Seidman CE, Seidman JG, Heyn H, Bruneau BG.
Developmental Cell. 2020 Dec 8:S1534-5807(20)30929-1. doi: 10.1016/j.devcel.2020.11.020. Online ahead of print. PMID: 33321106

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

Other publications, conference papers, and presentations

Preprint:

"Cell Layers: Uncovering clustering structure and knowledge in unsupervised single-cell transcriptomic analysis"
Andrew P. Blair, Robert K. Hu, Elie N. Farah, Neil C. Chi, Katherine S. Pollard, Pawel F. Przytycki, Irfan S.Kathiriya, Benoit G. Bruneau
bioRxiv 2020.11.29.400614; doi: https://doi.org/10.1101/2020.11.29.400614

National conferences:

- Keystone Symposium on Molecular Mechanisms of Heart Development, Keystone CO, March, 2017
- Weinstein Cardiovascular Development and Regeneration Meeting, Columbus, OH, May 2017
- Single Ventricle Summit, Stanford University, Palo Alto CA April, 2018
- Weinstein Cardiovascular Development and Regeneration Meeting, Indianapolis, IN May 2019
- Keystone Symposium on Heart Failure, Keystone CO, March, 2020
- International Society for Stem Cell Research Annual Conference (Plenary), (Virtual) June 26, 2020

International Conferences:

- Heart Failure Update Conference, Toronto, ON, May, 2018
- Weinstein Cardiovascular Development and Regeneration Conference, Nara Japan, May 2018

Other:

- Michael Potter & Family Cardiovascular Genetics Endowed Lectureship, University of Ottawa Heart Institute, October 26, 2020

Website(s) or other Internet site(s)
https://cardiac- differentiation.cells.ucsc.edu

Technologies or techniques
1. New classifier approach to categorize cell types. Methods in Kathiriya et al 2020.
2. New approach to cluster single cell RNAseq. Described in Blair et al preprint, code available upon request.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Benoit Bruneau*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-0804-7597*
Nearest person month worked:	1.68
Contribution to Project:	*Dr Bruneau led the overall project*
Funding Support:	*D0D and Gladstone Institutes*

Name:	*Kavitha Rao*
Project Role:	*Senior Research Associate*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-5401-6989*
Nearest person month worked:	*2.13*
Contribution to Project:	*Ms. Rao co-led the experimental team on this project*
Funding Support:	*Gladstone Institutes*

Funding Support:	*Gladstone institutes*

Name:	*Sarah Winchester*
Project Role:	*Research Associate II*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3705-4697*
Nearest person month worked:	*5.25*
Contribution to Project:	*Ms. Winchester bred and maintained the mouse colonies.*
Funding Support:	*Gladstone institutes*

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:
P01 HL089707 (Srivastava; Role: Project Leader) 9/1/08–07/31/18
NIH/NHLBI. Transcriptional Networks During Cardiac Differentiation

Salk Institute 02/01/16-01/31/19
Epigenomics of Human Cardiac Differentiation and Congenital Heart Disease

New award:
P01 HL146366 (Srivastava, PI. Role: Project leader) 04/01/19–03/31/24
NIH/NIHLBI Combinatorial Regulation of Gene Networks During Cardiac Development and Disease

R01 HL155906 01/01/21-12/31/26
Gene Regulatory Networks for Heart Development

U01HL157989 (MPI: Bruneau, Pollard) 09/01/20-08/31/25
Genetic Determinants of 4D Genome Folding in Human Cardiac Development

What other organizations were involved as partners?

- **Organization Name:** Harvard Medical School
- **Location of Organization:** Boston, MA
- **Partner's contribution to the projec**t: collaborator

- **Organization Name:** University of California San Diego
- **Location of Organization:** San Diego, CA
- **Partner's contribution to the projec**t: collaborator

- **Organization Name:** University of California Santa Cruz
- **Location of Organization:** Santa Cruz, CA
- **Partner's contribution to the projec**t: collaborator

- **Organization Name:** Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology (BIST)
- **Location of Organization:** Barcelona, Spain
- **Partner's contribution to the projec**t: collaborator

- **Organization Name:** University of California San Francisco
- **Location of Organization:** San Francisco, CA
- **Partner's contribution to the projec**t: personnel exchanges

SPECIAL REPORTING REQUIREMENTS
Nothing to report.

APPENDICES:
One publication and one preprint.