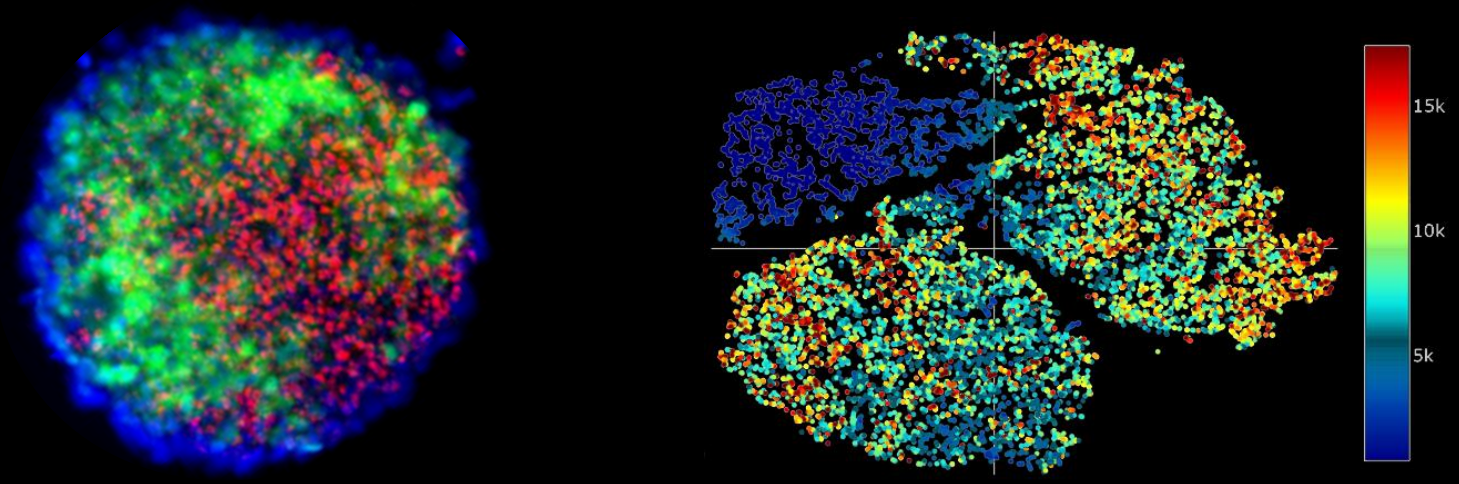
The omics of stem cell mediated regeneration:

A pilot single cell RNA-seq study of mechanotransduction



Presented by NASA Postdoctoral Fellow: Cassandra M. Juran

Presented on: May 31, 2019

Mentor: Eduardo A.C. Almeida[1] and Co-Investigator: Elizabeth A. Blaber[1,2] , Molly Coyne[1, 3], Justina Žvirblytė[1,3, 4]

[1]Space Biosciences Division, NASA Ames Research Center, Moffett Field, CA [2]University Space Research Program USRA, Mountain View, CA
[3]Blue Marble Young Scientist Program, Mountain View, CA [4]Life Sciences Center, Vilnius University, Vilnius, Lithuania

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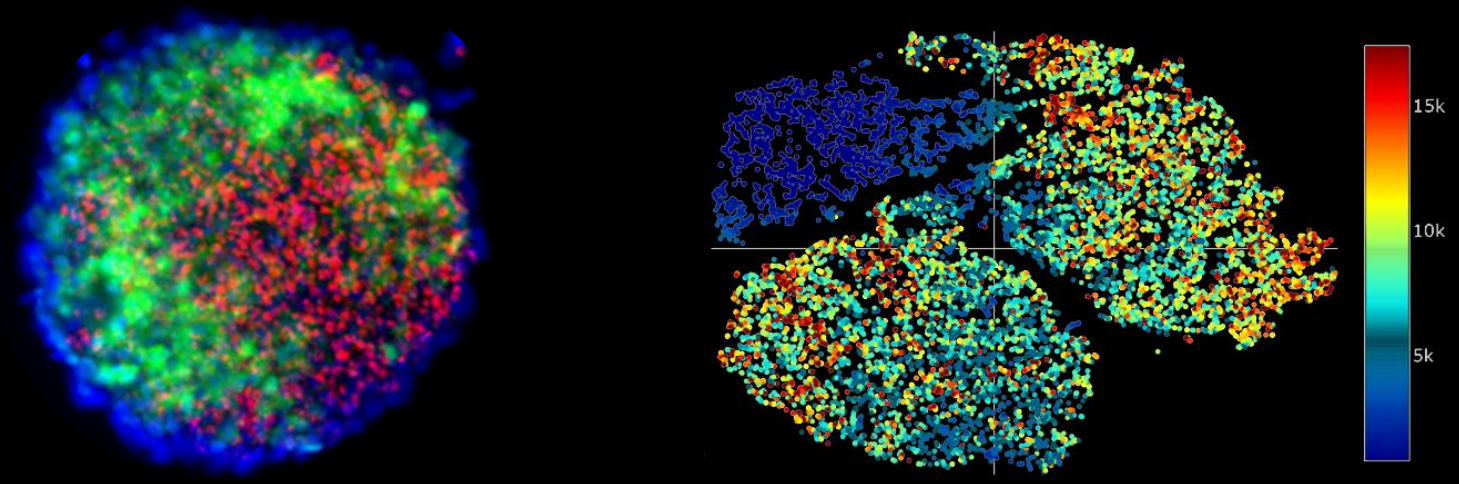

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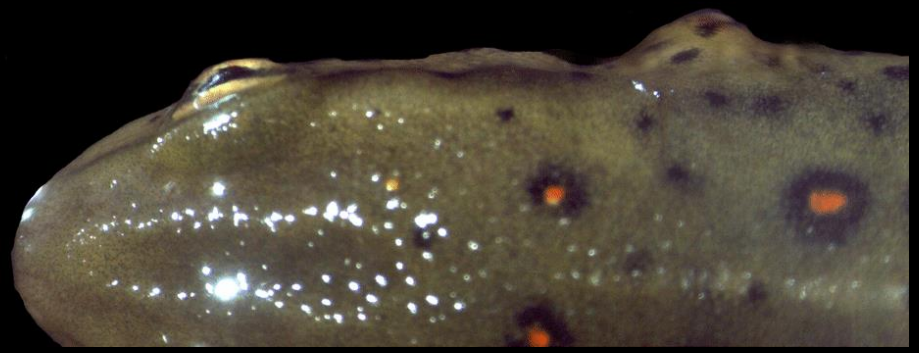
Regeneration

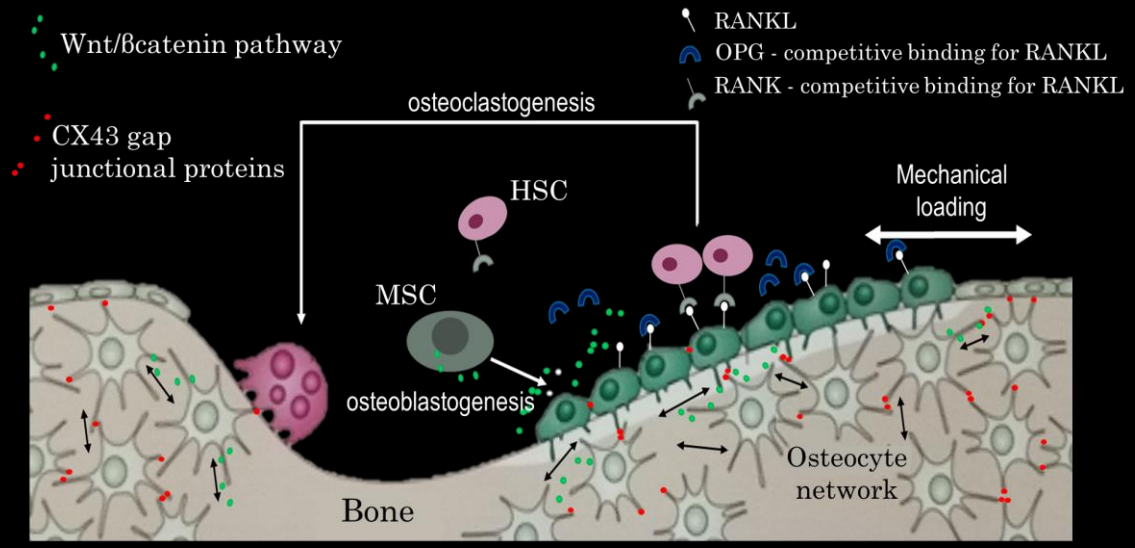
regeneration is the process of renewal, restoration, and growth that makes genomes, cells, and organisms resilient to natural fluctuations or events that cause disturbance or damage

Repair of Injury Deficit



Salamander limb regenerates from adult tissue de-differentiation and re-differentiation to replace the deficit.

Homeostatic



Many tissues undergo homeostatic regeneration, with deficits being replace by adult stem cell differentiation and functionalization.

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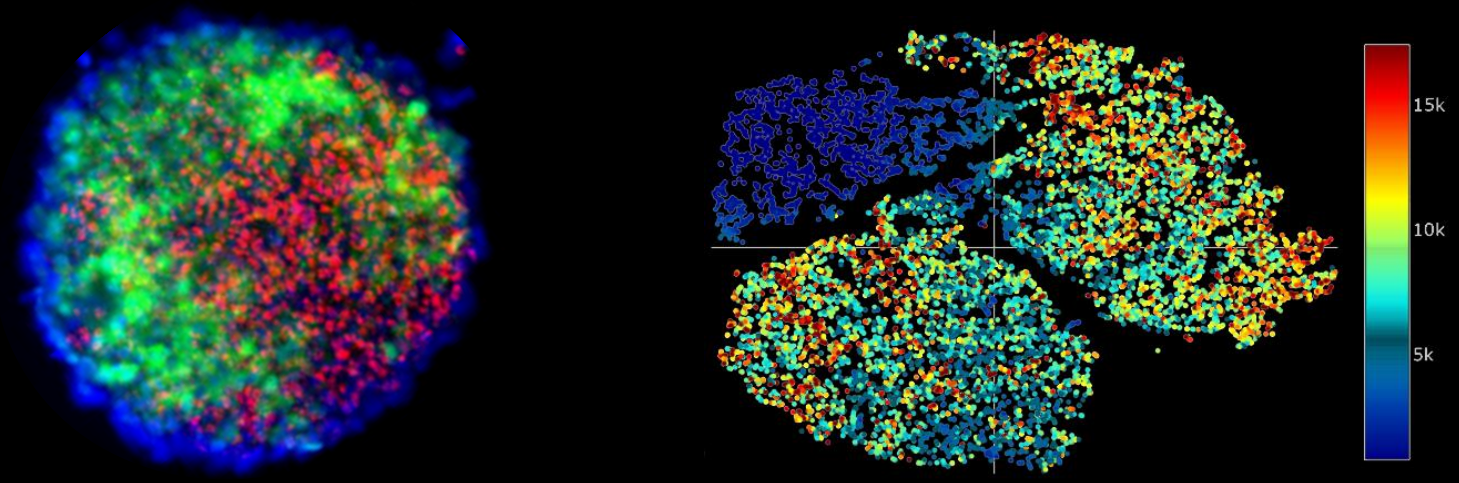

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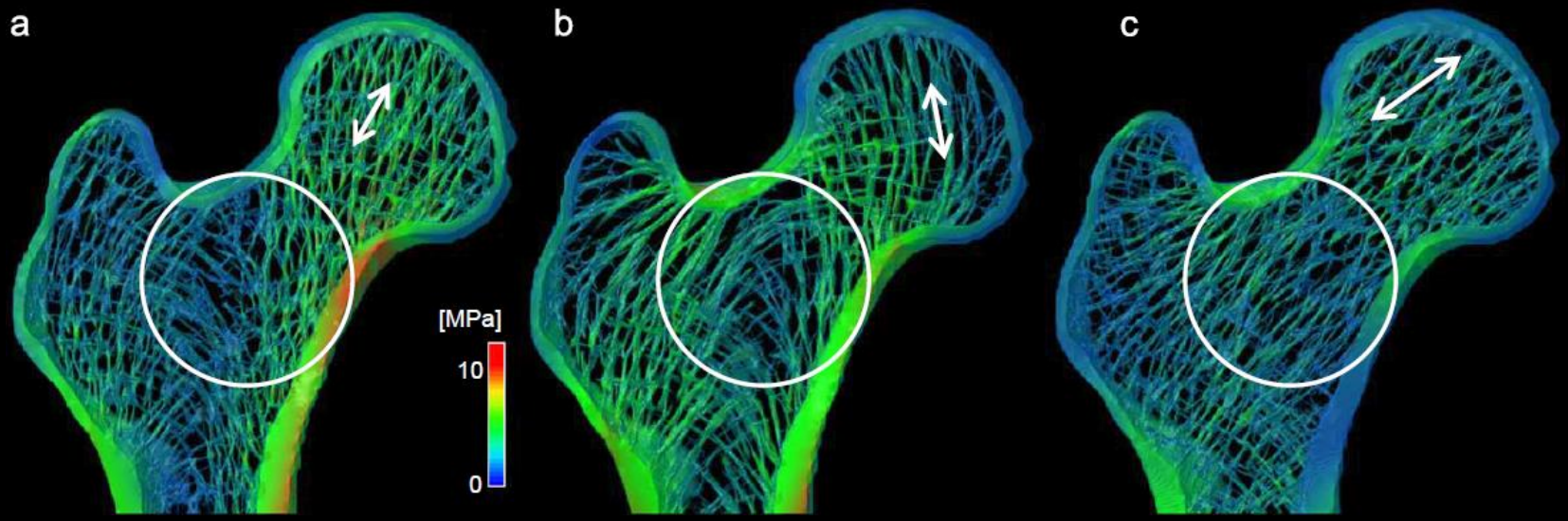
Mechanotransduction: Form Fits Function

- "Every change in the form and function of bones, or their function alone, is followed by certain definite changes in their internal architecture and equally secondary alteration in their external conformation, in accordance with mathematical laws." – Julius Wolff (1892)



K. Tsubota et al. Journal of Biomechanics 42 (2009) 1088 - 1094

- Mechanotransduction is a term which represents the combined processes of sensation of stress, transduction into biochemical signaling, and the subsequent sequences of biological responses these changes elicit.

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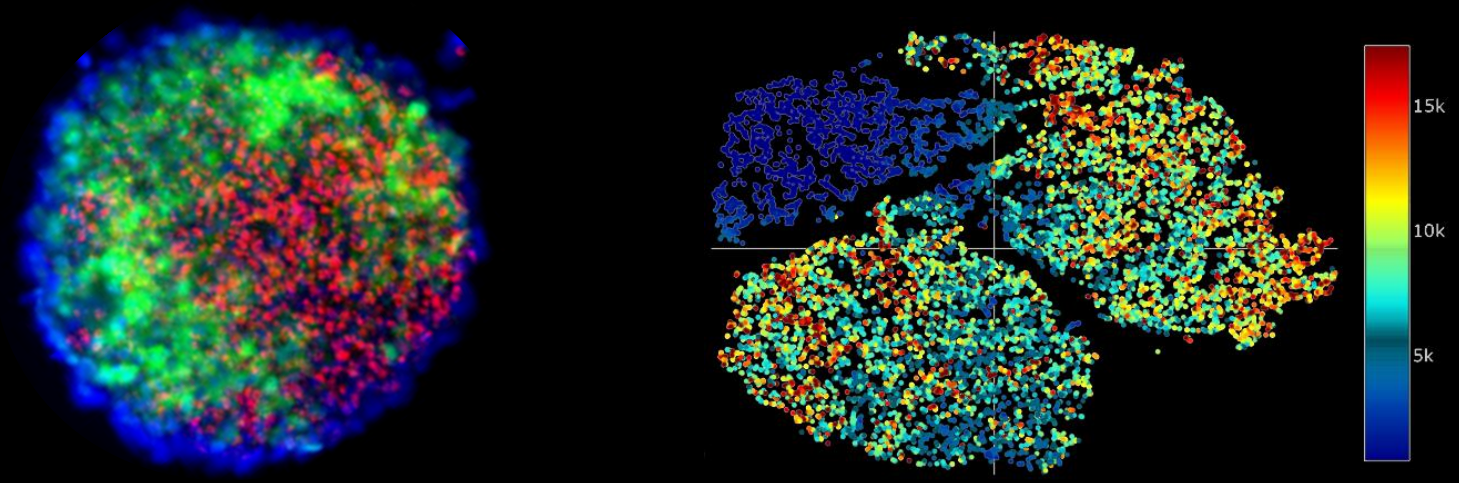

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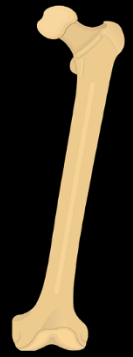
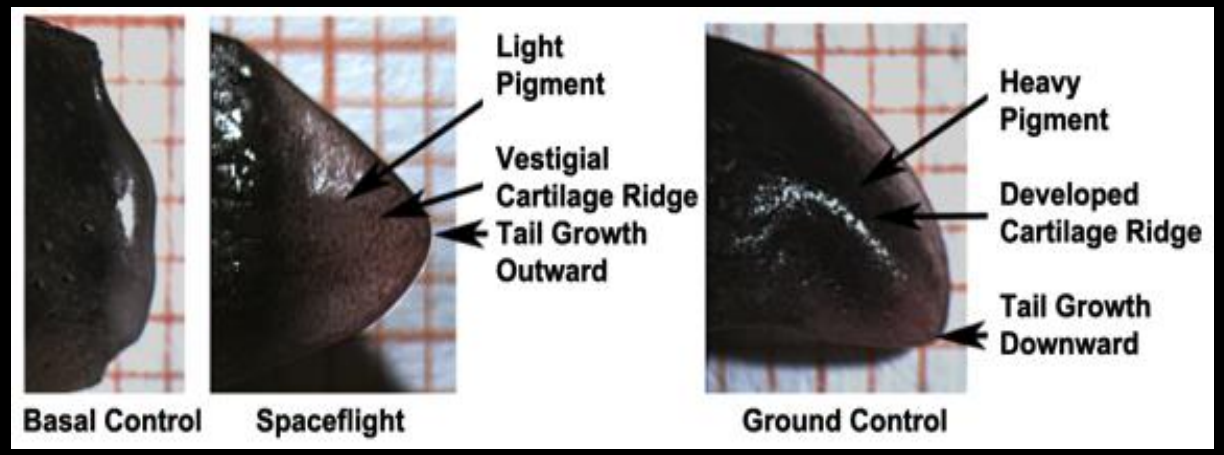
Why Single Cell Sequencing?

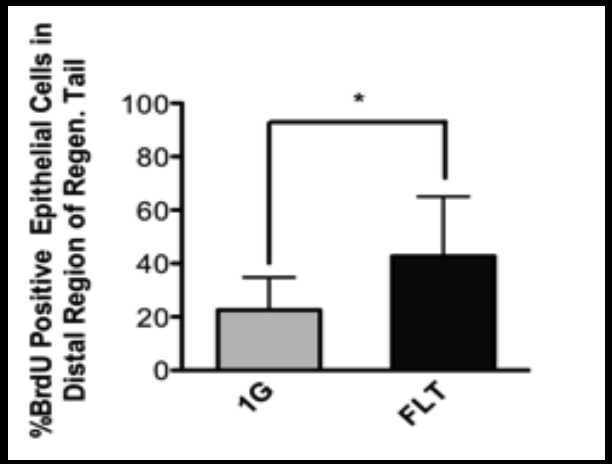


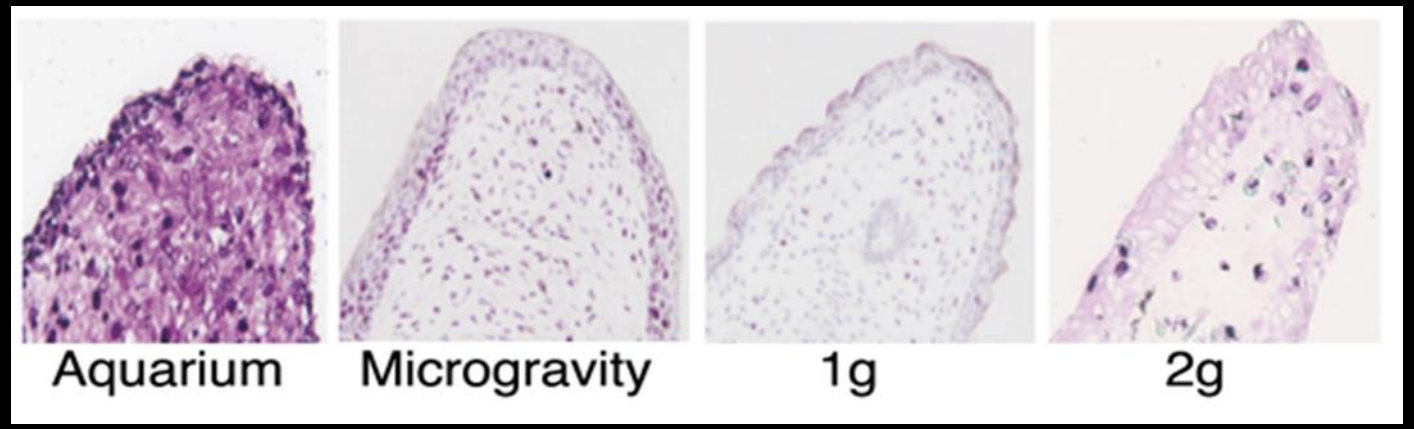
Spaceflight?

Space as a novel “mechanotransduction-null” Separate effects of loading and regeneration

Regenerative Species: Spanish Ribbed Newt Flown on Foton M2 & M3





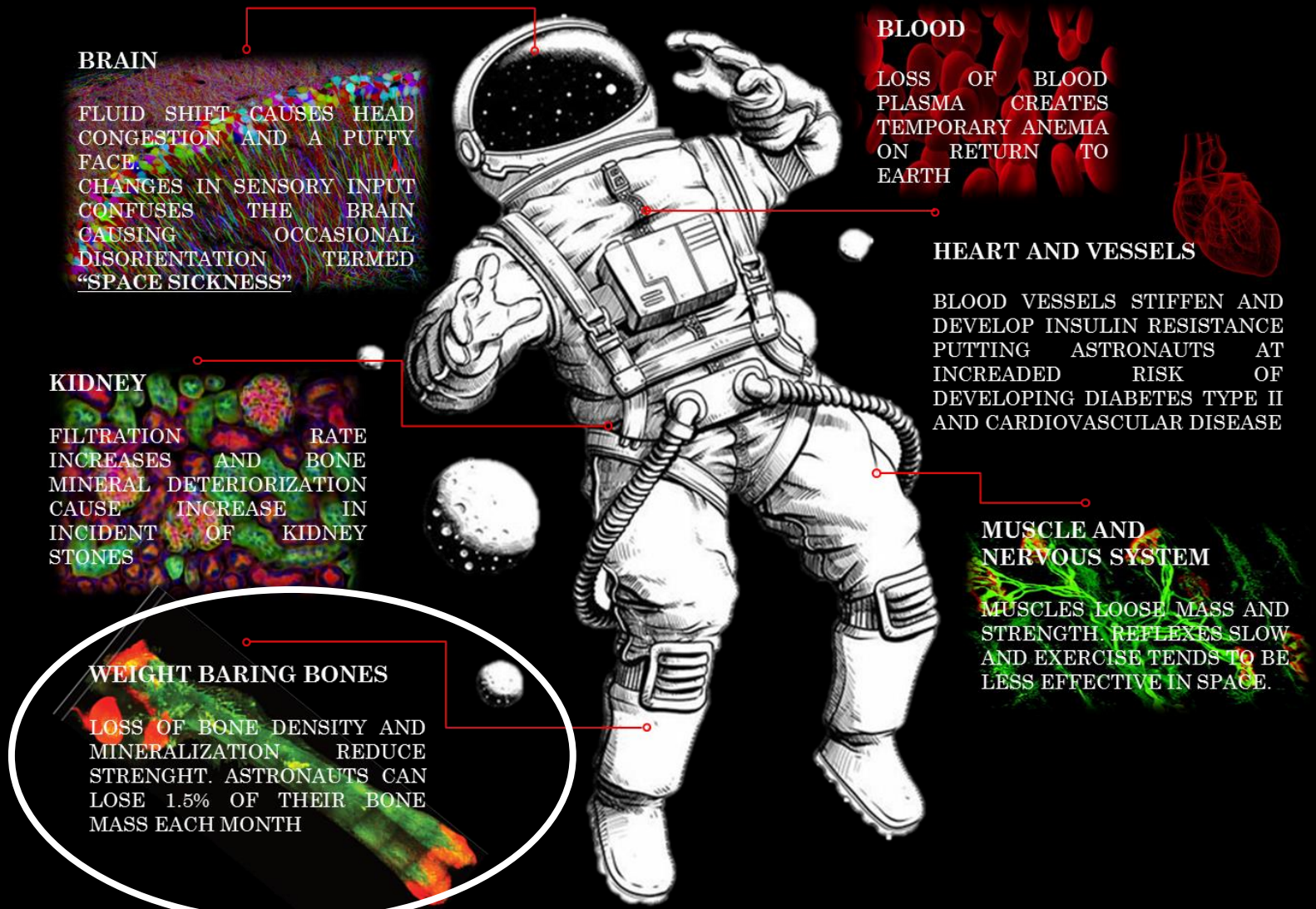


Cellular analyses of BrdU nuclear incorporation show regenerative deficit is related to blastema stem cells maintaining stemness longer, and failing to fully differentiate (more BrdU incorporation in microgravity)

Unloading in microgravity interferes with stem cell-based tissue regeneration in the newt model, and suggested that the critical step affected was the transition between proliferative stem cell populations and differentiated cells and tissues.

Detrimental Effects of Spaceflight to Biology

Hypothesis: Mechanical unloading in microgravity may inhibit the proliferation and/or differentiation of adult stem cells required for normal tissue repair and regeneration

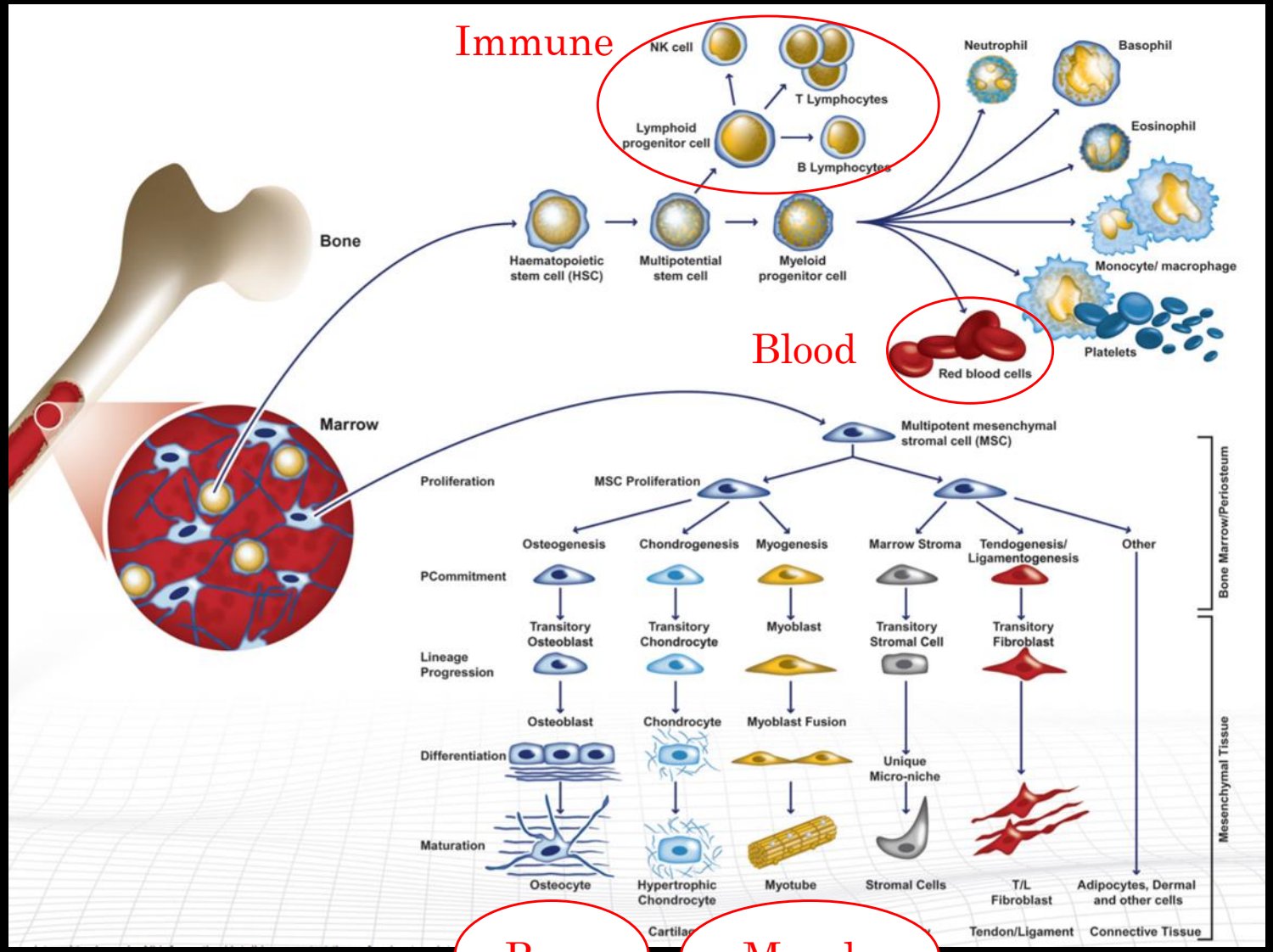


- Spaceflight has been shown to have detrimental effects on mechanisms of homeostasis in the human body.

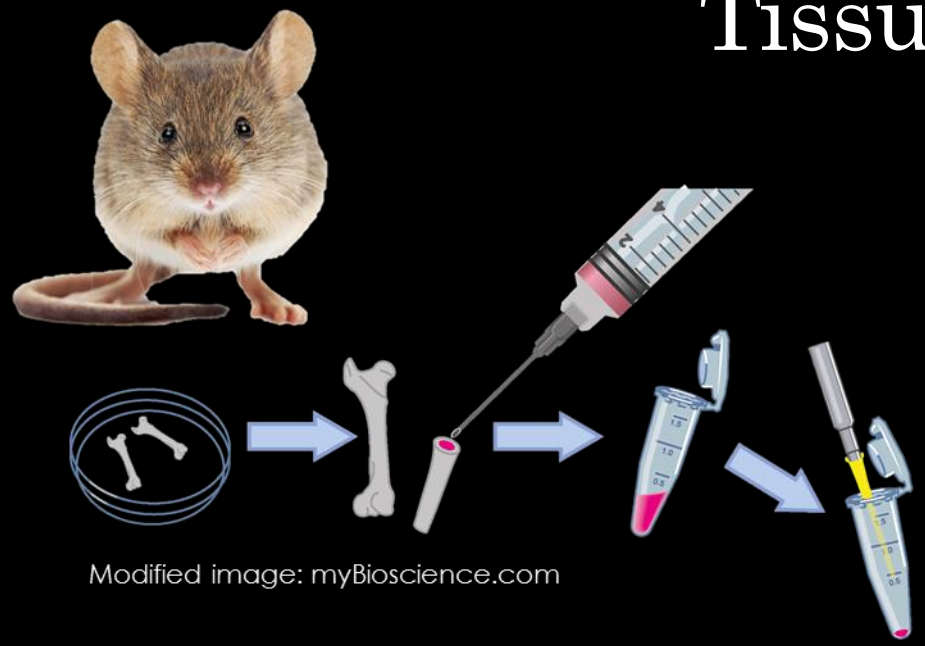
- Tissues with high cell turnover from **stem cell** populations like **immune** cell populations, **cardiovascular** cells, **intestinal** cells, **blood** and **bone** MSCs and HPCs are vulnerable to decreased primary cell proliferation during and post flight.

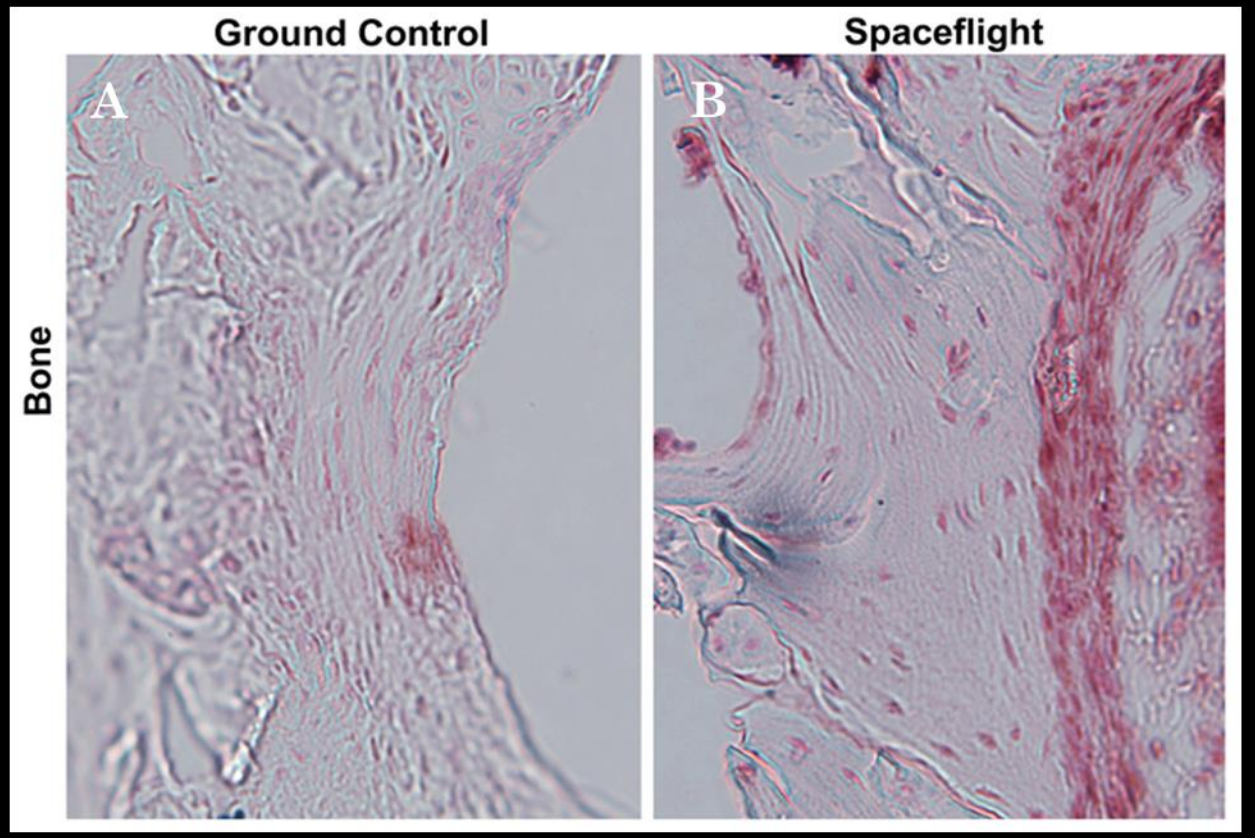
Why study bone in microgravity?

1. Main supportive structure of the human body at normal 1g Earth environment.
   - Changes in bone were observed and studied as early as the Gemini flights.
2. Estuary for several lineages of supportive stem cells
   - Mesenchymal stem cells
   - Hematopoietic stem cells



Molecular Analysis of Spaceflight Mouse **Bone Marrow** cells Show Down-Regulation of Key Pathways Related to Tissue Regeneration





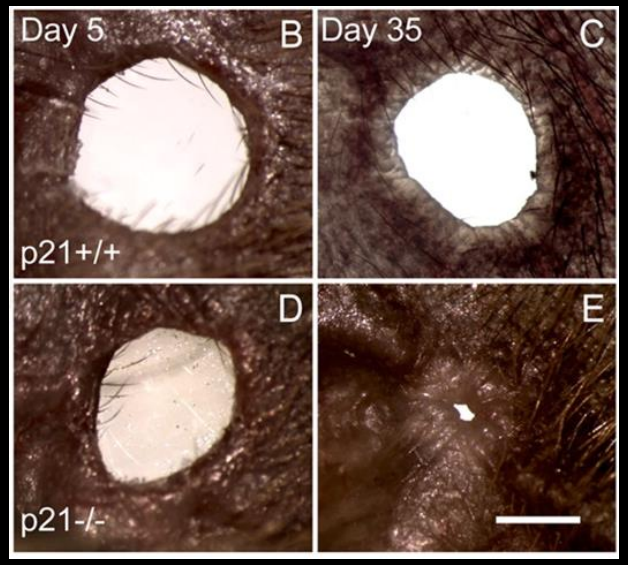
Of specific interest **CDKN1A/p21** is a **modulator of cell cycle progression** showed **elevated expression on spaceflight samples**.

Regeneration conclusions from spaceflight research

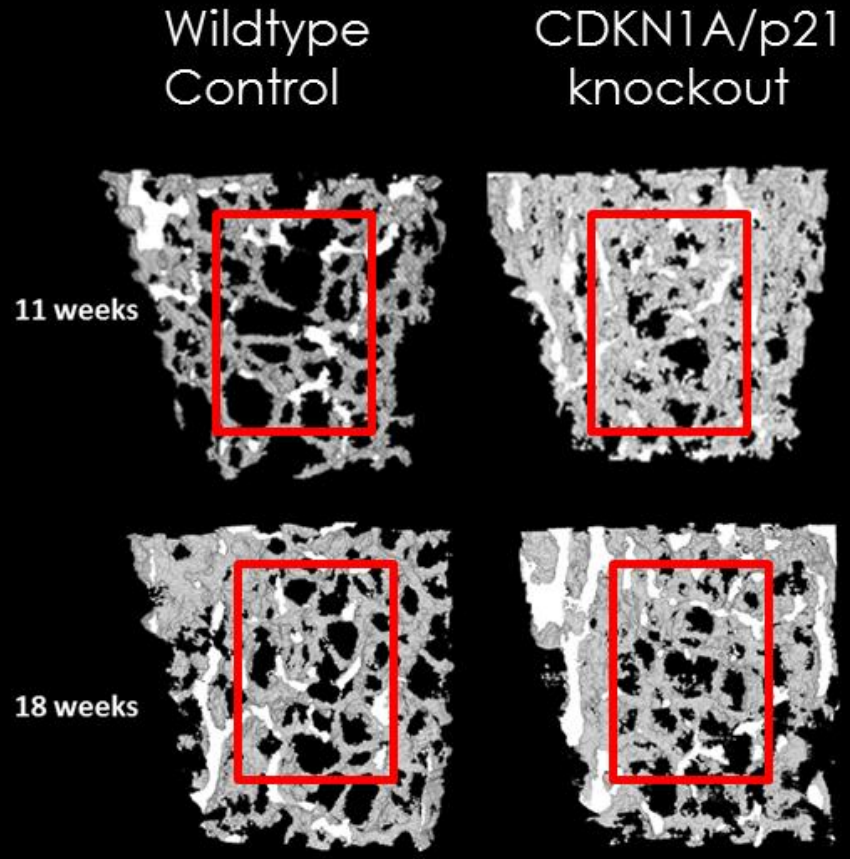
1. Spaceflight interferes with stem cell mediated regeneration at the transition from proliferation to differentiation.

2. CDKN1a/p21, a cell cycle moderator with influence of cell cycle exit for differentiation and senescence, is upregulated by spaceflight.

CDKN1a/p21 Role in Cell Cycle and what happens when its knocked out



Mice genetically modified to not express CDKN1a/p21exhibit regenerative abilities



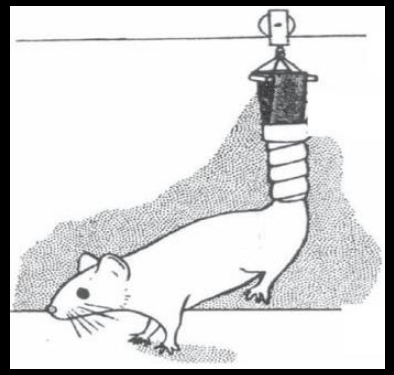
Blaber et al., Unpublished

- In juvenile **skeletal development greater trabecular bone volume** is seen at 11 weeks in the $p21^{-/-}$ knockout mice.
- Bone volume in the $p21^{-/-}$ decreases form 11 to 18 weeks indicating that the MSC/HSC osteogenic homeostasis process is upregulated in the knockout.

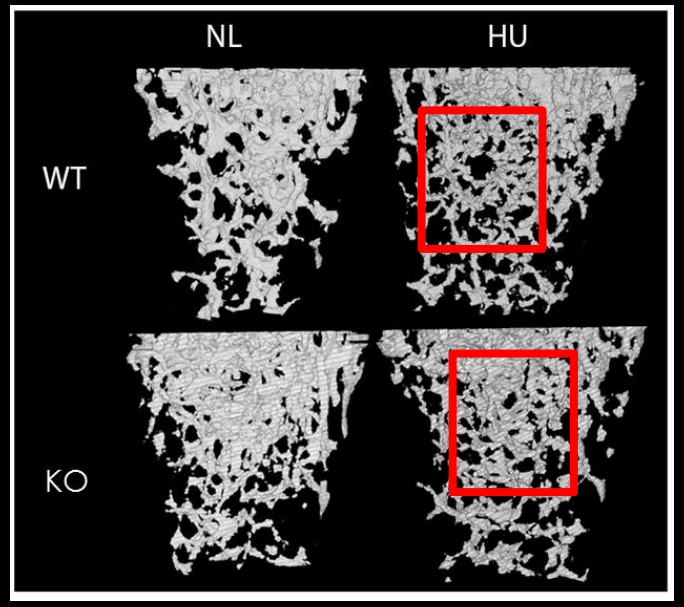
Does the presence of p21 in unloaded mice influence skeletal homeostatic regenerative mechanisms?

Ground based Spaceflight simulatory experiments: Bone Structure analysis and marrow osteoblastogenesis

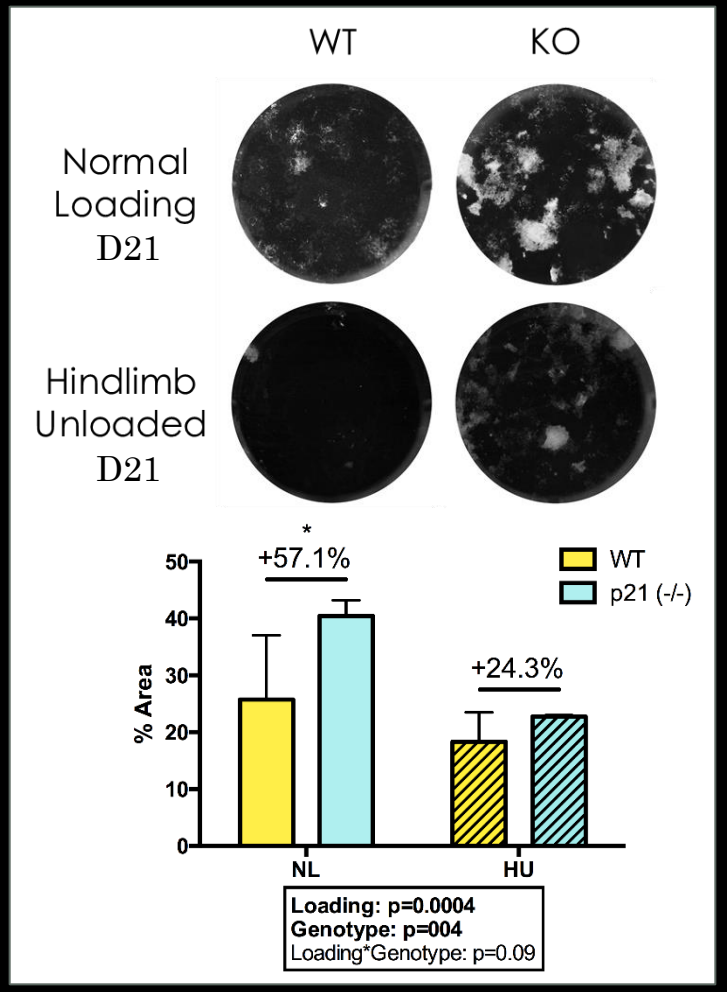
Hindlimb unloading (HU) is an established ground based model of spaceflight effects



MICROCT ANALYSIS OF THE TRABECULAR BONE OF THE FEMUR OF CDKN1A/P21 KNOCKOUT AND RELEVANT CONTROL MICE SHOW THE KNOCKOUT MICE MAINTAIN OSTEOBLASTIC BONE VOLUME DURING **UNLOADING**.



BONE MARROW FLUSH ISOLATED CELLS FROM THE HINDLIMB UNLOADED MICE DEMONSTRATE REDUCED MINERALIZATION FROM THE NORMALLY LOADED CONTROLS. HOWEVER, THE KNOCKOUT MOUSE CELLS BETTER MAINTAIN THE ABILITY TO FORM MINERAL NODULES AFTER UNLOADING.



Blaber et al., Unpublished

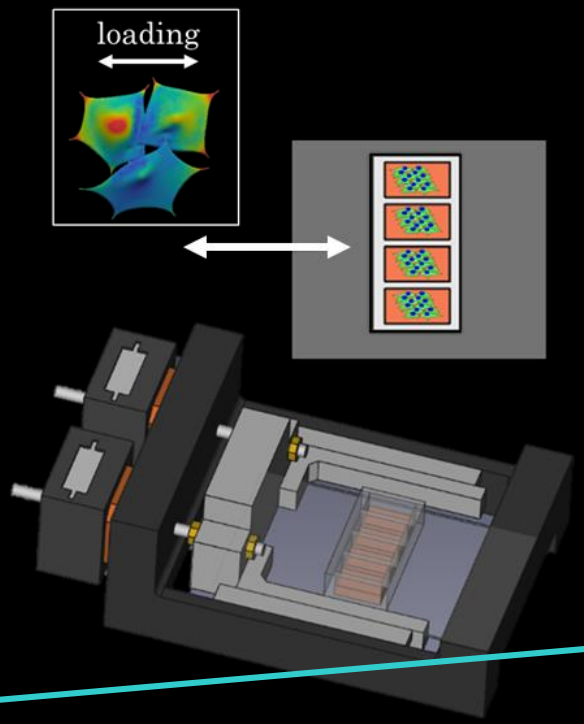
Question: ***Will mechanical stimulation in the absence of CDKN1a/p21 affect osteoblastogenesis of bone marrow primary cells and positively regulate proliferation and/or differentiation?***

Hypothesis: CDKN1a/p21 is mechanoregulated and influences downstream mechanotransduction biochemistry in mechanosensitive cells.

Experimental Design: Direct Cyclic Stretch Mechanical Loading System

Wildtype (Wt)

CDKN1a/p21-null ($p21^{-/-}$)



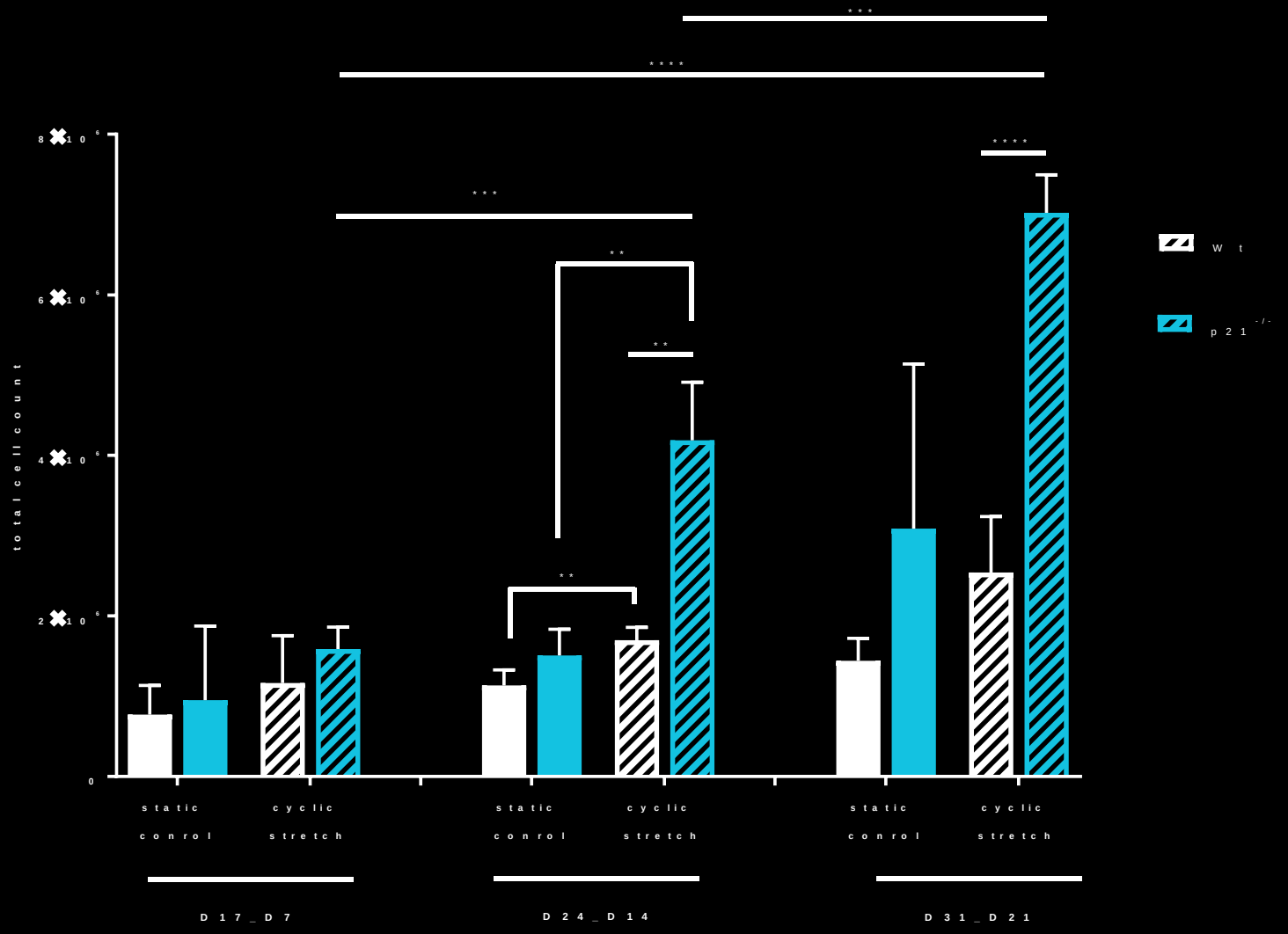
- Isolate bone marrow MSC and osteoprogenitor populations from p21-/- and Wt. Culture in OB media containing AA and BGPH to enable
- Assess cultures for Proliferation and Cell Cycle Activity.
- Evaluate cultures for Differentiation markers of functioning osteoblast cells.

system there is only 1 source of disruption to the system: the cyclic stretch.

Set-up → **10 day osteogenesis commitment** → **21 loading regime** → **Analysis**

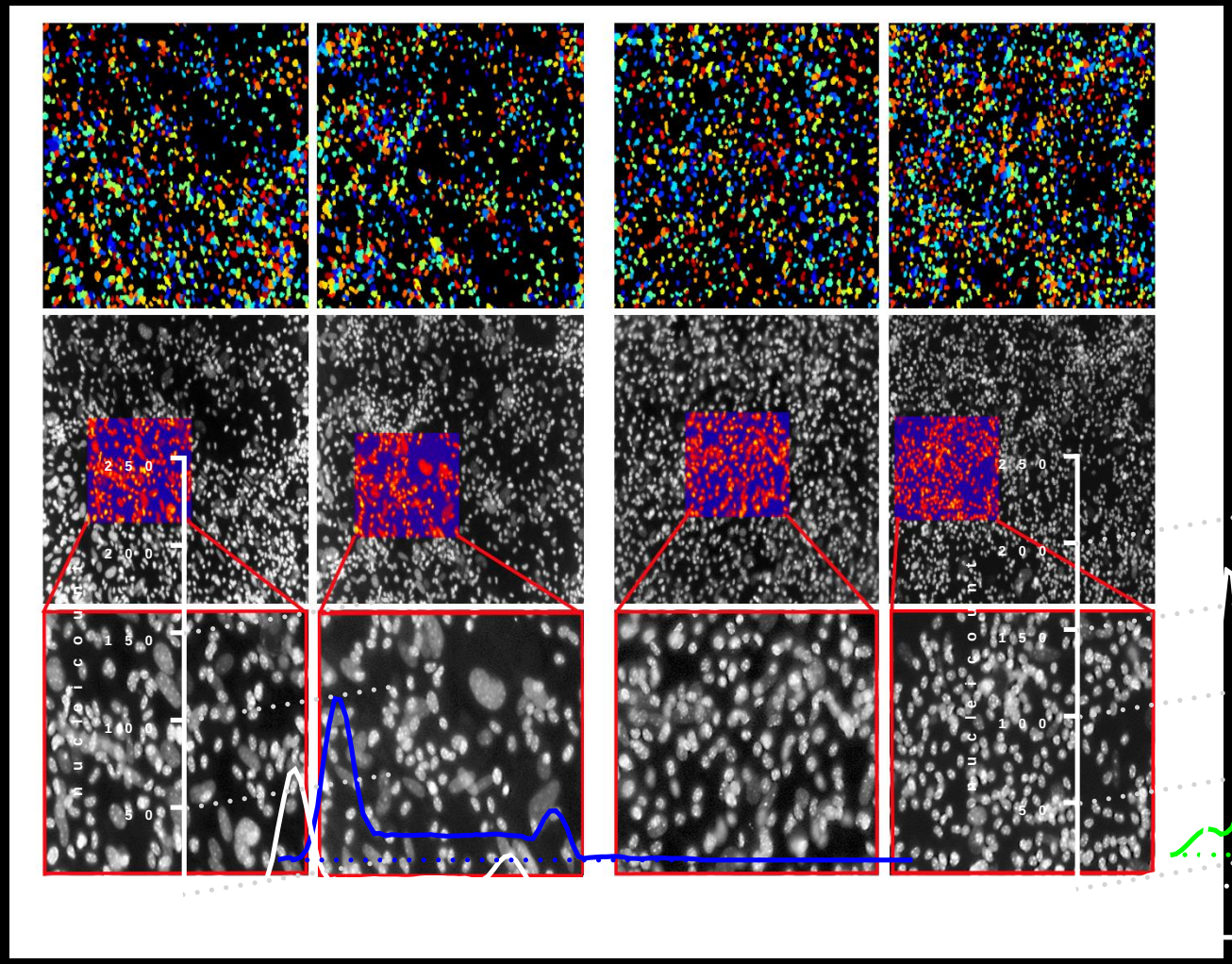
Cell Isolation and Plating	Start stretch culture	Time Point 1	Time Point 2	Time Point 3
D0	D10	D7 post-stretch	D14 post-stretch	D21 post-stretch

p21$^{-/-}$ cells have enhanced response to pro-proliferative mechanostimulation



- The p21$^{-/-}$ genotype have an innate proliferative advantage compared to the Wt genotype.
- Cyclic stretch increases cellular proliferation and reduces doubling time.
- In the absence of CDDKN1a/p21 the pro-proliferative response to mechanical stimulation of BMSCs is enhanced.

p21$^{-/-}$ cells have enhanced response to pro-proliferative mechanostimulation

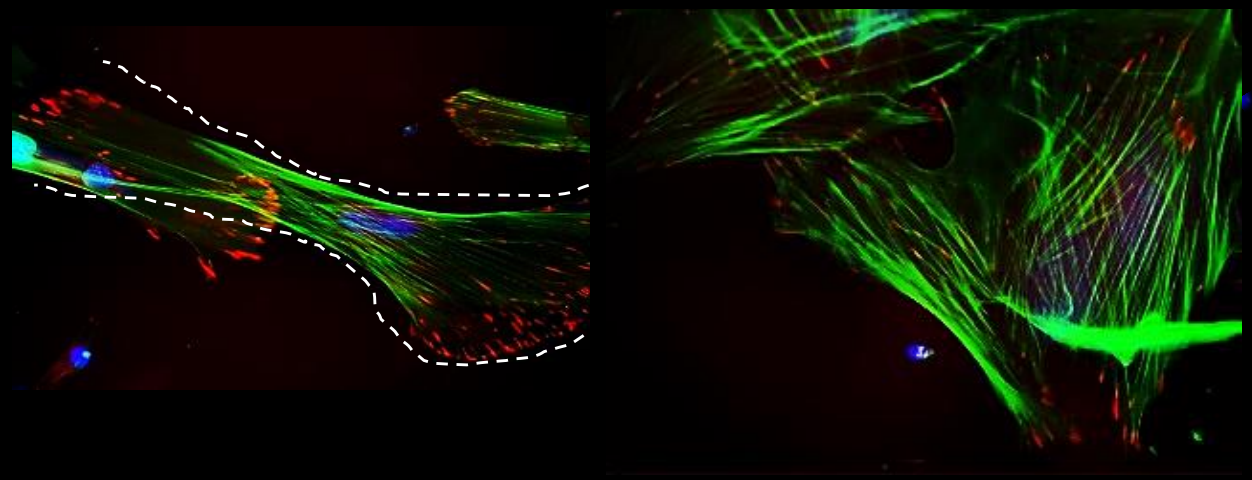


- Wt culture has more large adherent nuclei than the p21-/- cultures.
  - This indicates the cells are not actively undergoing mitosis or cell cycle.

p21$^{-/-}$ cells morphologic self-organization reactive to cyclic stretch stimulation.

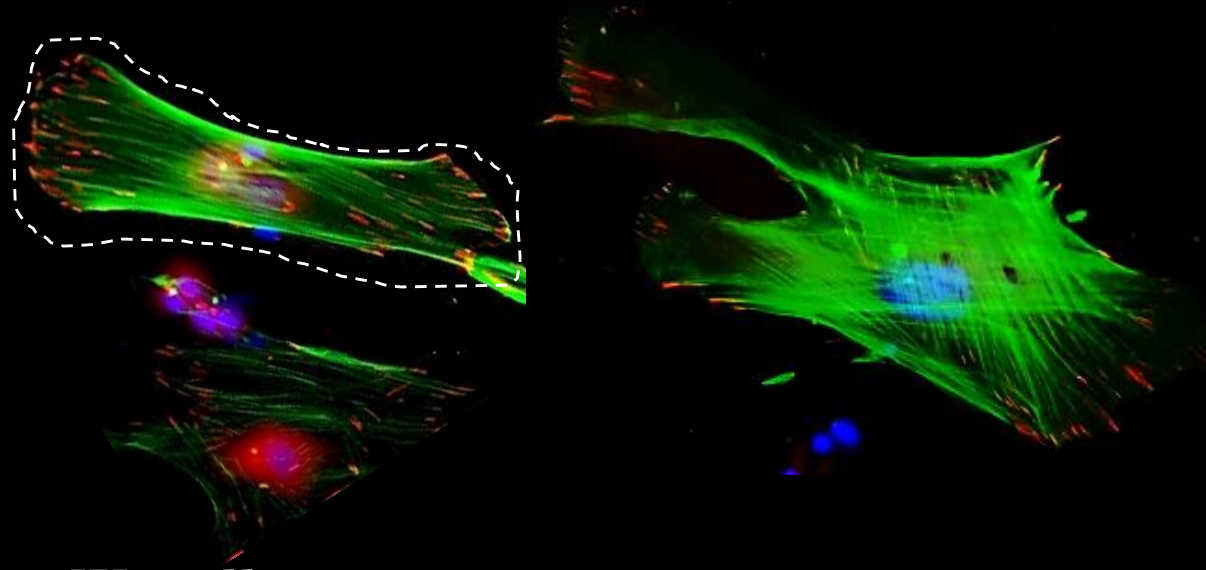
CDKN1a/p21-null Genotype

Cyclic stretch **Static control**



Wildtype control genotype

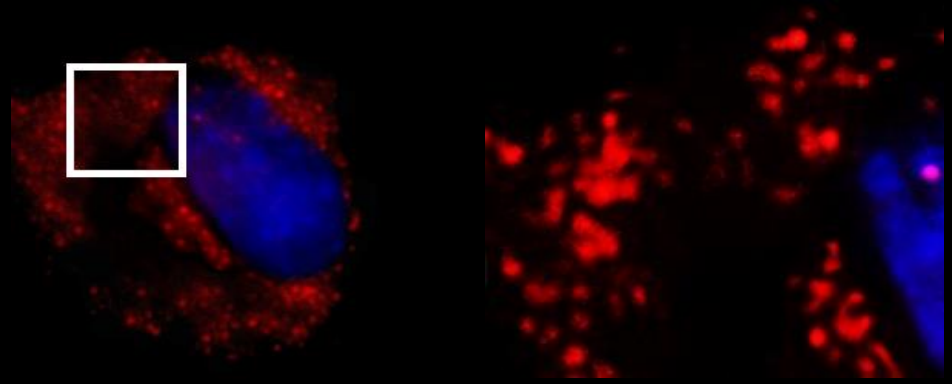
Cyclic stretch **Static control**



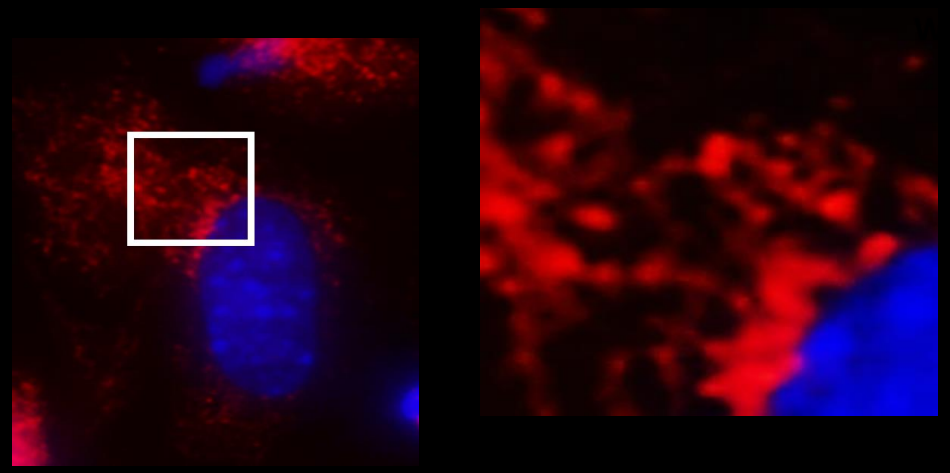
- The p21$^{-/-}$ cells are noticeably smaller than the Wt cells.
- Cyclic stretch causes cytoskeletal alignment and protruding bud fanned morphology which is exaggerated in the p21$^{-/-}$ genotype.
- The average number of focal adhesions per cell are greater in the p21$^{-/-}$ genotype.

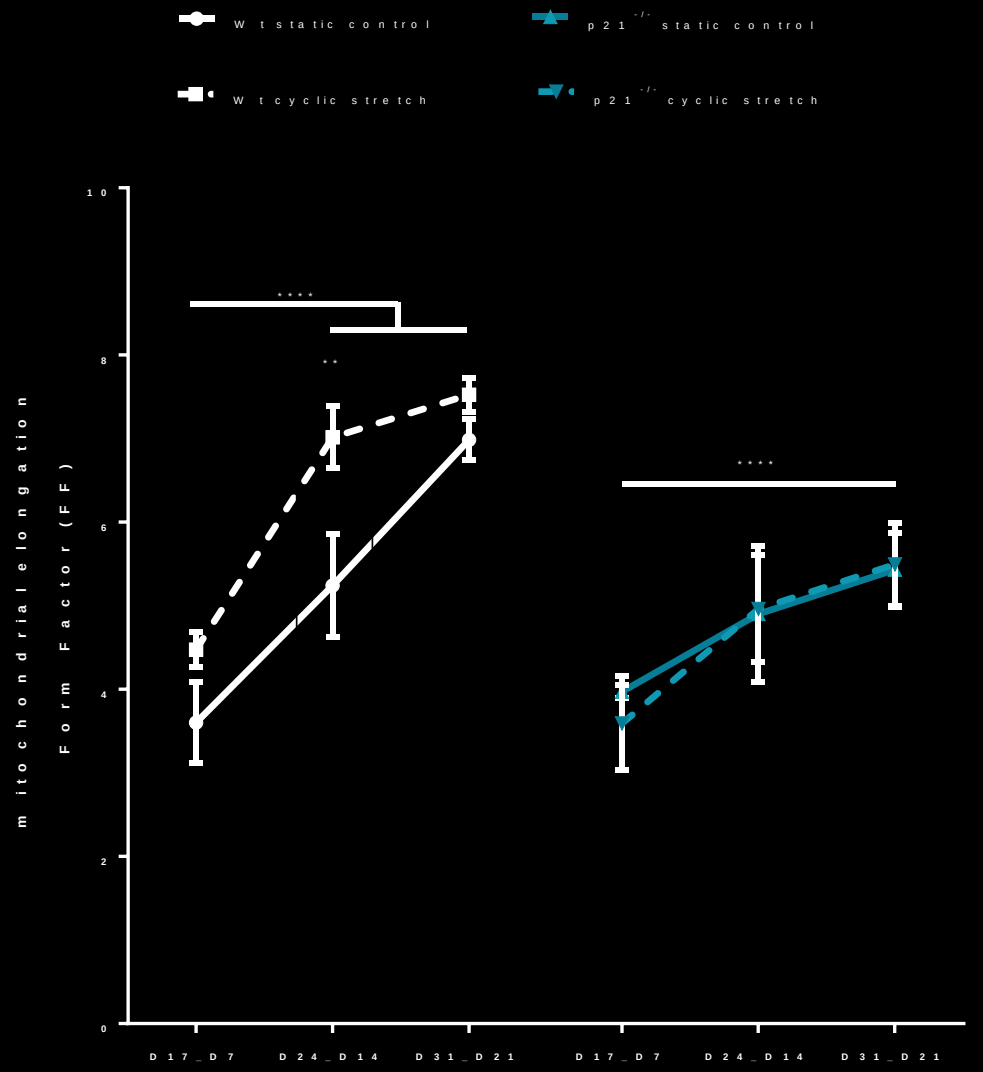
Metabolic glycolysis to oxidative phosphorylation switching delayed in the p21$^{-/-}$ cell cultures.

p21$^{-/-}$ cyclic stretch

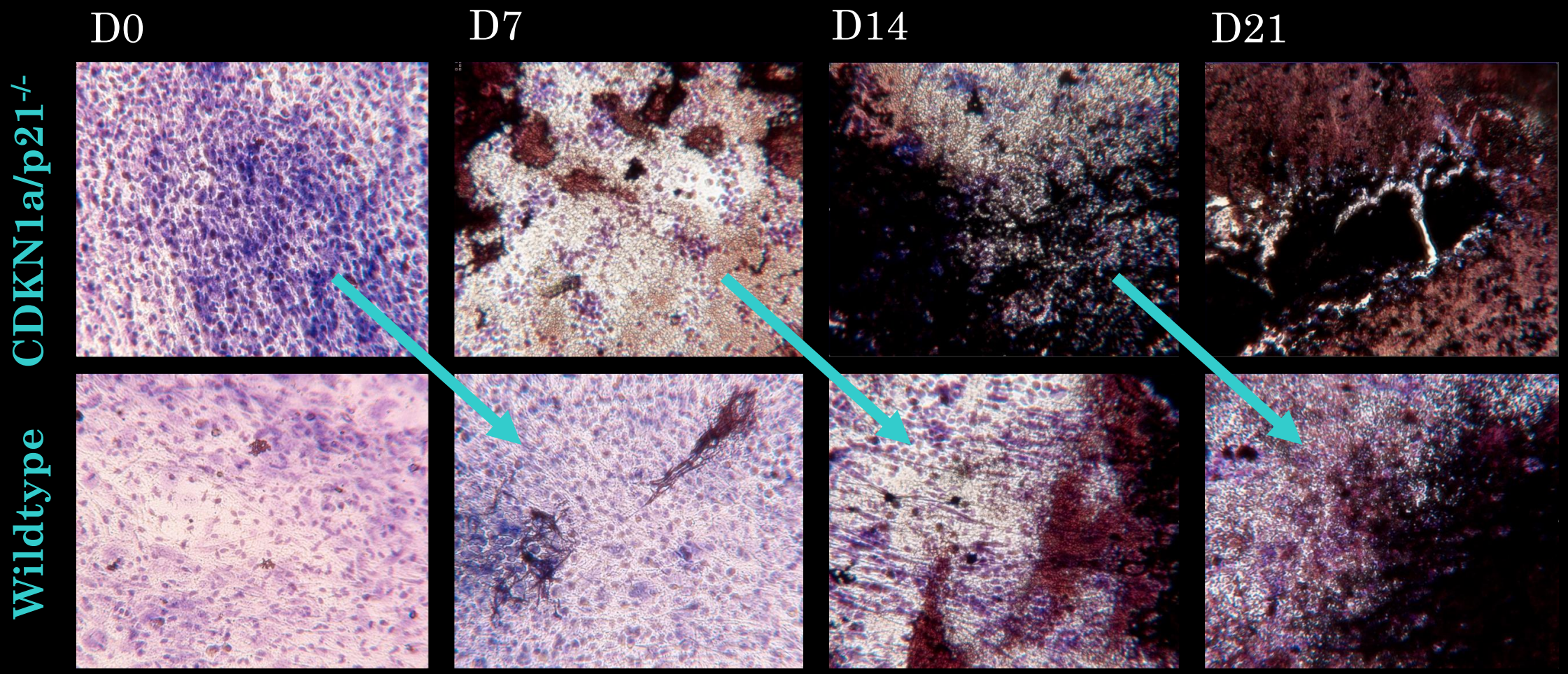


Wt cyclic stretch





Mineralization is an effect of loading response and not genotype dependent.



Role of CDKN1a/p21 in regenerative maintenance

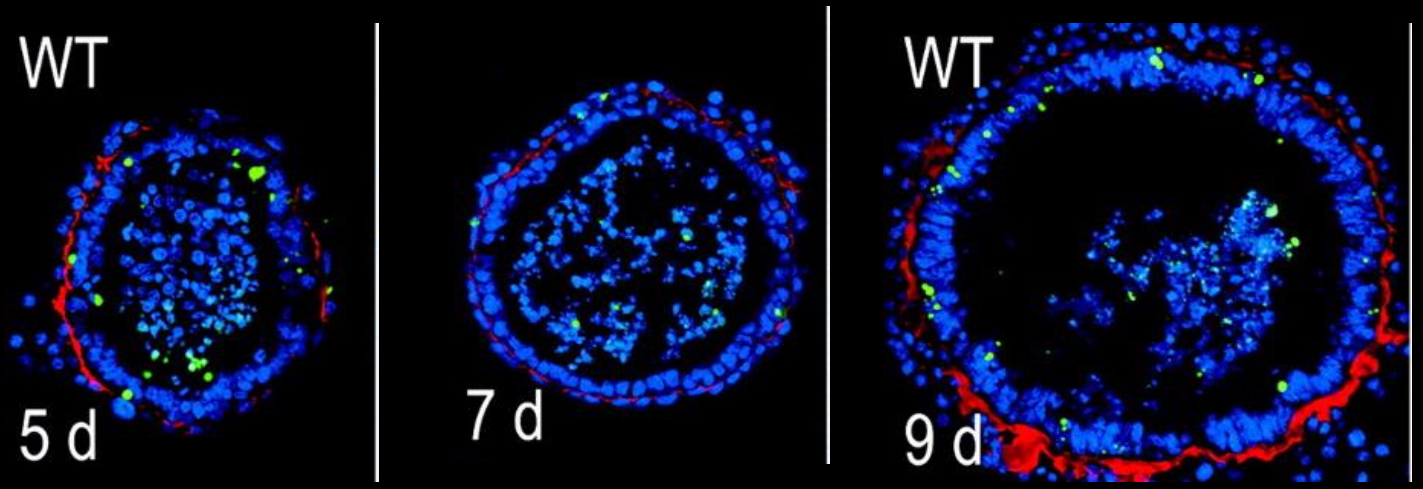
1. Deletion of CDKN1a/p21 releases osteogenic restrictions associated with stem cell activation causing greater bone turnover.

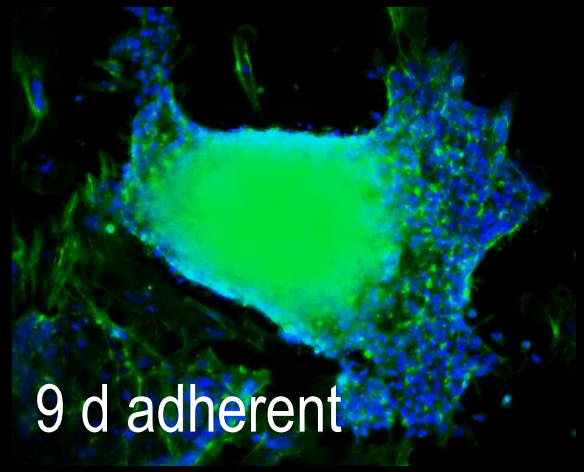
2. Deletion of CDKN1a/p21 increases mechanosensitivity of BMSCs and maintains the stem cells proliferative quality throughout the osteogenic process.

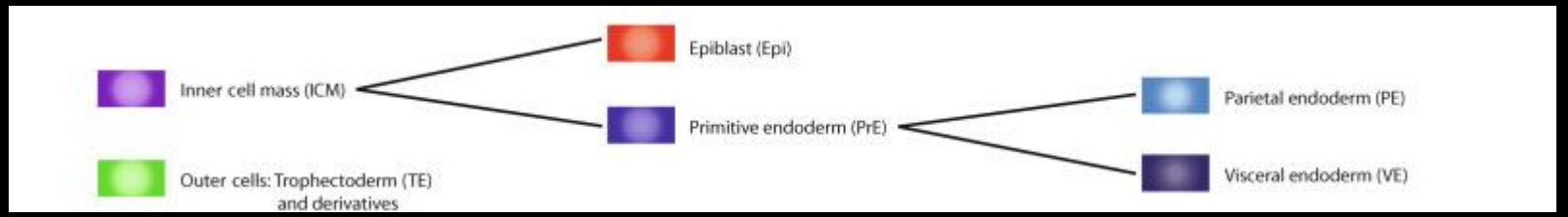
Question: Does CDKN1a/p21 have global relevance as a lynchpin regulator of regenerative stem cell mechano-responsiveness?

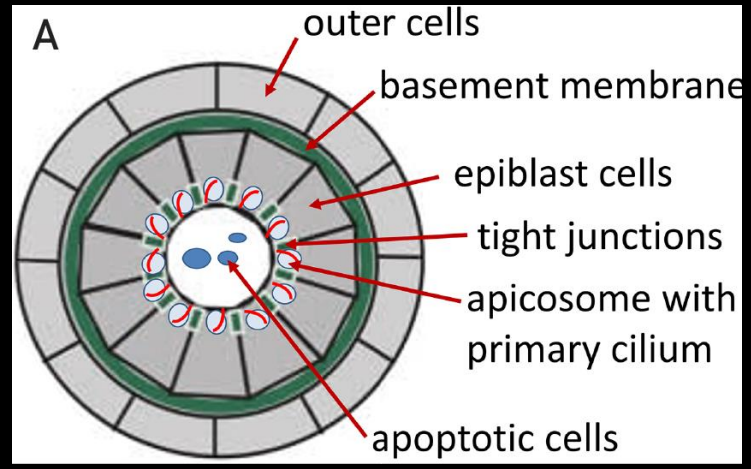
Hypothesis: Mechanical suppression of CDKN1a/p21 will retain stem cell self renewal capacity while not inhibiting differentiation commitment cascades.

Embryoid Bodies – Developmental Lineage Commitment, a model of differentiation transition







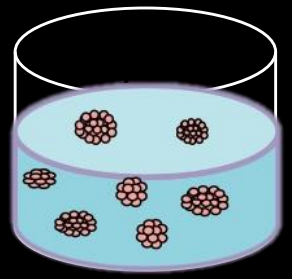


- We developed EBs for 5 days on ultra low adhesion plates
- After 5 days the EBs were transferred to collagen coated culture plates to simulate implantation and allow adherent cell outgrowth.

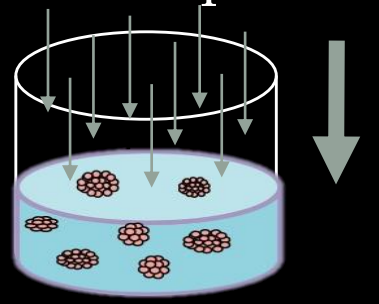
Lincon et al., Development 2017

Centrifugation Hypergravity Pulse to induce mechanostimulatory transcriptome changes

Ambient pressure



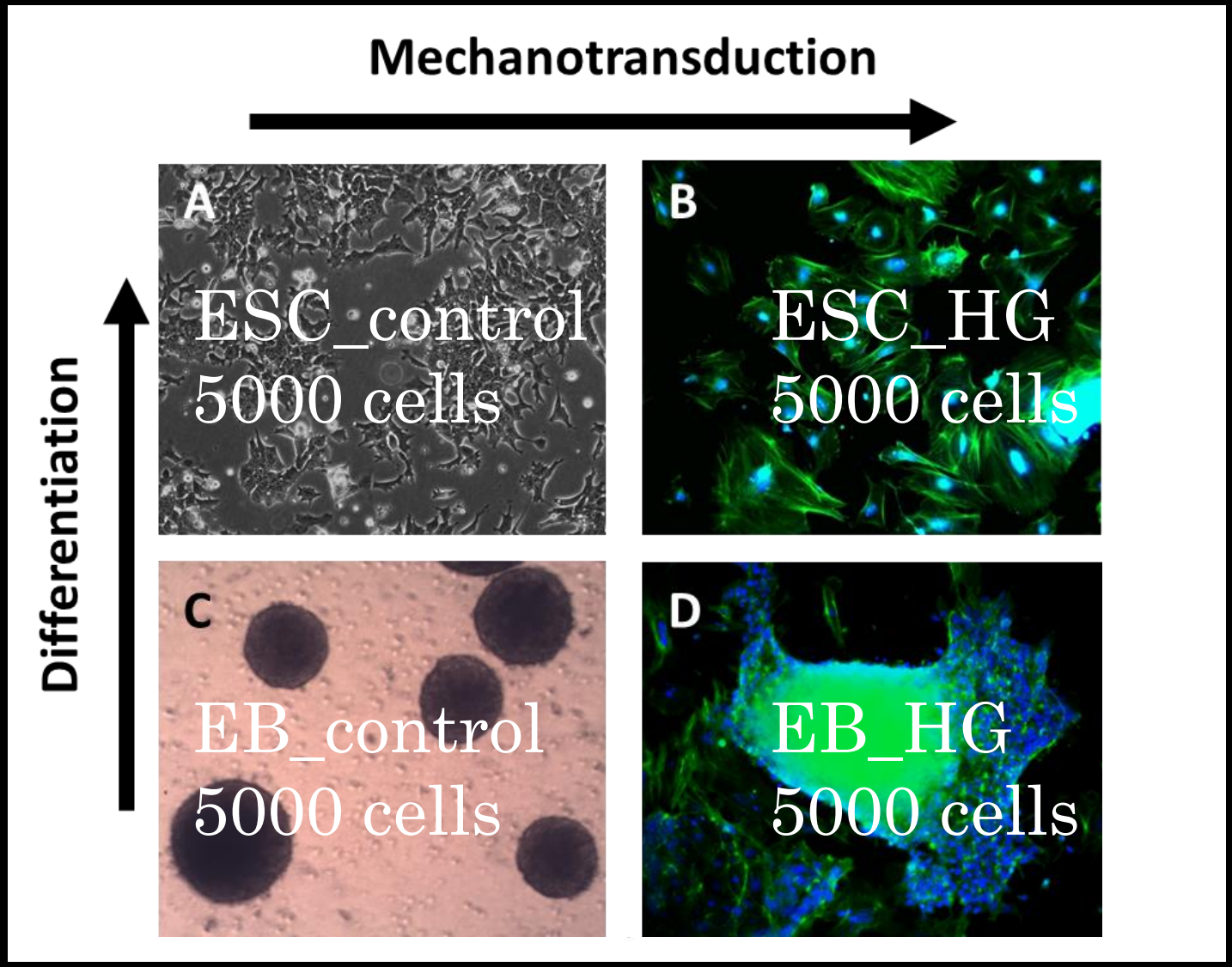
Hydrostatic pressure



$$\Delta P = \rho g h$$

- The centrifugal load increases the hydrostatic pressure experienced by the culture.
- The cultures were exposed to the increased hydrostatic pressure static load for 60 minutes at 50g.
- After the HG pulse load the cultures were given 6 hours for mRNA expression changes.

Experimental Design



Single Cell Library Preparation

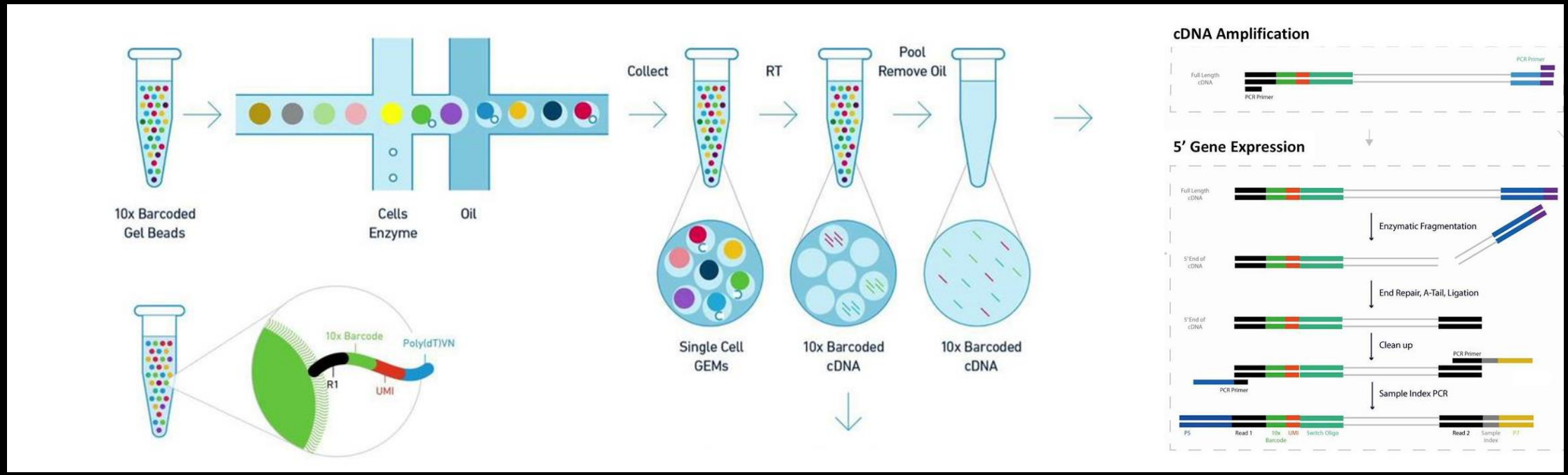


Image credit: 10x genomics

- A single cell is encapsulated in a GEM bead containing RT reagents.
- Thermal cycling within the GEM transcribe cDNA from the single cell mRNA expressome
- The GEMs can then be ruptured and traditional amplification and Illumina sequencing tagging conducted.

Sequencing Metrics and pre-processing

Estimated Number of Cells

23,181

Post-Normalization Mean Reads per Cell

27,918

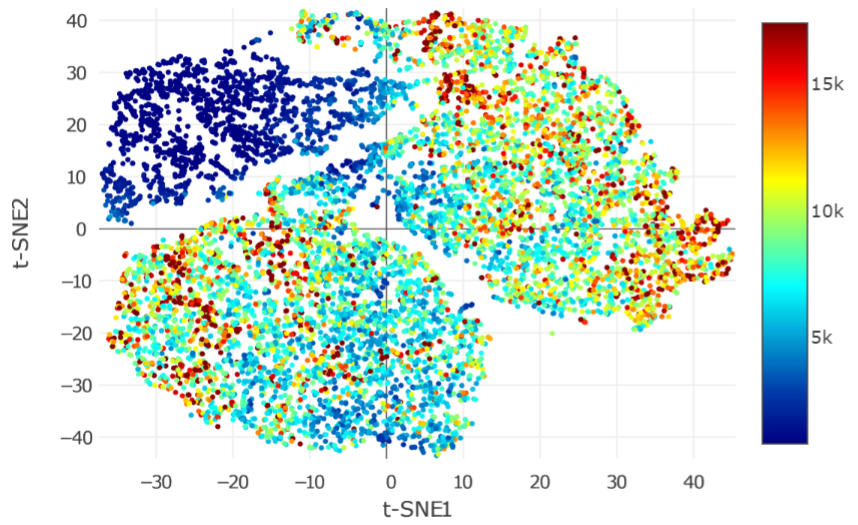
Median Genes per Cell

2,326

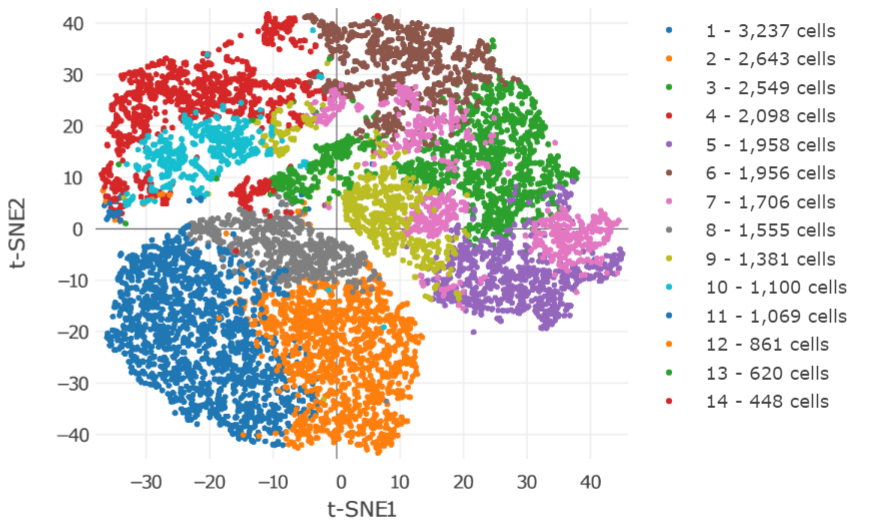
Cells

Estimated Number of Cells	23,181
Fraction Reads in Cells	83.9%
Pre-Normalization Mean Reads per Cell	33,474
Post-Normalization Mean Reads per Cell	27,918
Median Genes per Cell	2,326
Median UMI Counts per Cell	7,816

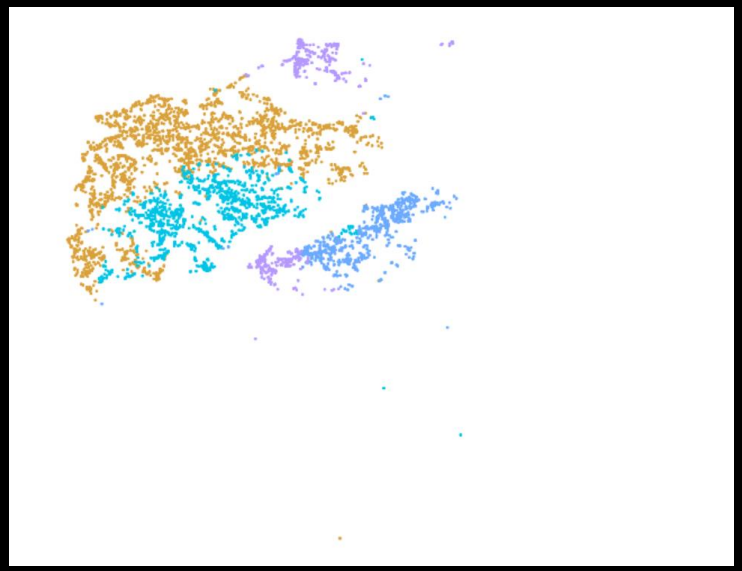
t-SNE Projection of Cells Colored by UMI Counts

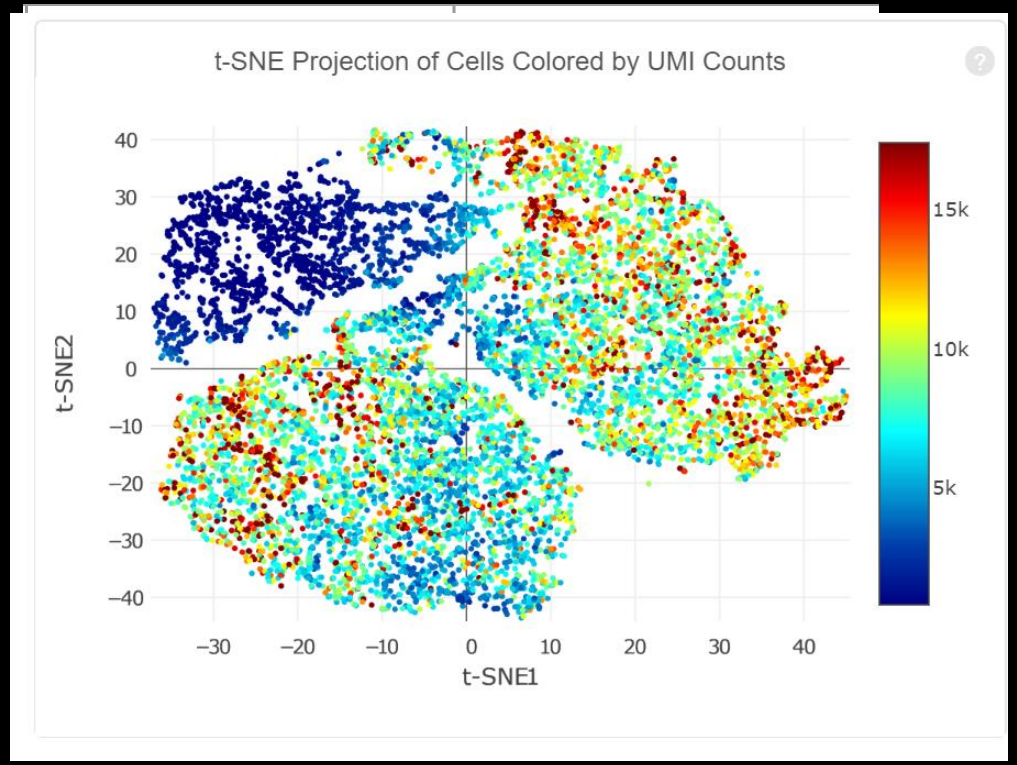


t-SNE projection of Cells Colored by Automated Clustering



Segregation of scRNA-seq by Library





Shared Clusters

- Cluster 4 (2098)
- Cluster 10 (1100)
- Cluster 13 (620)
- Cluster 14 (448)

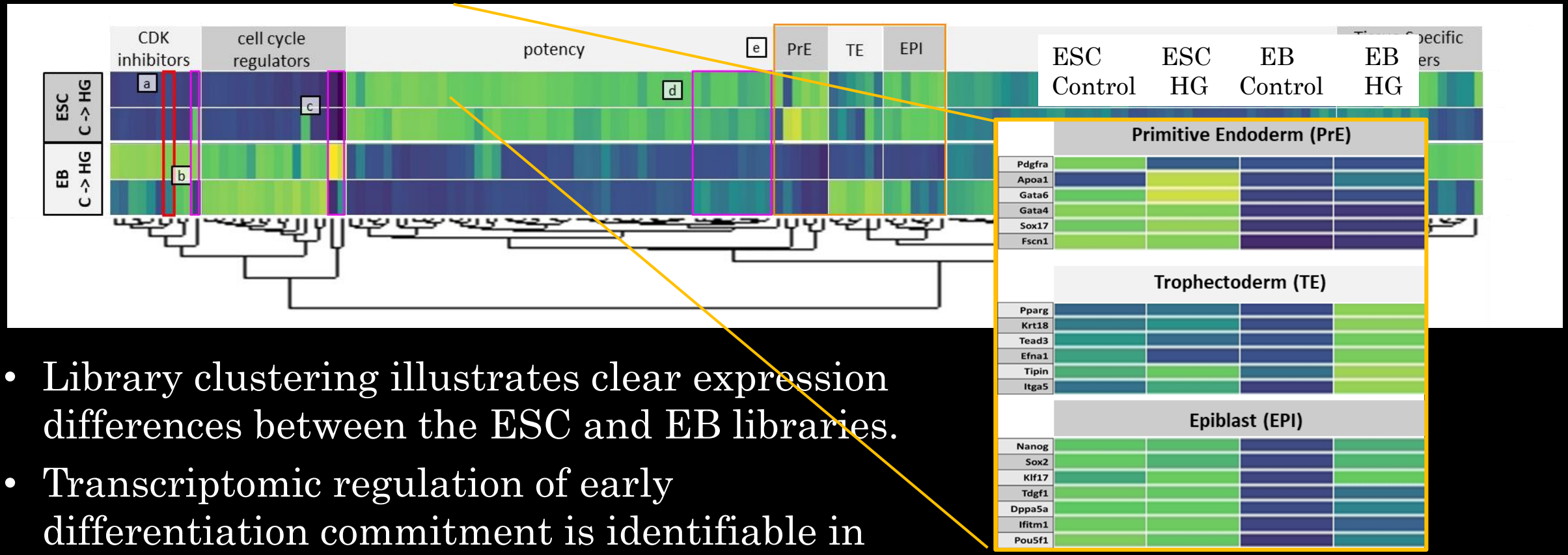
ESC exclusive

- Cluster 1 (3237)
- Cluster 2 (2643)
- Cluster 8 (1555)
- Cluster 11 (1069)
- Cluster 12 (861)

EB exclusive clusters

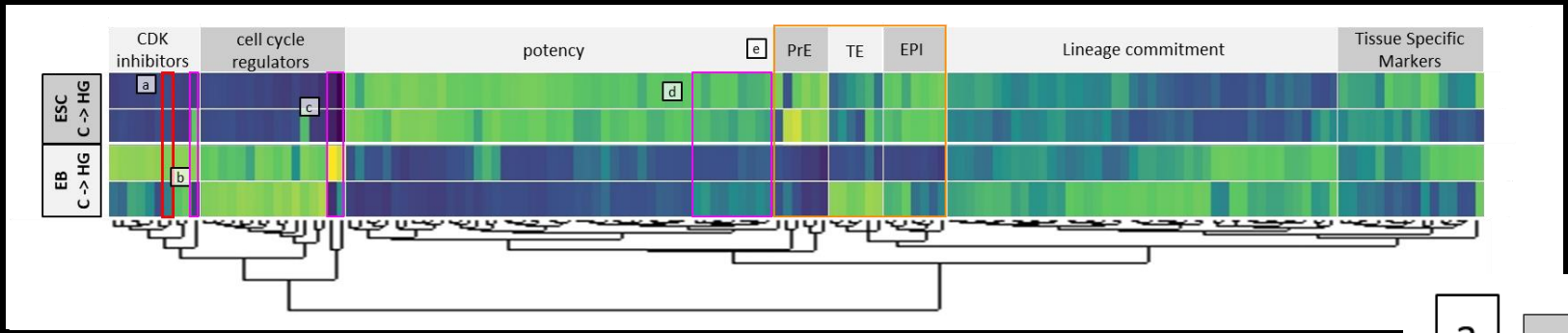
- Cluster 3 (2549)
- Cluster 5 (1958)
- Cluster 6 (1956)
- Cluster 7 (1706)
- Cluster 9 (1381)

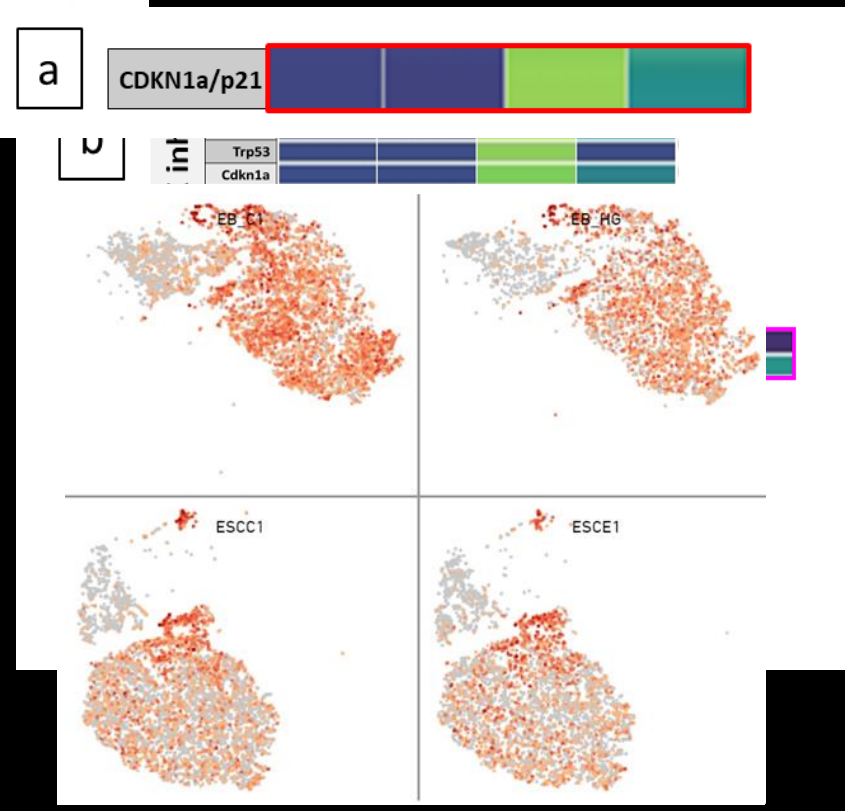
Library Analysis: Two Paths Developmental and Mechanotransduction



- Library clustering illustrates clear expression differences between the ESC and EB libraries.
- Transcriptomic regulation of early differentiation commitment is identifiable in both the ESC library and the EB library. Specifically the pre-implantation cell types have clear mechanoregulation.

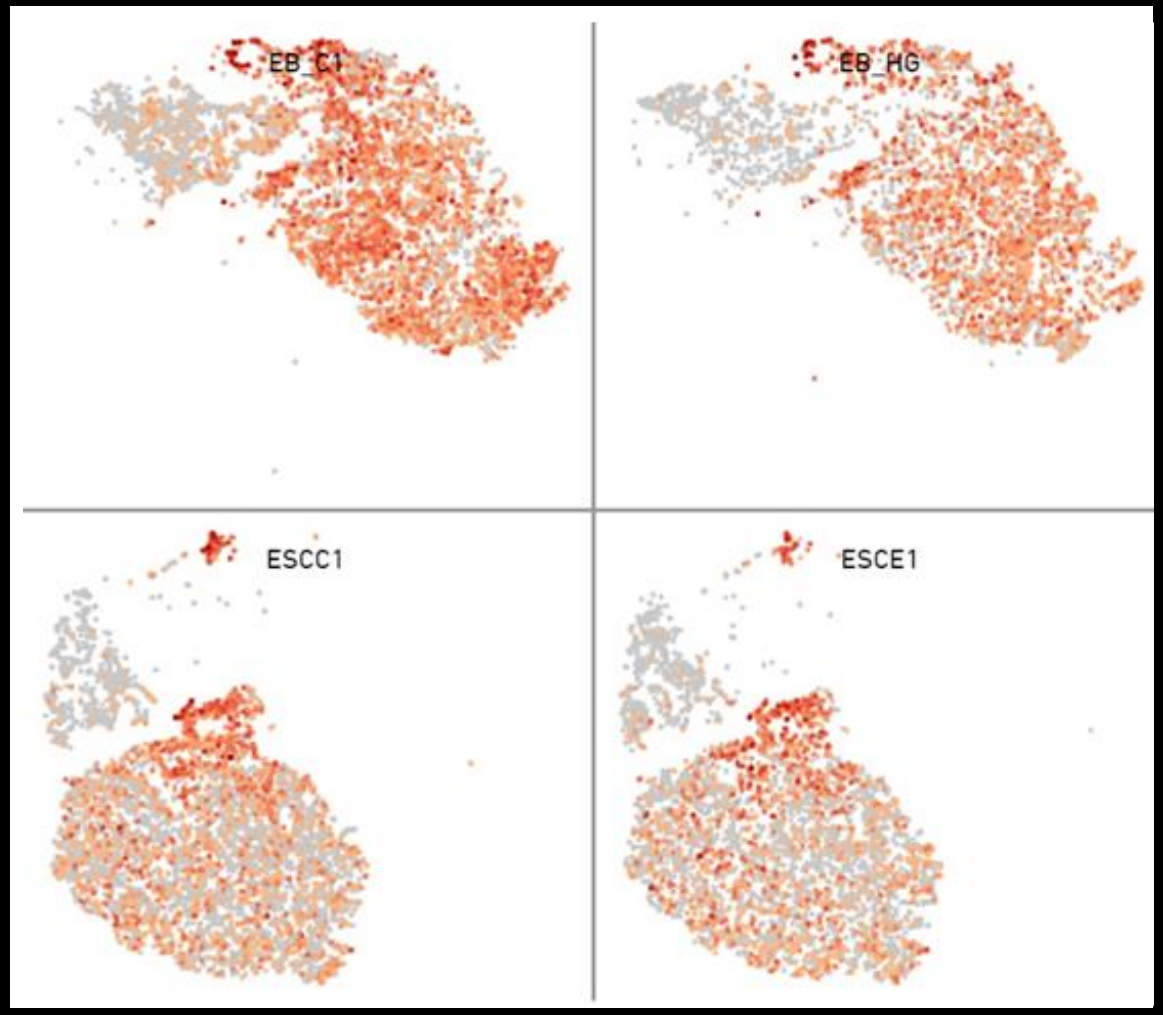
Library Analysis: Two Paths Developmental and Mechanotransduction

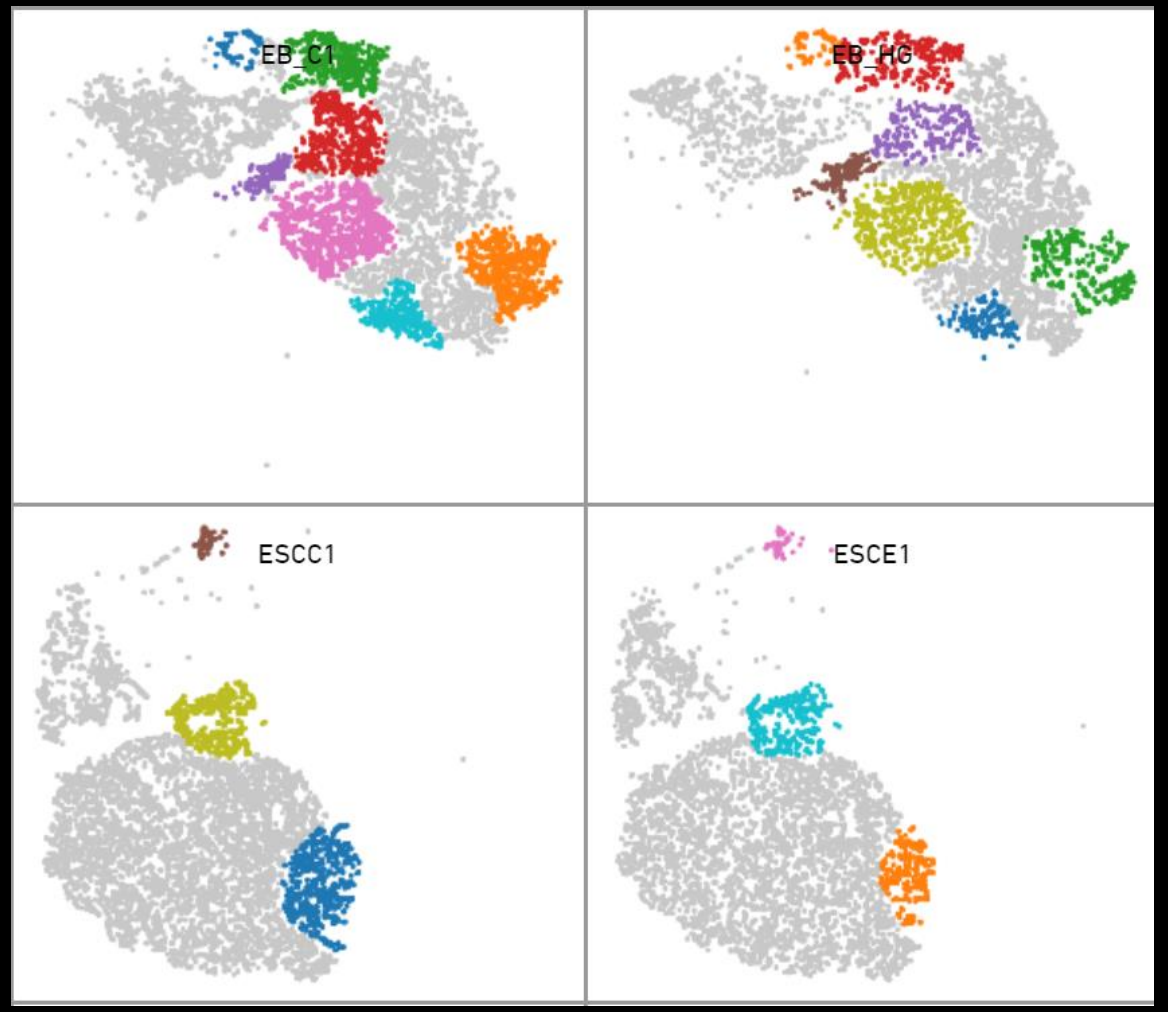




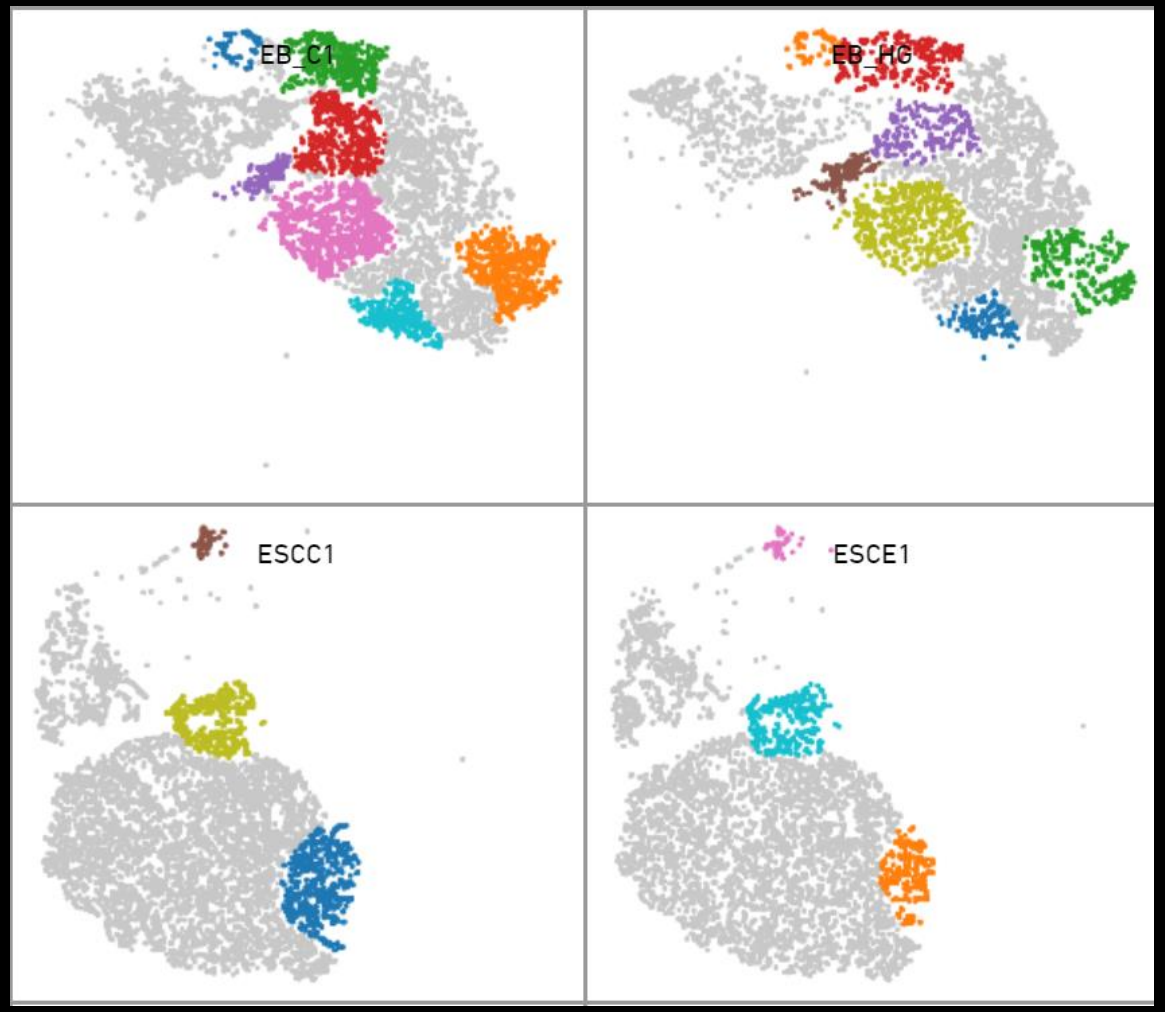
- CDK inhibitors, cell cycle regulators and potency genes also show mechanoregulation.
- p21, which was shown to play a large role in mechanotransductive modulation of proliferation to differentiation transition also has mechanically induced expression changes, with expression levels being suppressed toward levels of pluripotent ESCs after mechanical stimulation.
- However, this mechanoregulation is almost exclusive to the EBs.

p21 expression sub-clustering and mechano-regulatory lessons learned

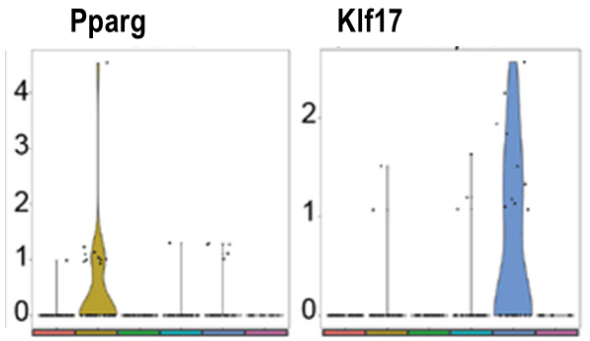




p21 expression sub-clustering and mechano-regulatory lessons learned



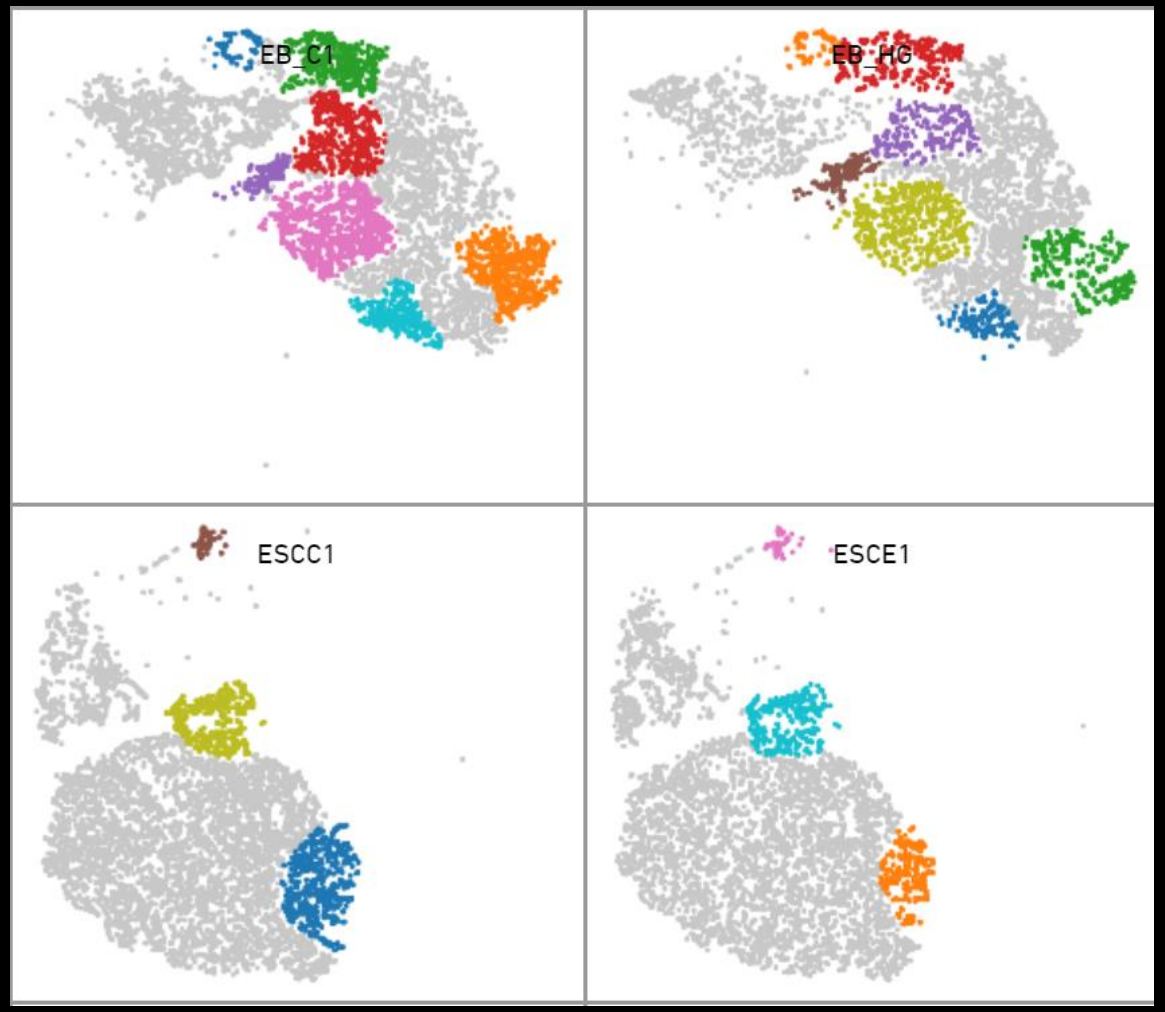
P21 sub-clusters EB exclusive



Pparg is a trophectoderm marker that is expressed in a single sub-cluster.

Klf17 is an epiblast marker that is expressed in a single sub-cluster.

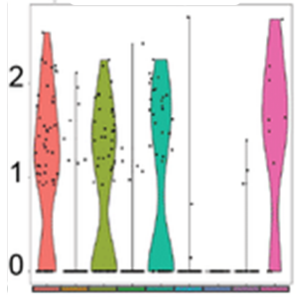
p21 expression sub-clustering and mechano-regulatory lessons learned



ESC Control	ESC HG	EB Control	EB HG

P21 shared sub-clusters

Cdkn1c



Cdkn1c/p57, a cell cycle regulator similar to CDKN1a/p21 shows mechanoregulstion in both the ESCs and EBs.

So what lessons have we learned about regeneration from studying mechanotransduction?

All together,

CDKN1a/p21 is a mechanotransductive lynchpin of stem cell mediated regenerative transition from proliferation to differentiation and a candidate molecule for development of spaceflight, aging, and general stem cell senescence countermeasures

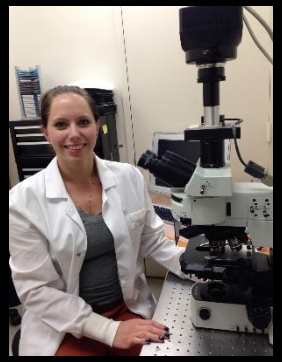
Associated Posters

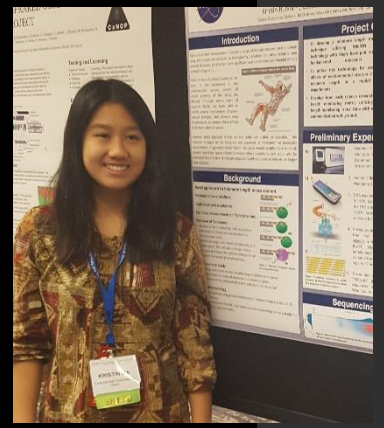
Molly Coyne

THE ROLE OF GRAVITY MECHANOTRANSDUCTION IN REGULATING STEM CELL TISSUE REGENERATIVE POTENTIAL AT THE SINGLE CELL EXPRESSOME LEVEL

Bone and Cell Signaling Lab in total, and especially

- **Eduardo Almeida, Ph.D. (Mentor)**
- Elizabeth Blaber, Ph.D.
- Margareth Cheng-Campbell
- Molly Coyne
- Olivia Stimpel
- Kristin Ma





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Questions?