# Research Plan

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**Qualified Scientist: Elena Brindley**

**Adult Sponsor: Angela Stone**

**Title:** How Loss of RASA3 Function Impairs Erythropoiesis and Epiphyseal Plate Development Through Dysregulation of Key Signaling Pathways

**Rationale:** Bone marrow failure syndromes (BMFS) are a group of inheritable disorders often characterized by compromised hematopoiesis and a reduction of mature erythrocytes. The complex interactions between genetic changes and alterations in the bone marrow itself that produce these disorders have yet to be identified, making it difficult to classify distinct etiologies in patients. Aplastic Anemia, a common BMFS, can be either acquired or inherited. Acquired Aplastic Anemia is the immune-mediated suppression of the bone marrow and responds well to immunosuppressive therapy.**[1,2]** The inherited variant of the disorder is less well understood, and thus cannot be treated to the same extent.

Models of bone marrow failure disorders- both spontaneous and engineered- allow for the focused study of these uncharacterized molecular pathways.**[1]** Better treatment of patients is a priority, and models that mimic the phenotype of BMFS are essential in determining important effects of the disorders. One such spontaneous model is the autosomal recessive *scat* (severe combined anemia and thrombocytopenia) mouse model; *scat* carries a missense mutation in the protein-coding *Rasa3* gene.**[1]**

RASA3, a Ras-GTPase Activating Protein (GAP), functions as a negative regulator of the Ras signalling pathway, which is often studied in cancer research. RASA3 has previously been found to play a key role in normal blood formation, with the specific mutation in *scat* resulting in the mislocalization of RASA3 to the cytosol and thus loss of RASA3 function. Homozygous s*cat* mice cycle between hematologic crisis and remission, in a way similar to some BMFS patients.**[2]** The first crisis begins *in utero* and lasts until ~P9. Mice in crisis are smaller, with bone abnormalities limiting their growth. The exact mechanism mediating the crisis-remission transition is not well understood, but it offers an opportunity to study the resolution of bone-marrow failure.

Bone marrow failure disorders may manifest with physical abnormalities. Bone structure and development may be affected, causing microcephaly and hypertelorism, among other malformations. At the end of each long bone lies cartilage known as the growth, or epiphyseal plate. The epiphyseal plate is composed of several sections, those being the proliferating, hypertrophic, calcification, and ossification zones. The chondrocytes that the growth plate is composed of are stacked in columns, and should theses columns be interrupted, debilitating defects can occur. Some of the physical abnormalities present in BMFS, such as stunted growth, can be attributed to such dysphoria in the proliferating zone.**[3]** Analyzing the phenotypes present in heterozygous and homozygous *scat* mice can aid in the diagnosis and treatment of BMFS patients who exhibit physical irregularities.

**Research Question:** Can the *scat* mouse model, allowing the signalling pathway of RASA3 and its cascade to be easily observed and characterized by its malfunction in the growth plate.

**Hypothesis:** If there are abnormalities in the growth plate then will exhibit collagen is the hypochondriac zone, despite that not the primary location of collagen in normal mice. Additionally, if the western blots exhibit a normal distribution of proteins, then they will show an increase in proteins downstream from RASA3.

**Materials and Methods:**

1) Growth Plate Development **[4]**
    a) Femurs and tibias will be taken from mice ranging from P5 to P35, fixed in 10% neutral buffered formalin overnight, decalcified with 10% ethylenediamine tetraacetic acid, and embedded in paraffin. Longitudinal sections will be prepared on slides. After deparaffinization and rehydration, the sections will be incubated with primary antibody overnight, then incubated with a secondary antibody and finally mounted using DAPI. The growth plates will be then imaged at 20X.
2) Western Blots
    a) Cells will be lysed with RIPA then loaded into wells along with a weight marker and separated using gel electrophoresis (10% gel). The resulting proteins on the gel will be transferred for 90 minutes at 90V onto a membrane and incubated with a primary antibody overnight, washed with TBST, and incubated with a

secondary antibody. Following another set of washes chemiluminescence substrate will be applied and the blot imaged.

3) Cell Cycle Analysis
    a) $CD34^+$ from umbilical cord blood will be separated and placed into a culture to differentiate into red cells. The cells will be incubated at 100,000/mL in 50μM EdU in normal culture media for 4 hours at 37°C, and the ClickIT manufacturer's protocol followed. The samples will then be resuspended in 250μL PI/RNAse staining buffer and analyzed using flow cytometry.

**Data Analysis:**

1) Experimental Setup
    a) Independent Variable: The knocked-down gene RASA3.
    b) Dependent Variables: The amount of protein detected on each western blot, and the concentration of collagen on the growth plate.
2) Analysis
    a) Flow cytometry will be used to determine what percentage of cells exist in each phase of the cell cycle. Distribution graphs will be created.
    b) Western blots will be performed, and analysed using the EVOS Imaging software that accompanies the imaging machine.
    c) The growth plates will be compared based on expression of each protein. The exact method is not standardized as of before the project begins.

**Risk Assessment:**

1) The use of mouse tissues (sacrificed prior to my involvement with the lab).
2) Live cells, while not diseased, should not come into contact directly with the skin or eyes.
3) Using a thin needle to extract bone marrow requires safety precautions.

These concerns will be mitigated by wearing the correct protective equipment such as a lab coat and gloves, as well as by observing the CPCSEA guidelines for laboratory animal safety.

**Vertebrate Animal Research:**

Conducting research using vertebrate animals should be avoided when possible, however, this project required the use of tissues collected from mice. The growth plates that will be used to

model a human disorder, allowing the physical deformities to be studied without putting human patients in danger. Finding more efficient ways to diagnose and treat patients who are suffering, particularly the developing children the mice model takes precedence over the lives of mice. However, treating the animals with the utmost respect and care is a priority.

**Potentially Hazardous Biological Agents Research:**

Human cord blood will be obtained from approved protocol with Feinstein Institute Biorepository, Biosafety Level 1. A sterile hood with laminar flow will be employed when blood is being used, as well as nitrile examination gloves. Sterile pipettes will be used to transfer blood. The blood will be disposed of through Feinstein Institute Biohazard bags and disposal bins. Prior to working in the lab training will be administered in human cell cultures, murine limb dissection, and histology preparation.

**Bibliography:**

**1.** *Hartman, E. S., Brindley, E. C., Papoin, J., Ciciotte, S. L., Zhao, Y., Peters, L. L., & Blanc, L. (2018). Increased Reactive Oxygen Species and Cell Cycle Defects Contribute to Anemia in the RASA3 Mutant Mouse Model scat. Frontiers in physiology, 9, 689. doi:10.3389/fphys.2018.00689*

**2.** *O'Brien, K. A., Farrar, J. E., Vlachos, A., Anderson, S. M., Tsujiura, C. A., Lichtenberg, J., … Bodine, D. M. (2017). Molecular convergence in ex vivo models of Diamond-Blackfan anemia. Blood, 129(23), 3111–3120. doi:10.1182/blood-2017-01-760462*

**3.** *Wheeless, C. R. (2016, October 10). Wheeless' Textbook of Orthopaedics. Retrieved from http://www.wheelessonline.com/ortho/growth_plate_anatomy*

**4.** *National Academies Press. (2011). Guide for the care and use of laboratory animals. Washington, D.C.*

**Post-Summary Addendum:**

After conducting all experiments, several changes were implemented. The focus of the project shifted from observing the effect of the knockdown of RASA3, to the analysis of growth plates. Due to the lab being a in the center for hematopoietic processes, the bone samples utilized had no standard for analysis. Observing the percent protein expression, and determining the dysplasia of chondrocytes became a priority. After much research, I developed three steps for

processing the images. These were color clustering, canny edge detection, and distance measurements. All was done using Javascript implementations of programs.

The title was changed to “Using Image Segmentation to Computationally Interpret Loss of RASA3 Function and its Impact on Erythropoiesis and Epiphyseal Plate Development Through Dysregulation of Key Signaling Pathways”, as that more accurately represents the research I conducted independently.

The procedure was as follows: Image segmentation is done with the intent of changing the representation of an image into something more easily analysed. The techniques used for the IHC images were color clustering, edge detection, and a linear regression followed by distance calculation. The Canny Edge Detector operator was used to segment the growth plate image into lines. The five steps of the algorithm are as follows:

1. Apply Gaussian filter to smooth the image in order to remove the noise
2. Find the intensity gradients of the image
3. Apply non-maximum suppression to get rid of spurious response to edge detection
4. Apply double threshold to determine potential edges
5. Track edge by hysteresis: Finalize the detection of edges by suppressing all the other edges that are weak and not connected to strong edges.

Images were processed through the first two steps, and the intensity gradient in Y was used as the final segmentation. The gradient is able to visualise abrupt changes in morphology, allowing the borders of the growth plate to become more easily visible. The skew lines composing the borders were approximated into two parallel lines, and the distance between them calculated in pixels. Each image was 512x512 pixels for consistency.

The original images were analyzed for proportions of color clusters to determine the expression of the protein that was stained for during each IHC procedure. K-means clustering was used, with 4 color clusters being the optimal segmentation.

The Javascript implementation of the Canny Edge Detector algorithm was first developed by the Biomedical Imaging Group. The color clustering algorithm is based off of the work of bioinformatician Martin Krzywinski.