

Abstract:

Complex organisms have hundreds of specialized cell types. Cellular competence, a specific but transient property of cells to respond dynamically to inductive cues, underlies how the diversity of lineages are established¹. While many genes have been shown to be specific for certain lineages or cell types, it is clear that many gaps in our knowledge exist, including how coordinated gene programs, required for cell fate specification, are achieved. The mechanistic basis of cellular competence and how cellular identity is maintained is poorly understood.

Emerging data suggest that genome organization in three-dimensions correlates with cell identity. *However, whether it is causal of lineage restriction is unknown.* **We hypothesize that spatial distribution of the genome in the nucleus establishes and maintains cellular identity.** We propose that dynamic organization exists between the regions of the nucleus – either peripheral (at the nuclear lamina) or central (the nucleoplasm)² - and that genome organization at the nuclear periphery provides an energetically advantageous way to restrict access to regions of the genome as cells progressively restrict fates. Regions of the genome held at the nuclear lamina are generally condensed (heterochromatic) and transcriptionally silent. We posit this provides a mechanistic explanation for cellular competence, and that genomic regions at the nuclear lamina are inaccessible to local inductive signals/morphogens and gene activation. Similarly, once a lineage choice is made, we hypothesize genomic regions relevant to alternate fates are repositioned to the nuclear lamina. Our most recent data demonstrates that genome organization at the nuclear lamina regulates lineage allocation during cardiac development. We believe that deciphering the rules underlying spatial distribution of chromatin in the nucleus will help us determine how this is achieved.

Specific Aims: The original aims of my CAMS proposal were:

1. Test the hypothesis that repression of alternate fates by Hopx is required for commitment to the myocyte lineage.
2. Test the hypothesis that Hopx physically interacts with the nuclear lamina to repress Hopx target genes in Lamina Associated Domains (LADs).

Results/Accomplishments/Plan

Aim 1: The goal of Aim1 is to broadly investigate the hypothesis that establishment of lineage identity requires active repression of alternative fates. A testable question which emanates from this hypothesis is that manipulating commitment may render cells susceptible to adopt alternative fates. My work suggests that an atypical protein, Hopx (Homeodomain Only Protein X), is expressed in multiple progenitor populations³⁻⁸ and regulates commitment during cardiogenesis⁹. We hypothesize that loss of Hopx renders lineage restricted cardiac progenitor cells (cardiomyoblasts) vulnerable to adopting alternate fates.

During cardiac development, multipotent cardiac progenitor cells give rise to cardiac myocytes, endothelial cells and smooth muscle cells. Recent *in vivo* clonal have demonstrated that progenitors are restricted to one or two of these lineages, while older ESC based studies have suggested these cells are tripotent (at the clonal level). Therefore, this raises the possibility that local inductive cues *in vivo* promote one fate over another, while the ESC studies suggest that in the appropriate media, these cells retain sufficient plasticity to adopt different fates. My previous work identified a pool of lineage-restricted progenitors *in vivo* that gave rise exclusively to cardiac myocytes, “cardiomyoblasts”⁹. Cardiomyoblasts are specified immediately after multipotent cardiac progenitor cells arose and clonally expand to give rise to cardiac myocytes in all 4 chambers *in vivo*. We identified these cells based on expression Hopx. Taken together, our work suggested the hypothesis *Hopx promotes myocyte fate commitment in cardiac progenitor cells by closing a window of competence* to adopt non-myocyte fates. We predict that loss of *Hopx* extends the window of competence to adopt non-myocyte fates. We are taking parallel approaches to address this hypothesis.

Consistent with our hypothesis, our preliminary data demonstrate that loss of Hopx in embryonic stem cell derived cardiac tissues results in an increase in the number of endothelial cells and an increase in endothelial gene expression (data not shown). However, this does not directly address our hypothesis, and could be due to a loss of myocytes in the cardiac cultures. Therefore, we have focused our approaches on lineage tracing

derivatives of *Hopx*^{+/-} and *Hopx*^{-/-} cells *in vivo* and *in vitro*. This is facilitated by an allele in which we have knocked a FlpO-ERT2 recombinase into the start codon of *Hopx*. The recombinase is expressed upon *Hopx* expression, and upon tamoxifen induction the recombinase translocates to the nucleus. The recombinase excises a Stop codon in an indicator allele and a fluorescent protein (TdTomato) is expressed.

Ricardo Linares, a graduate student in my laboratory, has optimized tamoxifen induction protocols to lineage trace *Hopx*^{+/-} and *Hopx*^{-/-} cardiomyoblasts specified at embryonic day (E) 8.5 and E9.5. We are harvesting embryos at E15.5 scoring the identity of derivatives using immunohistochemistry. However, this approach relied identifying derived cells which are expressing an intracellular reporter (RFP), while endothelial markers which work well for immunohistochemistry are membrane bound (Cdh5, CD31, Tie2). Therefore, we have more recently turned to RNA-FISH combined with reporter fluorescence immunohistochemistry as a complementary approach. Both approaches have demonstrated cells derived from *Hopx*^{-/-} cardiomyoblasts have adopted endothelial fate (Fig. 1). This is in contrast to cells derived from *Hopx*^{+/+} cardiomyoblasts, which are exclusively cardiac myocytes. The RNA-FISH signal is predominantly cytoplasmic, and hence can be colocalized with the reporter signal (indicating a derived cell). We have designed RNA-FISH probes against several endothelial specific genes. We will quantify the percentage of cells derived from *Hopx*^{+/-} and *Hopx*^{-/-} cardiomyoblasts that express endothelial specific transcripts and/or myocyte-specific transcripts. Based on our control experiments, we predict that *Hopx*^{+/-} derived cells in the heart are exclusively cardiac myocytes. We also plan to harvest embryos at E12.5, as embryonic lethality due to tamoxifen exposure is a significant barrier to accruing sufficient numbers.

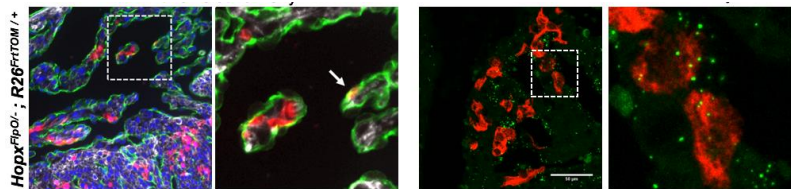


Figure 1: *Hopx*^{-/-} derived cells express non-myocyte markers. Pregnant dams were injected with tamoxifen at E8.5 and E9.5 and then embryos were analyzed at E15.5. *Hopx*^{FlpO/-}; *R26*^{Tom/+} embryos demonstrate fated cells expressing Pecam1 (left panels) and *Kdr* (right panels).

Ricardo has also been taking complementary *in vitro* approaches to test his hypothesis. Over the last several months, he has painstakingly generated ESC lines in which *Hopx*^{+/-} and *Hopx*^{-/-} cardiomyoblasts can be lineage traced upon differentiation of ESCs into cardiac tissues. However, we have discovered a major limitation of this system. *Hopx*⁺ cells give rise to the overwhelming majority of cardiac myocytes *in vivo*, however, in our hands, we do not detect significant numbers of *Hopx*-derived tissues during *in vitro* differentiation assay. Initially we posited this was because of inefficient recombinase activity of FlpO. However, more recently, we suspect that alternative pathways are active to generate cardiac myocytes *in vitro* and *Hopx* is not robustly expressed at early stages of cardiac specification *in vitro*. These data have led us to question the utility of the *in vitro* cardiac differentiation experiment to test our hypothesis. We are now optimizing our ability to isolate cardiomyoblasts from appropriately staged embryos and culture them *in vitro* for 24-48 hours while they differentiate. If successful, this would allow us to culture the cells in specific media which promotes cardiac myocyte or endothelial specification and score the identity of cells specified using an analogous combined immunofluorescence RNA-FISH approach as described above. By culturing both *Hopx*^{+/-} and *Hopx*^{-/-} cardiomyoblasts that can be lineage traced, we will be able to test the role of *Hopx* in specifying the myocyte and/or endothelial lineages and whether loss of *Hopx* renders cardiomyoblasts vulnerable to adopt endothelial cells. Our *in vivo* and *in vitro* studies are aided by the endothelial-specific reporter alleles we have bred into our lineage tracing studies.

Aim 2: The broad goal of Aim 2 is to understand how genome organization regulates cellular identity. Our original mass spectrometry data implicated an interaction between *Hopx* and the nuclear lamina, Ricardo is following up this observation. He has validated an interaction between *Hopx* and *Lap2β* and *LaminB*, two proteins found exclusively at the nuclear lamina (Fig 2A-C). Therefore, he is focusing on mapping LADs in *Hopx*^{+/+} and *Hopx*^{-/-} cardiac tissue. We are isolating embryonic myocytes and attempting to knockdown *Hopx*. Ricardo has optimized our ability to ChIP *LaminB* from cardiac myocytes, which we have confirmed using *LaminB* ChIP-western. We are now validating our knockdown approaches and then

will proceed with LaminB ChIP-seq (Fig. 2D-E). We recently published an important manuscript demonstrating that Hdac3 (Histone deacetylase 3) controls spatial organization of the genome via tethering of chromatin to the nuclear lamina to control cardiac cell fate choices and differentiation¹⁰. An additional key finding in our study was that chromatin marked by Histone 3 Lysine 9 dimethylation (H3K9me2) localized to the nuclear periphery. Therefore, we will complement our LaminB studies with H3K9me2 ChIP-seq.

We are expanding our efforts to understand lamina-genome interactions from the aforementioned studies in multiple directions. These efforts form the foundation for a newly awarded DP2/NIH New Innovator grant. First, we have confirmed using a ChIP-mass spectrometry approach and immunofluorescence that H3K9me2 is enriched on chromatin adjacent to the nuclear lamina in multiple species (most saliently, human, Fig. 3A). We are now mining the ChIP-mass spectrometry data (which was generated in a collaboration with Ben Garcia at Penn) to identify other modifications and/or epigenetic factors which are enriched on peripheral heterochromatin (Fig. 3B). Second, we are collaborating with Kyle Loh/Irv Weissman (Stanford University), Nicole Dubois (Mt. Sinai School of Medicine), and Katie Pollard (UCSF) to generate an atlas of nuclear lamina-chromatin interactions across multiple isogenic cell types. We have profiled ~15 cell types in total, including cell types from all three germ layers and at varying levels of lineage restriction. This will allow us to define the portion of the genome which changes during adoption of identity and what is encoded within that portion. Third, we are understanding how *LMNA* mutations alter genome organization at the nuclear lamina. *LMNA* mutations are associated with multi-organ phenotypes, including premature aging, lipodystrophy, and dilated cardiomyopathy. We have knocked in a specific mutation into a control iPS cell line (*LMNA T10I*), which is based on a patient seen by in the Penn Inherited Cardiomyopathy Clinic who had end stage heart failure and underwent an orthotopic heart transplant and profiled derived cardiac myocytes. We have defined

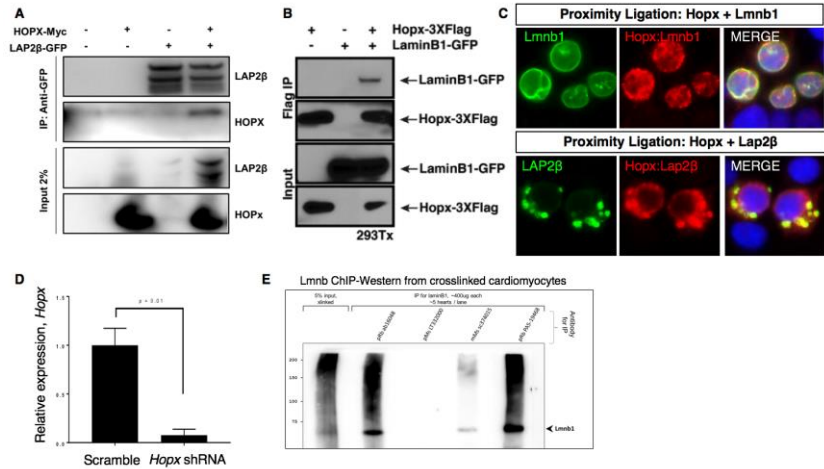


Figure 2: Hopx interacts with LaminB1 and Lap2β. (A-C) 293Tx cells transfected with Hopx-Myc and either GFP-Lap2β (A, C) or GFP-LaminB1 (B, C). (A,B) demonstrate we can co-immunoprecipitate Hopx:LaminB1 and Hopx:Lap2β. (C) Proximity ligation assays (C) performed as indicated using Hopx or Myc antibody and GFP antibody. Cells transfected with only one construct (but probed with both antibodies) did not yield signal (negative control, data not shown). (D) Efficiency of *Hopx* knockdown in neonatal cardiomyocytes. (E) Optimization of Lamin B ChIP.

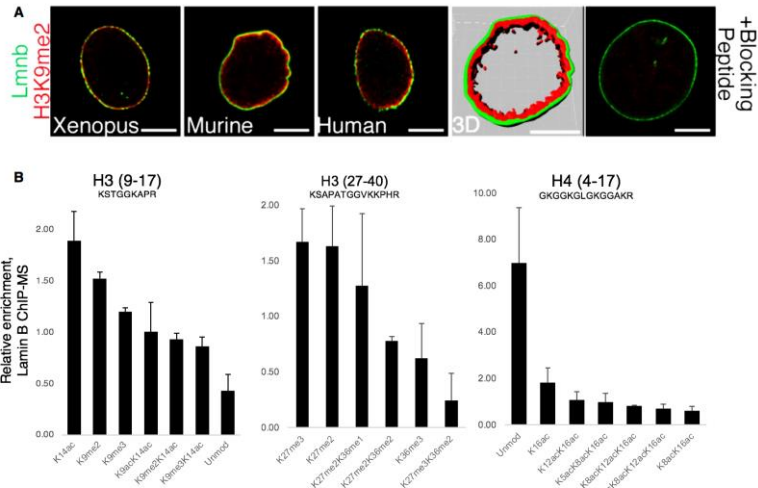
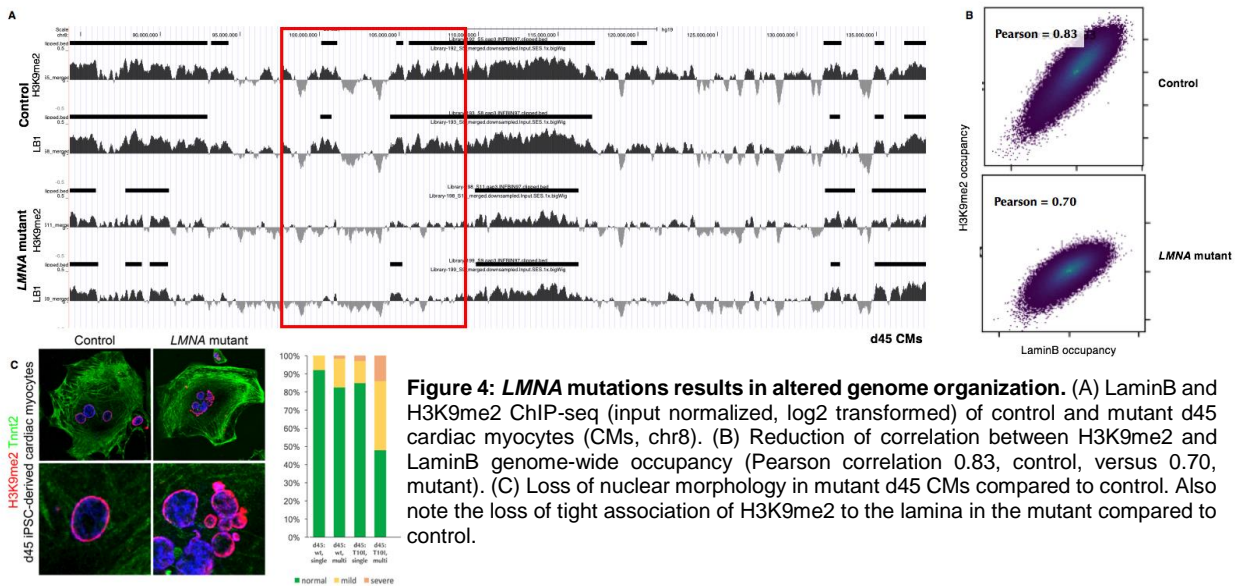


Figure 3: Enrichment of H3K9me2 at nuclear lamina. (A) Immunofluorescence of H3K9me2 in different species primary cells. Specificity confirmed by blocking peptide used in murine studies. (B) Lamin B ChIP-MS confirms enrichment for H3K9me2 at nuclear lamina. Analysis also shows enrichment for H3K27me2/3 and relative depletion of acetylated H4 chromatin at lamina.

LaminB and H3K9me2 occupancy in mutant and control iPSC derived CMs using ChIP-seq (Fig 4A). Our preliminary data demonstrates disarray of genome organization in mutant cardiac myocytes compared to control, and specifically a loss of lamina-associated chromatin in mutant cells. In addition, consistent with our murine studies, our control cells demonstrate a high correlation between lamina association and H3K9me2 occupancy. However, we have noted that there is a reduction in this correlation in mutant cells (Fig. 4B). Imaging studies have demonstrated severe lamina morphologic defects in differentiated cells and a loss of H3K9me2 localization at the nuclear lamina (Fig. 4C). We are currently performing transcriptome profiling of mutant and control myocytes. In addition, we are assaying the calcium flux and contractility in mutant and control isolated myocytes composed of myocytes and fibroblasts. This will allow us to correlate genome organization changes, transcriptional activity, and physiologic function.



Distinct from DP2 Award, I have received an extremely competitive score on an R01 (4.0 percentile). It focuses on the role of Bromodomain Extraterminal Protein 4 (Brd4) on cardiac development and progenitor specification. We have found that loss of Brd4 results in a loss of cardiac progenitor specification during cardiac development, and we are testing the hypothesis that loss of the co-activator function of Brd4 in progenitor cells results in vulnerability to adopt alternative fates. Separately, our preliminary data suggests that Brd4 instructs genome organization and is an architectural protein. We are eager to follow these data as they may reveal important principles of genome organization and fate decisions.

Mentoring/Environment/Career Development

Jonathan A. Epstein, MD is my primary mentor. Additional mentors that I meet with regularly include Michael S. Parmacek, MD (Chair of Medicine), Thomas Cappola, MD (Chief of Cardiovascular Medicine) and Garret A FitzGerald, MD FRS (Chair of Pharmacology, Director of the Institute for Translational Medicine and Therapeutics). They are all physician-scientists who I interact with regularly formally (as part of my “mentoring committee”) and informally about scientific progress and career development.

Other Activities/Award Administration:

- Faculty appointment details: Tenure track position, Salary: \$200,000/year, Laboratory start-up: 1.5M (no timeline) + Salary coverage X 3 years (after grant support), Bioinformaticist ¼ time X 2 years, Administrative assistant; 2 weeks of inpatient service/academic year.
- American Heart Association, Invited Talk (11/17, Anaheim, CA) Keystone Meeting: Heart Failure, Crossing the Translational Divide, (1/18, Keystone, CO, Oral presentation); Keystone Meeting: Gene Control in Development and Disease, (3/18, Whistler, BC, Poster presentation)
- Trainees Supported: N/A

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