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Exploring the Effects of Spaceflight on Mouse Physiology using the Open Access NASA GeneLab Platform

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Corresponding Author:	Afshin Beheshti, Ph.D. NASA Ames Research Center Moffett Field, CA UNITED STATES
Corresponding Author's Institution:	NASA Ames Research Center
Corresponding Author E-Mail:	afshin.beheshti@nasa.gov
Order of Authors:	Afshin Beheshti
	Yasaman Shirazi-Fard
	Sungshin Choi
	Daniel Berrios
	Samrawit G. Gebre
	Jonathan M. Galazka
	Sylvain V. Costes
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Afshin Beheshti, PhD
Assistant Professor
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

May 9th, 2018
To: JoVE

Dear JoVE,

We submit for your consideration a manuscript entitled: "*Exploring the Effects of Spaceflight on Mouse Physiology using the Open Access NASA GeneLab Platform".* This manuscript is in response to an invitation to publish from Dr. Jaydev Upponi. The manuscript has not been published and is not under consideration for publication elsewhere while under consideration. In addition, all authors declare no competing financial interests and conflict of interest with the data and information in this manuscript. We are more than excited for this opportunity to present the methodology of space biology experiments from rodent handling to data being processed on our GeneLab platform. We believe due to the wide appeal of this work to Systems Biology, animal handling for NASA experiments, GeneLab usage, and biology in general, *JoVE* is a most appropriate forum for this work, and we thank you for the invitation and taking this manuscript into consideration.

This paper brings awareness to the NASA GeneLab open science platform (genelab.nasa.gov). NASA has made all spaceflight omics data publicly available through the GeneLab platform and would like to bring awareness to the public to utilize this great resource to generate novel hypothesis-based research. For this manuscript we describe in detail how data is curated on GeneLab, using GeneLab, describing the interactive workspace, and a protocol for using the Galaxy GeneLab interface. In addition, we have also described a novel pipeline for an unbiased systems biology analysis to determine key driving pathways/factors when analyzing omics data. This manuscript not only provides the scientific community direction for designing optimal future microgravity experiments, but also demonstrates how publicly available spaceflight data from GeneLab can be utilized to generate hypotheses for future experiments

We also have provided how the rodent space biology experiments are performed. Although the procedures are trivial, the general public outside of NASA research will not be familiar with the procedures involved with rodent space biology experiments. This information will bring valuable insight on how rodent experiments are carried out and eventually translated to data which is available on GeneLab.

Due to the broad implications of these findings and the novel perspective utilizing systems biology to determine master regulators driving the biological response due to spaceflight, we believe this work will be of interest to your readership.

Please do not hesitate to contact me if I can provide any additional information.

On behalf of the authors,

Afshin Beheshti, PhD

TITLE:
Exploring the Effects of Spaceflight on Mouse Physiology using the Open Access NASA GeneLab Platform

AUTHORS AND AFFILIATIONS:
Afshin Beheshti[1], Yasaman Shirazi-Fard[2], Sungshin Choi[1], Daniel Berrios[3], Samrawit G. Gebre[1], Jonathan M. Galazka[3], Sylvain V. Costes[3]
[1]WYLE Labs, NASA Ames Research Center, Space Biosciences Division, Moffett Field, CA, USA
[2]NASA Ames Research Center, Space Biosciences Division, Moffett Field, CA, USA
[3]USRA, NASA Ames Research Center, Moffett Field, CA, USA

Email Addresses of Co-Authors:
Yasaman Shirazi-Fard (yasaman.shirazi-fard@nasa.gov)
Sungshin Choi (sungshin.y.choi@nasa.gov)
Daniel Berrios (daniel.c.berrios@nasa.gov)
Samrawit G. Gebre (samrawit.g.gebre@nasa.gov)
Jonathan M. Galazka (jonathan.m.galazka@nasa.gov)

Corresponding Authors:
Afshin Beheshti (afshin.beheshti@nasa.gov)
Sylvain V. Costes (sylvain.v.costes@nasa.gov)

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GeneLab, NASA, Animal Enclosure Modules, AEM, rodents, CO_2, RNA-sequencing, bioinformatics, transcriptomics, Rodent Habitats, spaceflight, microgravity

SUMMARY
The NASA GeneLab platform provides unfettered access to precious omics data from biological spaceflight experiments. We describe how a typical mouse experiment is conducted in space and how data from such experiments can be accessed and analyzed.

ABSTRACT
Performing biological experiments in space requires special accommodations and procedures to ensure that these investigations are performed effectively and efficiently. Moreover, given the infrequency of these experiments it is imperative that their impacts be maximized. The rapid advancement of omics technologies offers an opportunity to dramatically increase the volume of data produced from precious spaceflight specimens. To capitalize on this, NASA has developed the GeneLab platform to provide unrestricted access to spaceflight omics data and encourage its widespread analysis. Rodents (both rats and mice) are common model organisms used by scientists to investigate space-related biological impacts. The enclosure that house rodents during spaceflight are called Rodent Habitats (formerly Animal Enclosure Modules), and are substantially different from standard vivarium cages in their dimensions, air flow, and access to water and food. In addition, due to environmental and atmospheric conditions on the International Space Station (ISS), animals are exposed to a higher CO_2 concentration. We

recently reported that mice in the Rodent Habitats experience large changes in their transcriptome irrespective of whether animals were on the ground or in space. Furthermore, these changes were consistent with a hypoxic response, potentially driven by higher CO_2 concentrations. Here we describe how a typical rodent experiment is performed in space, how omics data from these experiments can be accessed through the GeneLab platform, and how to identify key factors in this data. Using this process, any individual can make critical discoveries that could change the design of future space missions and activities.

INTRODUCTION

The overall goal of this manuscript is to provide a clear methodology of how to use NASA's GeneLab platform[1] and how rodent experiments done in space are translated to omics data for analysis. Spacefaring humans are exposed to numerous health risks from altered gravity fields, space radiation, isolation from Earth, and other hostile environmental factors[2-6]. Biological experiments performed in space and on the ground have helped to define and quantify these risks[7-14]. In space, these experiments have been conducted on the International Space Station (ISS), the Space Shuttle, and other orbital platforms. Conducting these experiments requires specialized hardware and methodology given the unique concerns of performing experiments in space including limited crew time and the microgravity environment. Various platforms now exist for performing sophisticated experiments in space using plant, animal, and microbial models[15].

Rodent models have been particularly important to advancing our understanding of how mammals, including humans, respond to spaceflight. These include the impact of spaceflight on the muscle structure[16-18] and immune functions[19-21]. The standard vivarium cages used for housing rodents on Earth are not suitable for spaceflight experiments[22,23]. Therefore, over the years mice and rats have been flown and housed in various cages including the Japanese Aerospace Exploration Agency (JAXA) Habitat Cage[24], animal carrying space capsules used on the BION-M1 unmanned Russian satellite[25-27], the Mice Drawer System (MDS) designed by the Italian Space Agency[28-30], the NASA Animal Enclosure Module (AEM), and now the NASA Rodent Transporter and Habitats[23]. Rodent experiments first started on board the Space Shuttle using cages referred to as the Animal Enclosure Module (AEM). This hardware was used in 27 rodent experiments on the Space Shuttle[23]. The AEM was originally developed for relatively short experiments on-board the shuttle (< 20 days). Since the development of the ISS, the AEMs have been modified for longer duration experiments and are now referred to as Rodent Habitats[22,23]. The new Rodent Habitats are designed to support long-duration missions in the ISS using the EXpedite the PRocessing of Experiments for Space Station (EXPRESS) Rack interface. Rodent Habitats are substantially different from standard vivarium cages in their dimensions, air flow, filter and exhaust system, and access to food and water (**Figure 1**). Nevertheless, this hardware has proven to be an effective research platform, enabling key insights into the spaceflight-induced changes to mammalian physiology[19,31-36].

Large volumes of omics data can now be generated from biological spaceflight experiments including those performed with rodents. Recently, data from these omics experiments have been made publicly available through the NASA GeneLab platform[1] which is a comprehensive data repository and analysis platform that allows anyone to develop hypotheses from spaceflight experiments. GeneLab provides tools for the discovery, access, sharing and analysis of data. We utilized GeneLab datasets to show that differences between the standard vivarium cages and specialized Rodent Habitats used in space cause massive differences in the transcriptome of mice[36]. We analyzed four different publicly available datasets, comparing different tissues from rodents housed in either the Rodent Habitats or standard vivarium cages. Using an unbiased systems biology analysis, we determined that the main drivers and pathways that were changed were consistent with a hypoxic response due to the high CO_2 levels caused by higher CO_2 concentrations on ISS, which leads to higher CO_2 concentrations in the Rodent Habitat given that they are passive systems that take in the ambient air. This demonstrates how scientists can use open source tools and data to generate novel findings with implication on how the environment of the ISS impacts astronaut health.

Here we describe how rodent experiments are performed in space and how data from these experiments can be accessed through an open-source, omic platform related to space biology. We discuss the configuration of the Rodent Habitats used for space missions, and how spaceflight tissues are processed. We also describe how spaceflight omics data can be discovered and accessed on GeneLab and how key factors driving the overall response to spaceflight can be identified[36]. The specific example we will present on how this protocol is implemented will be comparing the biological differences occurring in rodents housed in Rodent Habitat and the vivarium controls that were published by Beheshti *et al.*[36]. It is important to note that ground controls are essential for spaceflight rodent experiments. As described in this protocol, these controls are done with both identical conditions (*i.e.,* CO_2 conditions, humidity, temperature, cage dimensions, *etc.*) in the Rodent Habitats on the ISS and in standard vivarium cages that have the standard environmental (*i.e.,* CO_2 conditions, humidity, and temperature) conditions on Earth. The rodents housed in the Rodent Habitat ground controls allow for the direct comparison to rodents in space. While rodents housed in vivarium cages allow for the biological comparison between the different housing (*e.g.,* vivarium cages *vs.* Rodent hardware). The Rodent Habitat is different than vivarium cages in that it has constant air flow (0.1-0.3 m/s), a long duration, and a secondary exhaust filter that captures and absorbs the animal waste guided to the exhaust filter by continuous air flow in microgravity. In addition, Rodent Habitats have passive systems and intake ambient air; therefore, they also have higher CO_2 concentrations due to elevated levels in the ISS cabin (~5000 ppm).

PROTOCOL:

The animal protocols for housing and tissue processing follow standard guidelines for laboratory animal care and have been approved by NASA’s flight and ground Institutional Animal Care and Use Committees (IACUC).

1. Configuration of Rodent Habitats

Note: The NASA Rodent Habitats (previously AEMs) have different features from the vivarium cages to accommodate for operations in space (**Figure 1**).

1.1. House 10 mice in each Rodent Habitat (up to 30 g per mouse). House 5 mice per compartment when the habitat is configured into two compartments or 10 mice if there is a single compartment.

Note: NASA Rodent Habitats have a larger accessible surface area per rodent than the standard vivarium cages.

1.2. For ground controls animals, house mice in Rodent Habitat inside the ISS Environmental Simulator (ISSES) under identical environmental conditions as the flight animals including CO_2 concentrations, temperature, and relative humidity.

1.3. Provide animals with *ad libitum* access to custom made NASA Nutrient Upgraded Rodent Foodbars (NuRFB) in accordance with National Research Council (NRC) nutritional requirements for mice[37], and to water through pressure activated lixits.

1.4. Monitor the animals' health and behavior which will be enabled in the Rodent Habitats with the 12:12 h light cycle similar to vivarium cages in standard facilities with LED lighting during the day and infrared lighting during video health checks that take place during the dark cycle.

1.5. Place four cameras in the Rodent Habitat cages for the daily monitoring of animals' health and behavior and collect videos during the night with infrared lighting.

1.6. Deliver the rodents to the ISS in a Transporter (**Figure 2B**) aboard the Dragon Capsule or similar launch vehicle.

1.7. Ensure that the rodents are observed and examined by the NASA flight veterinarian before being loaded into the Transporter for launch, and by the trained crew members upon arrival to ISS and before transfer to Rodent Habitats.

1.8. For this transition period, house up to 20 mice (10 on each side) or 12 rats in the transporter.

Note: Similar to the Rodent Habitat, the Transporter is a passive unit for environmental conditions. During this short transition period, this single-unit can house up to 20 mice.

2. Rodent Handling for Spaceflight Experiments

2.1. Procure rodents from standard vendors.

Note: Following delivery, group rodents within standard vivarium cages and have the animals acclimate to NASA NuRFB, lixits, and raised wire floors until the animals are loaded into the Transporter. Leaving the rodents in the cages will allow the animals to adapt naturally. The handling of mice in and out of both the Rodent Habitats and vivarium cages follows protocols commonly used for all rodent experiments[12,27,28]. The Rodent Habitat system (**Figure 1A**) will be utilized for both spaceflight mission on the STS and ISS, respectively, and for ground controls simulating ISS or STS environmental conditions.

2.2. For some missions use standard vivarium cages (**Figure 1B**) for the vivarium control. Use 5 or 10 mice per standard vivarium cage.

2.3. For Rodent Habitats, place 10 mice in two different compartments with 5 mice per compartment. Remove the cage divider to house 10 mice per Habitat in a single compartment.

2.4. Utilize three components of the Rodent hardware during spaceflight missions as described below (**Figure 2**).

2.4.1. Place rodents in a Transporter (**Figure 2B**) for the travel between the Earth and the ISS or vice versa at double density (10 mice per side, 20 mice per Transporter).

2.4.2. Once on ISS, attach the Animal Access Unit (AAU) **(Figure 2C**) to the Transporter. Transfer rodents from the Transporter to the Habitats using Mouse Transfer Boxes (MTB) (5 mice per MTB) (**Figure 2D)**.

Note: The AAU is used to contain any animal products (*e.g.,* feces, urine, fur) from getting to the ISS cabin.

2.4.3. Detach the AAU from the Transporter and attach to the Rodent Habitat. Then transfer the animals from the MTB to the Rodent Habitat (**Figure 2A**) where they reside for the duration of the mission.

Note: The CO_2 concentration due to elevated levels in the ISS cabin for all Rodent Habitats is at ~5000 ppm.

2.5. Monitor the temperature and humidity of the Rodent Habitats, but there are no active thermal controls. Ensure that the Rodent Research team works with ISS to maintain and control the cabin temperature, which determines the temperature in the Rodent Habitat.

Note: The light and dark cycle in the Rodent Habitats occurs every 12 h (*e.g.,* 5:00 – 17:00 GMT, lights on) and the ISS crew performs regular and frequent change out of the food (weekly or biweekly) and refills the water (every ~28 days).

3. Euthanasia of Rodents and Processing Tissue

3.1. For euthanasia, give rodent an overdose of a general anesthetic (Ketamine/Xylazine up to 150/45 mg/kg body mass diluted in phosphate-buffered saline for a total volume of 0.3 mL) paired with a secondary method of euthanasia (cervical dislocation or thoracotomy).

3.2. For experiments conducted on the ISS:

3.2.1. Return rodents either live, or

3.2.2. Euthanize on ISS.

3.2.2.1. Freeze rodent carcasses at -95 ± 2 °C in the freezers on the ISS and return to Earth on the available return vehicle (currently SpaceX Dragon capsule).

3.2.2.2. Once the rodents are returned to Earth, dissect all organs and tissues (*i.e.,* liver, kidney, skin, muscles, heart, spleen, eyes, adrenal glands, lungs, and brain) and store at -80 °C or in RNA stabilizing solution.

3.3. Follow the same procedures and timings for all control ground experiments as the flight experiment with a 3-5 day offset to match the ISS telemetry data.

3.4. From the preserved tissues isolate RNA, protein, and DNA isolation using standard protocols that are described in detail associated with each dataset on the GeneLab platform (genelab.nasa.gov).

Note: Rodent tissues not utilized by the primary investigator(s) become part of NASA's Institutional Scientific Collection. These samples are stored in Ames Research Center's (ARC) Non-Human Biobank where they are cataloged and made available for request by the science community. Available tissues can be found on the Life Sciences Data Archive Public Website at: https://Lsda.jsc.nasa.gov/Biospecimen.

4. Generating Omics Data from RNA, DNA, and Protein Extracts

4.1. From the extracted macromolecules (RNA, DNA, protein) use standard protocols to generate omics data. These are described in detail in the respective study metadata on GeneLab.

5. GeneLab Repository and Submitting Data

Note: Space biology related omics data are submitted to the GeneLab Data Repository. GeneLab accepts and hosts space-related omics data funded by multiple space agencies around the world.

5.1. Generate omics related data that can be hosted on the GeneLab repository.

5.1.1. Submit generated data to GeneLab, either when analysis is complete or based on the discretion of the investigator.

Note: Data submitted to other public omics databases are imported and published into the GeneLab repository. GeneLab generated data are curated and published without an embargo period. GeneLab, specifically the Sample Processing Lab, generates data from various spaceflight experiments using optimized extraction protocols and techniques to increase the omics data from spaceflight experiments.

5.2. When the data are ready to be submitted, format and transfer the metadata and data to GeneLab with the following method (**Supplemental Figure 1**):

5.2.1. Use the ISAcreator tools to define an experimental study and store the metadata.

Note: The ISAcreator tool is available for download with a guided tutorial here[38].

5.2.2. Refer the data listed here[39] to understand accepted data types and formats for raw and processed data files.

5.2.2.1. To optimize upload and storage, compress data files.

5.2.3. Transfer the metadata and raw and/or processed data to GeneLab data curators through the workspace[40].

5.2.4. Create a username and password and upload the data.

5.3. Once the data have been uploaded to the workspace, share data to a GeneLab curator.

Note: Detailed steps on how to upload and share files can be found in the Data Submission Guide[41].

5.4. Each submission is verified by a curator and published in the GeneLab repository[42].

6. Finding Datasets for Analysis using Search Features on GeneLab

6.1. Search for different datasets on GeneLab by going to the link (**Supplemental Figure 2)**[38].

6.1.1. Specifically related to a previous publication[36], search for the following terms: GLDS-21, GLDS-111, GLDS-25, and GLDS-63.

6.2. Access the GeneLab homepage by clicking on "GeneLab Data System" on the left-hand side of the screen.

6.3. Enter the keywords in the "search data" box to search for specific areas of interest. In this case enter each of the following dataset identifiers separately: GLDS-21, GLDS-111, GLDS-25, and GLDS-63.

6.4. In addition to searching the GeneLab repository, search across other databases including NIH GEO, EBI Pride, and ANL MG-RAST by selecting the desired check boxes under the search bar.

Note: Currently only for the GeneLab repository, a user can search by using the following filter categories: Organisms, Assay Type, Factors, and Project Type.

7. Storing and Transferring Files of Interest for Analysis

Note: The GeneLab Workspace is designed to store and transfer files directly from the GeneLab database (**Supplemental Figure 3**).

7.1. Click on "Workspace" on top of the Data Systems menu.

7.2. If new user, register for a new account.

Note: The GeneLab Workspace is powered by GenomeSpace[43].

7.3. Access detailed instructions on how to use the workspace by selecting "Help" on the top menu and clicking on User Guide.

7.4. For each user, access all the datasets in the GeneLab repository by selecting the "Public/genelab" folder on the left-hand menu.

7.5. Copy the datasets of interest to a local directory workspace by going to the folder with the data of interest. Right click on the specific file, select "copy/move" in the menu that appears, select the folder to copy the file to, and then click on "copy".

7.5.1. Find the following datasets related to a previous publication[36] as instructed above and copy over to the local workspace: GLDS-21, GLDS-111, GLDS-25, and GLDS-63.

8. Accessing Metadata and Description of Each Study

Note: Metadata files for each dataset in the GeneLab repository are in the "Public/genelab" dataset subfolder on the left-side menu.

8.1. Find the metadata information for the dataset of interest by accessing one or more metadata files contained in a "metadata" subfolder of each dataset. For example, for GLDS-100, there are 2 files in the "Public/genelab/GLDS-100/metadata" subfolder: "GLDS-100_metadata_RR1_BIOBANK-Eye-ISA.zip" and "GLDS-100_metadata_RR1ExpDesign.pdf".

8.1.1. Ensure that every dataset has a single zipped file that provides metadata according to the ISATab specification (which subsumes the MIAME, MIAPE, and other MIBBI framework standards for minimum metadata requirements). Always end this type of file name in “ISA.zip”. For example, for GLDS-100, this file is “GLDS-100_metadata_RR1_BIOBANK-Eye-ISA.zip”.

8.2. Use the ISACreator tool[44] or a text editor to visualize and access the ISATab metadata, which contains the text description for the study and assay metadata for each dataset.

Note: Within the ISATab metadata, samples are described and associated with bioassays, and bioassays are described and associated with output data files.

8.3. Check for the presence of the output assay data files that are located within each dataset in subfolders by type of assay. For example, for GLDS-100, RNA-Seq output assay files are located in the “Public/genelab/GLDS-100/transcriptomics/” folder.

9. Analysis of GeneLab Data

Note: Various pipelines can be implemented for various omics data. Here, the specific example focuses on an unbiased systems biology transcriptomic pipeline which is used to determine the “key drivers” of the system being studied.

9.1. Check previously published literatures[36,45-50] to understand this pipeline.

9.2. Once a specific dataset of interest is selected for analysis, download the data to a local machine with the following method:

9.2.1. Click on the specific dataset.

9.2.2. Click on the “Study Files” tab on the far left of the headers.

9.2.3. Ensure that all datafiles and metadata are available in this menu.

9.2.4. To download each file, click on the specific file names.

9.3. For the microarray datasets that will be downloaded from GeneLab, use the following pre-processing steps.

9.3.1. Process the raw data for each dataset separately using background subtraction and Quantile normalized using RMAExpress[51] for the commercial microarrays.

9.3.2. Create principle component analysis (PCA) plots using R to determine how closely the biological replicates grouped together.

9.3.3. Import data into MultiExperiment Viewer[52] and calculate significant genes first using the false discovery rate (FDR) statistics starting with FDR < 0.05. If no significant genes appeared with FDR statistics, then use standard t-tests starting with a p-value < 0.05 to determine the significant genes.

9.3.4. Once the statistically significant regulated genes have been determined, implement a fold-change cut-off of ≥ 1.2 or ≤ -1.2 to compare the experimental samples with the controls.

9.4. Use Gene Set Enrichment Analysis (GSEA)[53] for pathway and functional predictions.

9.4.1. Use GSEA either through GenePattern[54,55], directly through GSEA, or using R programming environment.

9.4.2. Determine the significantly regulated pathways using the following gene sets: C2, C5, and hallmarks.

9.4.3. Perform leading-edge analysis on the significantly regulated gene sets and determine leading edge genes associated with each experimental comparison and Gene Set.

9.4.4. Find the leading-edge genes that overlap between all the gene sets for each experimental condition.

9.5. Use another platform to determine predicted functions and pathways that are being significantly regulated. In this case use ingenuity pathway analysis (IPA) to determine the significant upstream regulators, biofunctions, and canonical pathways.

9.5.1. Upload the list of genes with fold-change values for the statistically significant genes determined in step 9.4.4.

9.5.2. Follow IPA's instructions to generate upstream regulators, biofunctions, and canonical pathways for each experimental comparison.

9.5.3. Determine the gene associated for upstream regulators, biofunctions, and canonical pathways which have an activation z-score ≥ 2 (indicated activation) or ≤ -2 (indicating inhibition).

9.5.4. Find the overlapping genes related to all the predictions above.

9.6. Determine common/overlapping genes between steps 9.4 and 9.5.

Note: These genes are considered as the key/driver genes controlling the majority of the predicted functions and activity with the experimental conditions being analyzed. Previous studies have shown that knocking out or promoting these genes will make the experimental condition or system being studied non-functional[45,46,49].

9.6.1. Construct networks through IPA (or any network assembly software) to determine the connectivity of the genes.

9.6.2. Consider the most connected gene as central hub driving the key genes.

9.6.3. To determine the connectivity between the datasets, group all key genes in one network and repeat connectivity test to determine the central hub that is occurring among all key genes from all datasets being analyzed.

10. Using Galaxy[56] Interface on GeneLab to Analyze Transcriptomic Data

Note: Here a protocol for using the GeneLab Galaxy interface (available Fall 2018) to analyze transcriptomic data from GeneLab is described. Galaxy tutorials abound. Example tutorials on how to use Galaxy in general are available elesewhere[57,58].

10.1. Users can sign in to GeneLab using Google or NASA credentials. GeneLab Galaxy tools are located under the "Analyze" menu.

10.2. Follow these three ways to bring data into the GeneLab Galaxy platform.

10.2.1. Upload data from the local file system using the "Upload data" function.

10.2.2. Import data from GeneLab GenomeSpace using the GenomeSpace importer tool under "Get Data" section.

Note: All GeneLab data files are available in the "public" folder, organized by the dataset accession number (see above).

10.2.3. Import data appear in the "history" of analysis section on the right-hand side. Users can have multiple histories, which are managed using either "History Options" or "View All Histories" buttons at the top of the history pane.

10.3. Tools for analysis are listed and searchable on the left side of the interface.

10.4. Check for the appearance of datasets that have been imported on the current history.

Note: Many details regarding the data are available for inspection for each dataset.

10.5. Select a tool on the left-hand side to populate a form in the center panel, with options for analysis and specification of data inputs. Create jobs for executing the analysis by completing the form and pressing "Execute".

10.6. Check for jobs submitted which are represented in the history and color-coded to indicate status of execution (queued, executing, completed with or without errors).

10.7. Link the tools into complex workflows. Manage workflows through tools located in the "Workflows" menu. **Figure 3** shows an example workflow created for processing RNA-seq data.

10.8. Share datasets, workflows, and histories with others using the "Shared Data" menu.

REPRESENTATIVE RESULTS:

Determining key drivers from spaceflight transcriptomic data will assist NASA with determining health risks and developing potential countermeasures to combat negative effects on astronaut health. In our recent publication, we have followed the steps above and utilized GeneLab datasets to successfully show a novel finding that CO_2 concentrations on the ISS can impact health[36]. We have also used the technique above in other studies to successfully determine the key factors driving the system being studied[45-50]. Here we will show how the results from using this protocol can be successfully used to determine the key drivers.

In this study, we primarily focused on the biological differences that occur in rodents housed in the Rodent Habits ground controls and the vivarium controls. As described above, it is the key to better understanding these two habitats, which will provide us information on possible confounding factors that can impact health due to the environment on the ISS. For all rodent spaceflight experiments, these ground controls are also essential to determine which biological factors are associated directly with spaceflight or due to the environmental conditions on the ISS. As stated in the protocol, the environmental condition for the vivarium habitat is not exposed to the higher CO_2 level that is present for the Rodent Habitat. The vivarium habitat has the normal CO_2 level that is present on Earth (currently being ~300 to 380 ppm). The temperature and humidity for both habitats are similar.

We used the following datasets from the GeneLab platform to determine the key genes between the rodents housed in the Rodent Habitat ground controls and vivarium ground controls that are responsible for driving the differences between the two habitats: GLDS-21, GLDS-111, GLDS-25, and GLDS-63. Analysis to determine the significant genes was carried out as described above between the Rodent Habitat (previously AEM) and vivarium controls independently for each dataset. PCA plots showed grouping of the biological replicates (**Figure 4** shows the PCA plots for GLDS-21). From the pre-processed data, we determined the leading-edge genes from the different GSEA gene sets. Using the genes with 1.2-fold-change (log_2), we were able to predict the genes involved with predictions for upstream regulators, canonical pathways, and biofunctions. For each dataset we then found the common/overlapping genes involved for all the genes (**Figure 5**). These genes are now believed to be driving the response between the rodents in the Rodent Habitats (or AEM) and vivarium controls. Network representation of how these key genes connect shows the central hubs for each dataset being analyzed (**Figure 6**). For example, MAPK1 is the central hub for STS-108 skeletal muscle tissues from mice (**Figure 6A**). This would be interpreted as the gene that is driving the key genes and most likely the central player for causing biological differences for mice housed in Rodent

Habitats *versus* the vivarium cages. In our previous work, we discuss how these key genes are associated with CO_2 response from the existing scientific literature and how these genes can be responsible for biological changes observed in the mice[36].

Taking a systems biology approach, we next determined a “master regulator” that connects all the datasets/tissues and is potentially responsible for universal biological effects in rodents housed in AEMs compared to vivarium cages. This was done by determining the gene from all the datasets that is the most connected when constructing a network from all the key genes. We were able to show that MAPK1 is the most connected gene and central hub from all the key genes (**Figure 7**). To confirm if MAPK1 might be responsible for biological changes in mice from the higher CO_2 levels in AEMs, we looked through the scientific literature for supporting evidence. We found several studies indicating the correlation of MAPK1 with CO_2[59] and hypoxia[19,60,61].

FIGURE AND TABLE LEGENDS:

Figure 1: The Rodent Habitat (previously AEM) compared to the vivarium cages. A. Image of the AEM cage provided by NASA (Credits: NASA/Dominic Hart). **B.** The standard vivarium cage that is currently used (photo taken by our laboratory). This figure has been modified from Beheshti *et al.*[36].

Figure 2: The Rodent Habitat Hardware System with the three different modules involved during transportation to and from the space missions. The left module (**A**) is the Rodent Habitat module (previously AEM), the center module (**B**) is the Transporter, and the right module (**C**) is the Animal Access Unit (AAU). **D.** The Mouse Transfer Box (MTB). (Credits: NASA/Dominic Hart).

Figure 3: Example analysis workflow which can be used in the GeneLab Galaxy interface to process RNA-seq data.

Figure 4: Principal component analysis (PCA) of representative dataset after pre-processing steps. GLDS-21 dataset for AEM *vs.* vivarium cage is shown for the murine skeletal muscle from the STS-118 mission.

Figure 5: Venn diagram representing what key genes are determined using different pathway prediction tools.

Figure 6: The key genes determined for all conditions and murine tissues between the AEM *vs.* vivarium cages. A-E. Network representation of the key genes for each dataset/rodent tissue. Log_2 fold-changes (with a cutoff of 1.2-fold-change) to the gene expression were used to obtain different shades of green for fold-change in downregulated genes, while different shades of red depict fold-change in upregulated genes. The darker the shade of green or red, the greater the fold-change. This figure has been modified from Beheshti *et al.*[36].

Figure 7: Determining the "master regulator" for rodents in Rodent Habitat housing compared to vivarium cages. Connections between all individual key genes (**Figure 6**) were determined and displayed as a network through IPA. Network is represented as a radial plot with the most connected key gene, MAPK1, in the center.

Supplemental Figure 1: GeneLab-GenomeSpace Integration with ISACreator for Streamlining Data Processing Operations.

Supplemental Figure 2: Screenshot of GeneLab searches using federation/integration with heterogeneous bioinformatics external databases (GEO, PRIDE, MG-RAST).

Supplemental Figure 3: Screenshot of the GeneLab collaborative workspace showing the user account management, and access controls (*e.g.,* private, shared, public folders).

DISCUSSION:

The NASA GeneLab platform is a comprehensive omics database and analysis platform that will allow the scientific community to generate novel hypotheses related to space biology. Here we have presented a comprehensive procedure for rodent experiments from the beginning of spaceflight to the generation of novel hypothesis from analyzing data utilizing a publicly available space biology platform. In addition, we have also provided an extensive protocol on an unbiased systems biology analysis for identifying key genes driving the system being studied. We have used our recent study[36] as an example of how this protocol is effectively utilized to generate a novel hypothesis for space biology. We hope that this helps investigators better understand how spaceflight experiments are performed and how data from them lead to the data available on GeneLab, and ultimately allow for clearer interpretation of publicly available space biology omics data.

There are several critical steps within our protocol regarding both rodent spaceflight experiments and analysis of the data produced. Understanding the Rodent Habitat setup is critical to develop and design the optimal experiment for spaceflight. This would specifically entail the protocol and description we have provided in step 1 of our protocol. Once an investigator fully understands the different conditions existing in the Rodent Habitats compared to vivarium cages, the biological results being interpreted can be correlated properly to the environmental conditions in space. In additions, modifications to the Rodent Habitat cannot be done, since the Rodent Habitat has been optimally designed and approved by NASA for use of spaceflight.

To interpret the biological results, we have provided a thorough protocol on every step involved from uploading your data to GeneLab to analysis of the data to generate novel space biology hypothesis. Although all the steps are important in understanding how to generate data, the most critical steps for data analysis are steps 9 and 10. Step 9 provides a protocol to analyze transcriptomic data using an unbiased systems biology method to determine genes/pathways that are truly driving the experimental condition being analyzed. Step 10 is critical as it provides users with an easy methodology to analyze omics GeneLab datasets using the GeneLab platform. Modifications to the protocol provided can be done for some steps regarding analyzing data. Specifically, steps 9.4 – 9.6 can be done using R programming or any other favorite tools that the user prefers. Depending on the dataset, different statistics and fold-change cutoffs can be used to determine the significantly regulated genes. In addition, for determining the key genes in steps 9.5 and 9.6, the user can modify this protocol and use any tool that utilizes the significantly regulated genes to predict functions. The important concept is that using multiple predictive functional omics tools allows for determination of genes involved with the majority of functions being regulated in the system being studied.

The GeneLab platform continues to develop, and while the analyses described here were performed after data download, the next phase of GeneLab will allow for analysis of omics data directly on GeneLab platform, which will provide an easy workflow to generate processed data for higher-order analysis. Moreover, whereas we have focused on a protocol for interpreting transcriptomic data, GeneLab contains a wide variety of omics data including proteomic, genomic, metabolomic, and epigenomic data. The eventual platform will contain pipelines and guidelines for analysis of these different types of omics. The last phase of GeneLab will also implement a system level visualization interface to allow the basic user to easily generate space biology hypotheses.

Lastly, our systems biology analysis provides a unique and unbiased method to determine the key driving genes/pathways in any system being studied using omics datasets. We have used this methodology in several different independent studies with great success to determine the key drivers involved[36,45-50]. In a cancer related omics study, using this methodology we experimentally validated that our predicted key genes/pathways were actually driving the drug treatment response by knocking out the key genes *in vitro*[45]. We observed, as we had predicted through this protocol, that the treatment was not effective anymore due to the absence of the key genes. We believe that this unbiased systems biology protocol can be a useful tool to determine key pathways for any omics study.

This protocol provides a quick and efficient method for the generation of novel space biology hypotheses. The data generated from GeneLab can be leveraged by investigators for future funding opportunities, experimental validation, and potential targets for development of countermeasures against microgravity and space radiation. The protocol provided here will permit for future space biology investigations to occur with optimal efficiency to allow for safe long-term space missions.

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DISCLOSURES:

The authors have nothing to disclose.

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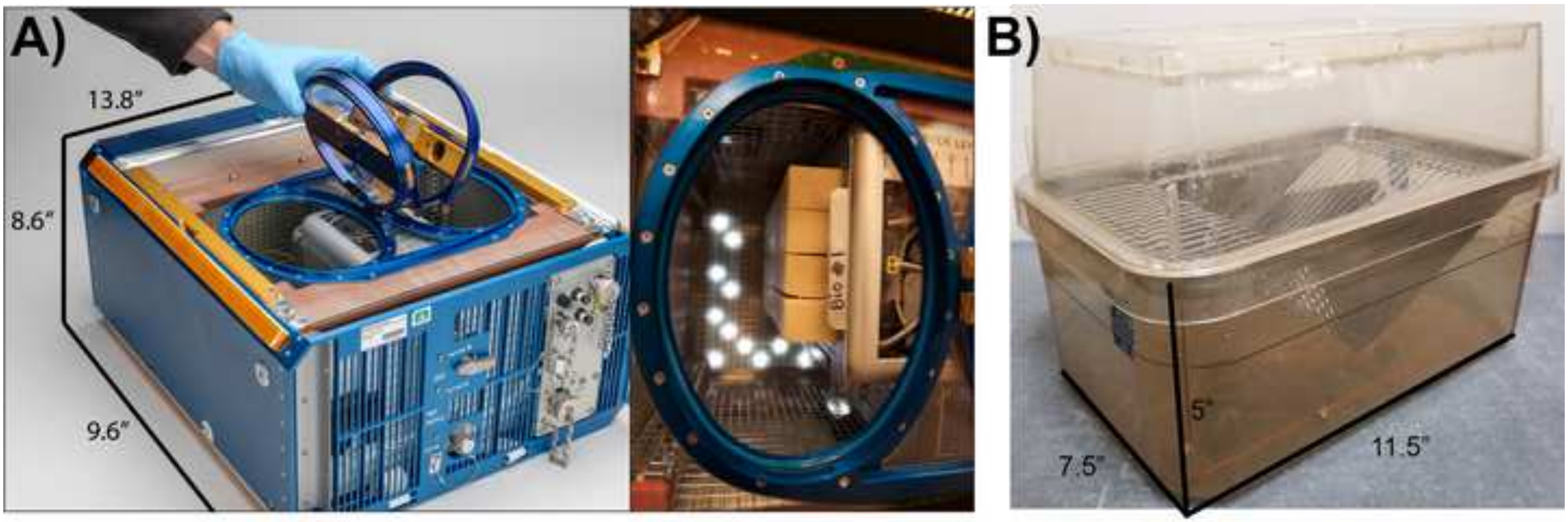

Animal Enclosure Module (AEM)

Sample vivarium cage

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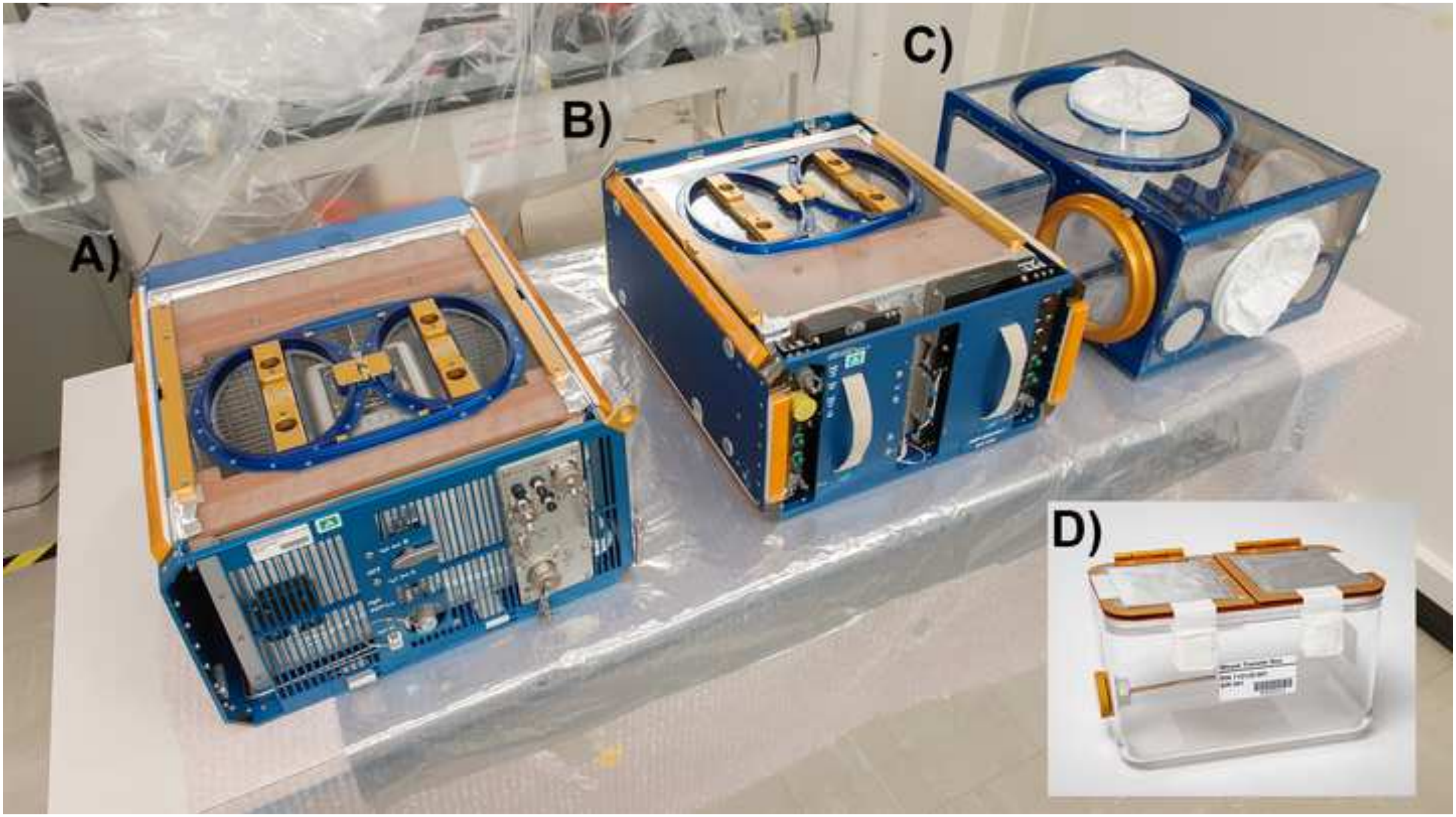

Figure 3

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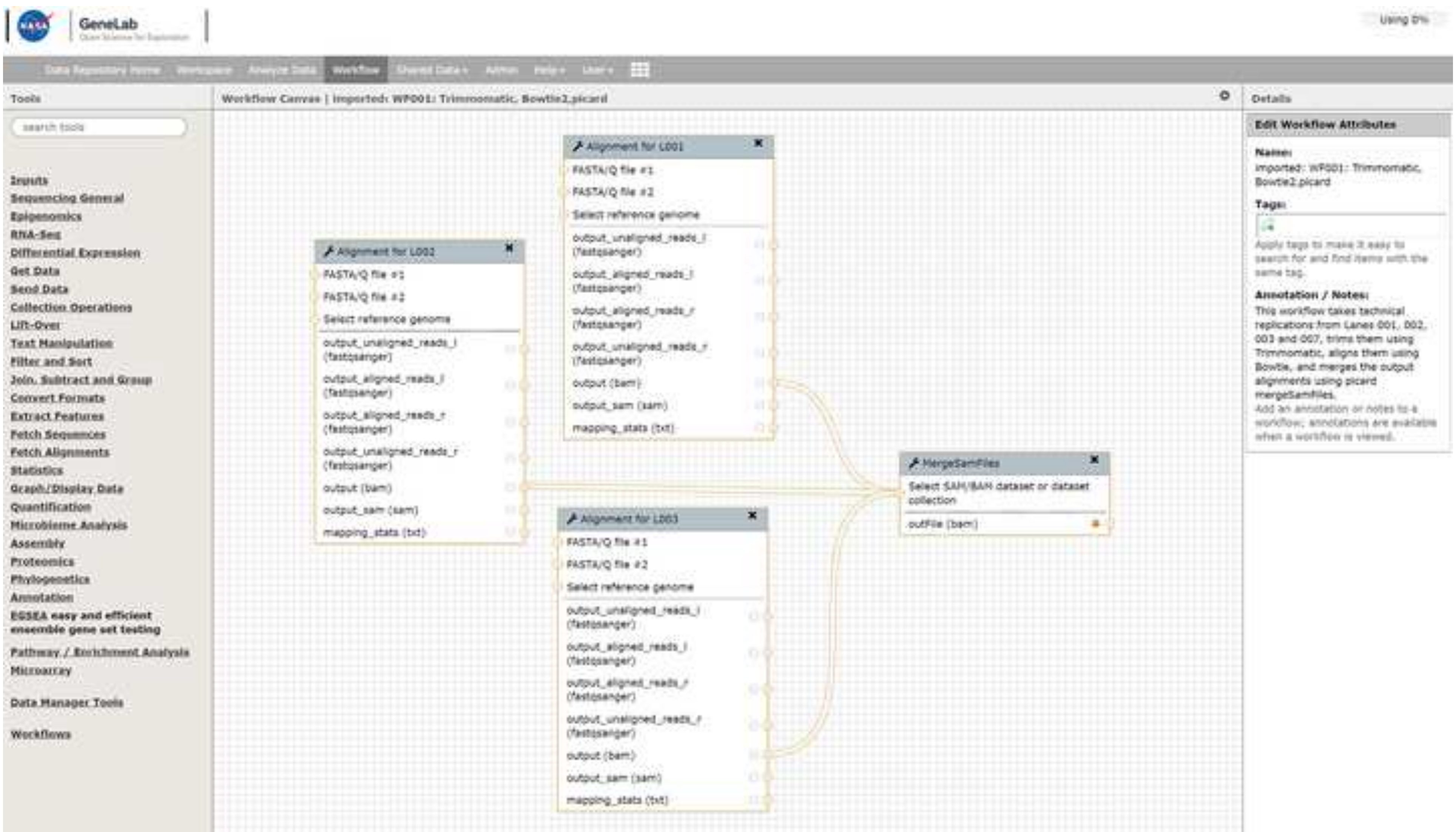

Figure 4

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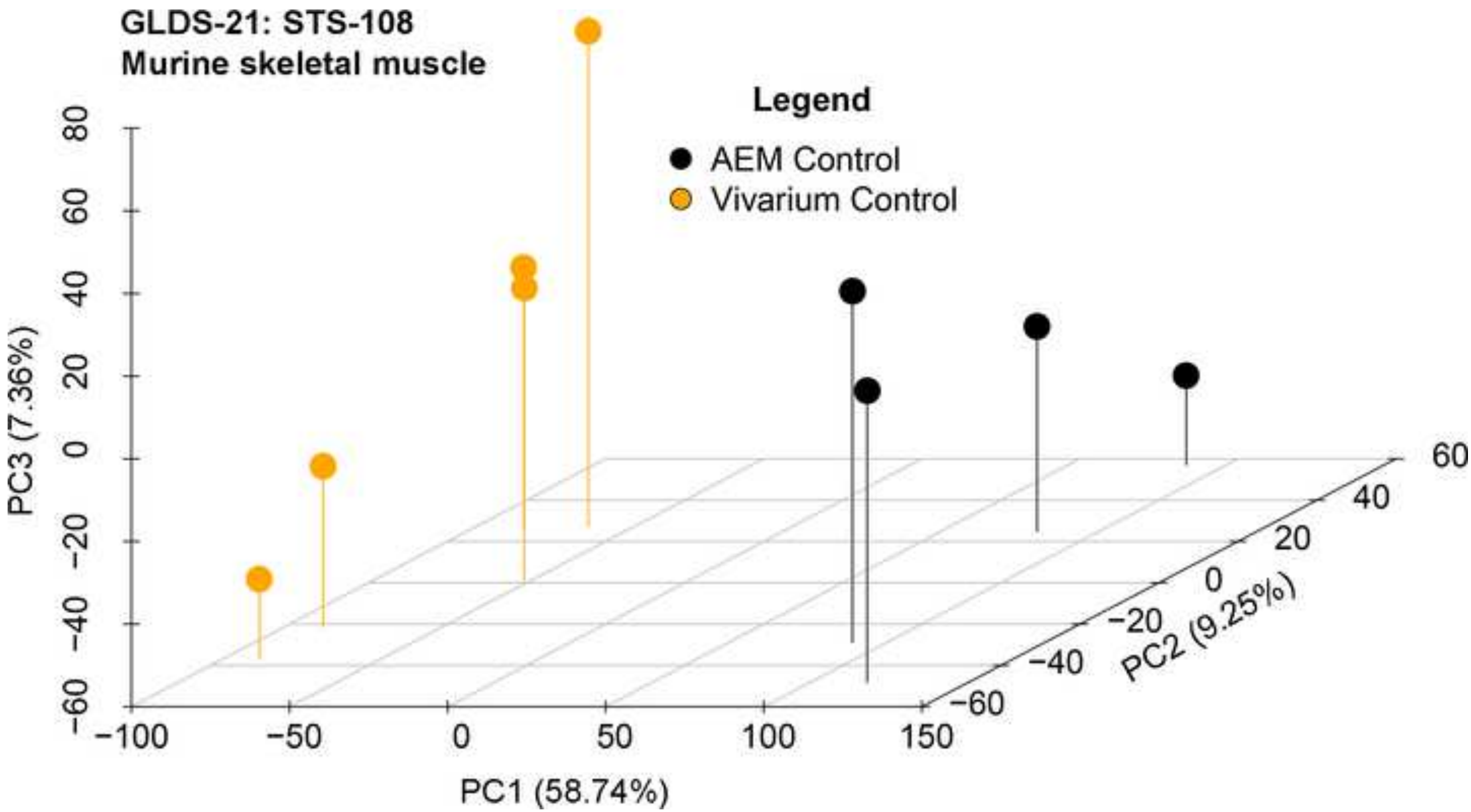

Figure 5

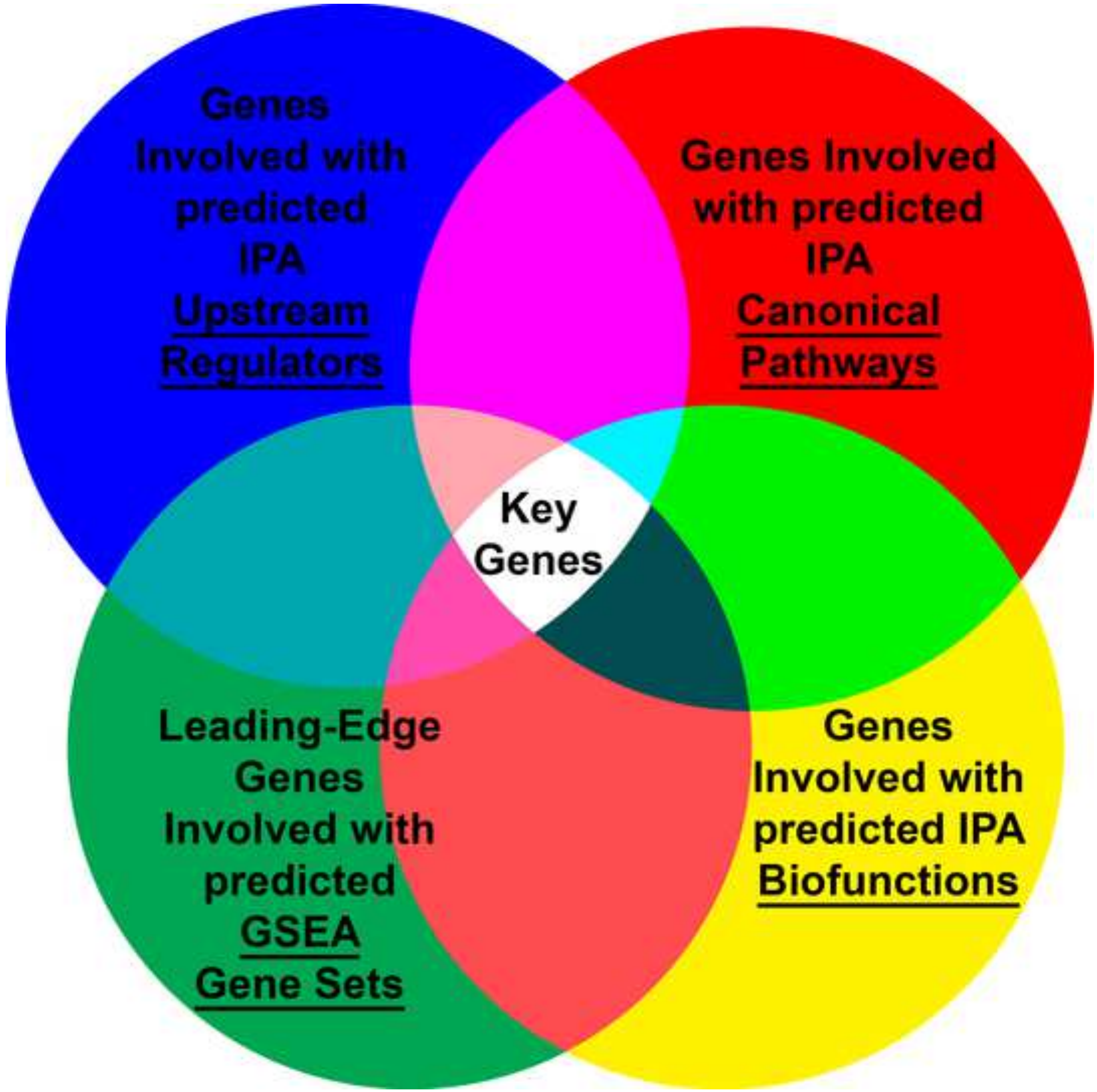
Genes Involved with predicted IPA Upstream Regulators
Genes Involved with predicted IPA Canonical Pathways
Key Genes
Leading-Edge Genes Involved with predicted GSEA Gene Sets
Genes Involved with predicted IPA Biofunctions

Figure 6

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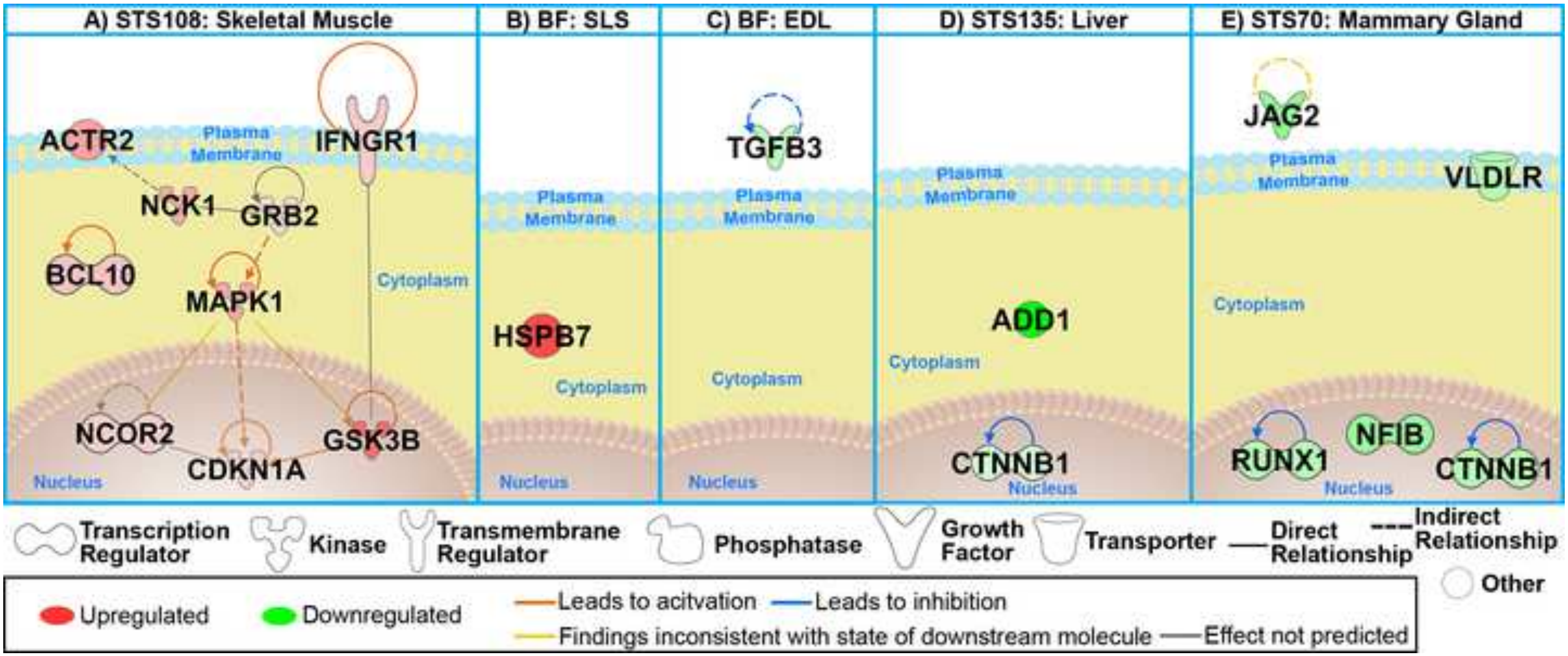

Figure 7

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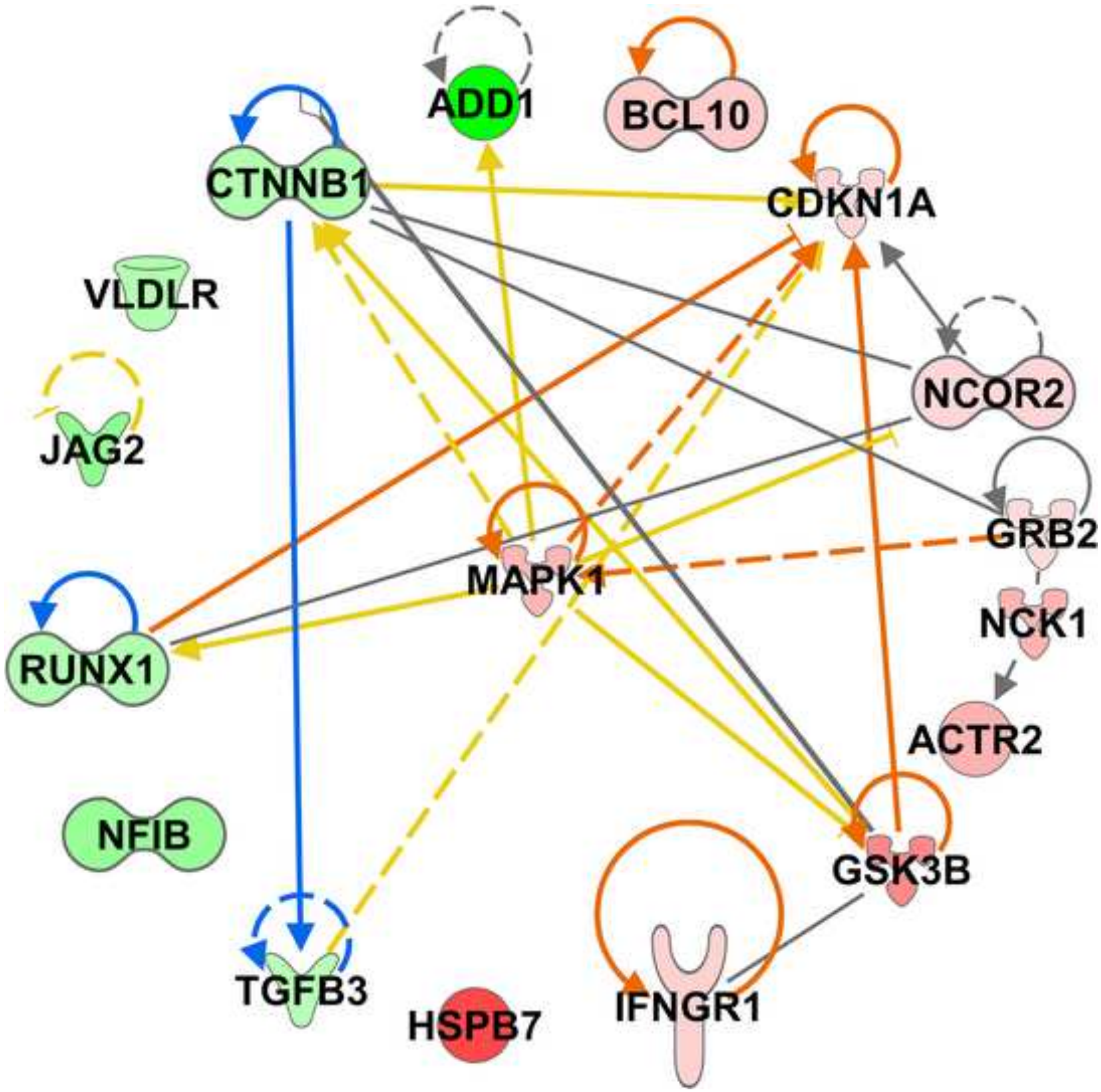

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
C57BL/6 Mice	The Jackson Laboratoy	C57BL/6J	C57BL/6 mice were used for datasets related to
BALB/C Mice	Taconic	BALB	BALB/C mice were used for datasets related to R
Vivarium Cages	Charles River Laboratory		Standard murine cages purchased from Charles I
Rodent Habitat	NASA		This cage and all components are built internally
RNAlater	ThermoFisher Scientific	AM7020	RNAlater is used to store the tissue for further R

Rodent Research-1 experiments
:odent Research-3 experiments
River Laboratory
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A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Afshin Beheshti
Department:	Space Biosciences Division
Institution:	Wyle Labs, NASA Ames Research Center
Article Title:	Exploring the Effects of Spaceflight on Mouse Physiology using the Open Access NASA GeneLab Platform
Signature:	
Date:	05/09/2018

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Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

Dear JoVE,

We are looking forward for this opportunity to have our manuscript published in your journal. We believe that the comments provided by the editorial staff and also the reviewers have greatly strengthened the manuscript. We thank both the editors and reviewers for their comments. Below we have provided the original comments that were provided to us and below each comment/suggestion we have placed our response in red text. We look forward to hearing back from you and hopefully moving to the next step of this manuscript.

Please don't hesitate to contact me for any additional information.

Thank you,

Afshin Beheshti, PhD

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have gone through the manuscript and thoroughly made edits from our proofreading.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Figures 1 and 6 were modified from figures originally from our publication: Beheshti et al, Scientific Reports, 2018 (reference # 35 in the manuscript). According the Scientific Reports and their licensing agreement and copyrights, users are free to share modify the figures in the publication. Here are the links of their editorial policy addressing this:

https://www.nature.com/srep/journal-policies/editorial-policies#license-agreement
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3. Figure 3: Please provide a figure with higher resolution if possible.

We have provided a higher resolution image for figure 3.

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

4. Please provide an email address for each author.

We have provided email addresses for all authors.

5. Abstract: Please do not include references here.

We have removed the reference from the abstract.

6. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We have added sentence at the beginning of the introduction to provide a clear goal of the methods for this manuscript. The following was added:

"The overall goal of this manuscript is to provide a clear methodology of how to use NASA's GeneLab platform and how rodent experiments done in space is translated to data to be analyzed in GeneLab."

In addition, the last paragraph of the introduction also discusses the goal of the manuscript.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have gone through the protocols text and removed all personal pronouns.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We adjusted the numbering of the protocol to follow JoVE instructions. We also removed all indentations.

9. Lines 97-113: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have modified this section to be discrete steps.

10. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be"

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We have revised the protocol section so no imperative tense is present.

11. For computational steps, please provide software screenshots as supplementary files to match each step.

We have provided additional supplemental figures from throughout the computation steps.

12. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have provided single-line spaces between all paragraphs, headings, and steps.

13. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted in yellow the 2.75 pages that should be filmed.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

The highlighted steps match the guidelines listed above.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have edited the discussion and added 2 more paragraphs that include the critical steps of our protocol, how potential modifications can be done to the any of the steps, any limitations the technique, significance, and future applications. We believe that these additions to the discussion have provided for an overall stronger manuscript.

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

16. References: Please do not abbreviate journal titles.

We revised the references so the journal titles will not be abbreviated.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The NASA GeneLab platform provides access to omics data from biological spaceflight experiments. The authors described how a typical mouse experiment is conducted in space and how data from such experiments can be accessed and analyzed using GeneLab.

Major Concerns:
none

Minor Concerns:
The manuscript, although complete and very clear, presents the need for a minor revision oriented as suggested below:
1. There are several typos: it is suggested to reread the whole manuscript carefully to correct them.

We thank the reviewer for this comment and have gone through the manuscript thoroughly to correct for the typos.

2. Rows 52-55: please include also refs to bone studies (example but not exclusive: Giuliani A, S, Ruggiu A, Canciani B, Cancedda R, Tavella S. (2018) High-Resolution X-Ray Tomography: A 3D Exploration Into the Skeletal Architecture in Mouse Models Submitted to Microgravity Constraints. Frontiers in Physiology. 2018;9:181. doi:10.3389/fphys.2018.00181.)

We have added the reference the reviewer has suggested.

3. Rows 55-58: Please include and discuss also on the MIS habitat. Ref. Blottner D, Serradj N, Salanova M, et al. (2009) Morphological, physiological and behavioural evaluation of a 'Mice in Space' housing system. J Comp Physiol B. 2009 May;179(4):519-33. doi: 10.1007/s00360-008-0330-4.

We have added the reference the reviewer has suggested.

4. Rows 290-291: "Upload the list of genes with fold-change values for the statistically significant genes determined in step 3.3.". Please verify if you really referred to step 3.3.

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

We thank the reviewer for catching this mistake. We have updated this step to refer to step 9.4.4.

Reviewer #2:

Manuscript Summary:

The manuscript gives an example of how to use Genelab to analyze data from spaceflight. The strength of the article is the thorough explanation on how to use Genelab. The use of the specific example of vivarium versus rodent habitats makes this explanation of Genelab clear and interesting.

Minor Concerns:

The article aims to accomplish two goals: spread the word on how to do spaceflights experiments using rodents, and describe to new users how to use Genelab to make new discoveries. The article works well for these two objectives for the initiated user, i.e. scientists who have a passing or intimate familiarity with doing science in space. However for scientists new to space science, I recommend that the authors add text to circa line 108 , and possibly to the section 'Representative Results' to expand on the crucial importance of ground controls for space research, and the importance of continuously mirroring as many environmental factors as possible in the ground controls.

We thank the reviewer for the above the comment. This is a very good point and we have added the following text in the sections the reviewer suggested:

This text was inserted after what was formerly line 108 (currently line 134):
"1.3.1 Note: It is important to note that ground controls are essential for spaceflight rodent experiments. As described in this protocol, these controls are done with both identical conditions (i.e. CO_2 conditions, humidity, temperature, cage dimensions, etc.) in the Rodent Habitats and also in normal vivarium cages that have standard environmental conditions on Earth. The rodents housed in the Rodent Habitat ground controls allow for direct comparison to rodents in space. While rodents housed in vivarium cages allow for biological comparison between the different environmental conditions between habitats."

We also added in the 'Representative Results' section the following as the reviewer has suggested (starting from line 502):

"For this study we primarily focused on the biological differences that occur in rodents housed in the Rodent Habits ground controls and the vivarium controls. As described above, it is key to have better understanding of these two habitats, which will provide us information on possible confounding factors that can impact health due to the environment on the ISS. For all rodent spaceflight experiments these ground controls are also essential to determine which biological factors are associated directly with spaceflight or due to the environmental conditions on the ISS."

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

My second recommendation is then to add text to the section 'Representative results' that explains clearly that the data that was analyzed was from the two ground controls, i.e. Rodent Habitat control and the vivarium control. Also, I assume that the vivarium control was not exposed to elevated CO2. But were other conditions (e.g. temp and humidity) identical? This needs to be made crystal clear.

We have added the following starting on line 509 to address the above comment and make it clear that only the CO_2 is different between the Rodent Habitat and vivarium control:

"The vivarium habitat has the normal CO2 level that is present on Earth (currently being ~300 to 380ppm). The temperature and humidity for both habitats are similar."

There is a risk, because of the detail give to protocols for doing animal experiments in space that readers (viewers of the video) will assume that spaceflight data were analyzed in the example. Because of this potential for confusion, it might be worth considering changing the structure to the following order: 1) importance of doing research on spaceflight risks; 2) example of rodent research and importance of good ground controls; 3) example of vivarium data compared to rodent habitat data using genelab; 4)details on how to use Genelab to do this kind of analysis; 5) detailed protocol for doing rodent research in flight.

We have added the following at the end of the introduction (line 103) to clarify the data we present as an example for the analysis will be specifically related to differences between the Rodent Habitat and vivarium controls:

"The specific example we will present on how this protocol is implemented will be comparing the biological differences occurring in rodents housed in Rodent Habitat and the vivarium controls that was published by Beheshti et al.[35]"

We have also reorganized the 'Representative Results' as the reviewer has suggested. The text that we have added to address the above comments are incorporated and organized into this suggestion.

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

Dear Dr. Bajaj,

We have gone over the manuscript and have made revisions based on your recommendations. The uploaded manuscript, labelled "58447_R1_RE_AB.docx", includes all track changes and comments to provide for an easier way to track my changes. Below I have provided the editorial original comments and below each comment I have provided a response to address the comments in red. Please don't hesitate to contact me if you need anything else and we hope we have addressed all the comments.
Thank you,

Please don't hesitate to contact me for any additional information.

Thank you,

Afshin Beheshti, PhD

Editorial comments:

1. The editor has formatted the manuscript as per the journal's style. Please retain the same.

We thank the editor for the formatting and all the have made all the new changes in the new version of the document.

2. Please address all the specific comments marked in the manuscript.

We have addressed all the comments with either the revisions that were made in the manuscript or provided a response below each comment.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, please remove the term GeneLab from your manuscript. The term may be introduced and used 2-3 times max but not a lot.

I had contacted you regarding this issue through email. In the email I had stated the following:

"GeneLab is a tool provided by NASA that will assist people to generate hypothesis related to space biology. It is not a product, but a way for people to analyze omics data related to space research. Like any other protocol the analysis for space biology related experiments using this method cannot be written with only mentioning GeneLab 2 or 3 times in the paper. Originally when Jaydev Upponi (who I have CC'd with this email) had contacted me with an invitation to

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

submit a paper to JoVE we had discussed what the paper should be about. I stated that we would like to have the majority of the paper be about GeneLab and how to analyze data using GeneLab and the beginning of the paper about how the rodent experiments are done in space. So basically a paper showing how omic data is generated for GeneLab to how data will be analyzed and a hypothesis can be generated using GeneLab."

Based on the above comments I have tried to reduce the instances which we state GeneLab and I hope that this will be enough reduction of the usage of GeneLab to address this comment.

4. For the protocol section, please use one specific experiment for which the results are shown and explain how you do the experiment using the GeneLab platform.

I have placed references in the protocol section to the experiment we discuss in the results section that is related to the original publication which I was contacted about to publish in JoVE.

5. Please use imperative tense throughout the numbered step as if directing someone how to do your experiment using specific graphical user interface.

We have gone through the manuscript and revised the manuscript with imperative tense throughout the numbering steps of the protocol.

6. You may use note wherever applicable but please use notes sparingly.

I have added a few more notes sections, but overall this was used sparingly.

7. We cannot have paragraphs of text in the protocol section.

I have removed the paragraphs of text in the protocol section.

8. Once all the formatting is done, please ensure that the protocol is no more than 10 pages and highlight for the video is no more than 2.75 pages including heading and spacings. These are our hard cut limit for protocol and video respectively.

The protocol for filming is highlighted in yellow and is at 2.75 pages from my estimate.

9. Section 5 of the protocol can be converted to a supplementary file.

I have revised section 5 to be more in line with the protocol using the imperative tense. I believe that this is more in line with the protocol and would hopefully fit in the text. If you still believe that this should be as a supplementary file, please let me know and I will include it as you have recommended.

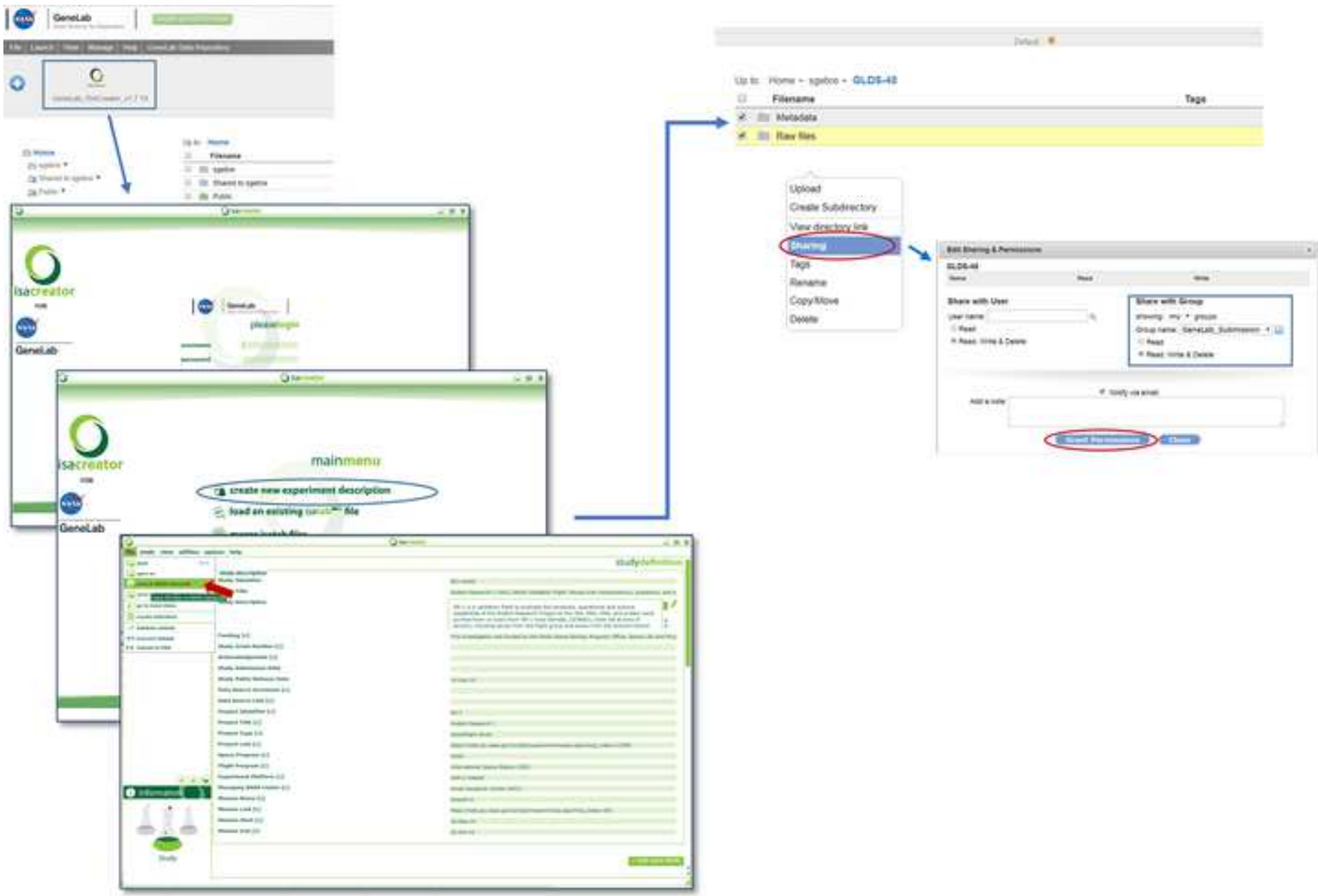
10. Please also remove all the web links and put it in the reference section. These can be referenced using in-text citations.

Afshin Beheshti, PhD
Assistant Professor
Bioinformatician and Data Analyst
Phone: 617-308-2540

KBR Wyle Labs, Space Biosciences Division
NASA Ames Research Center
Moffett Field, Mountain View, CA 94035
e-mail: afshin.beheshti@nasa.gov

I have removed all web links and put it in the reference section. These references are now cited in the text.

Supplemental Figure 1



Supplemental Figure 2

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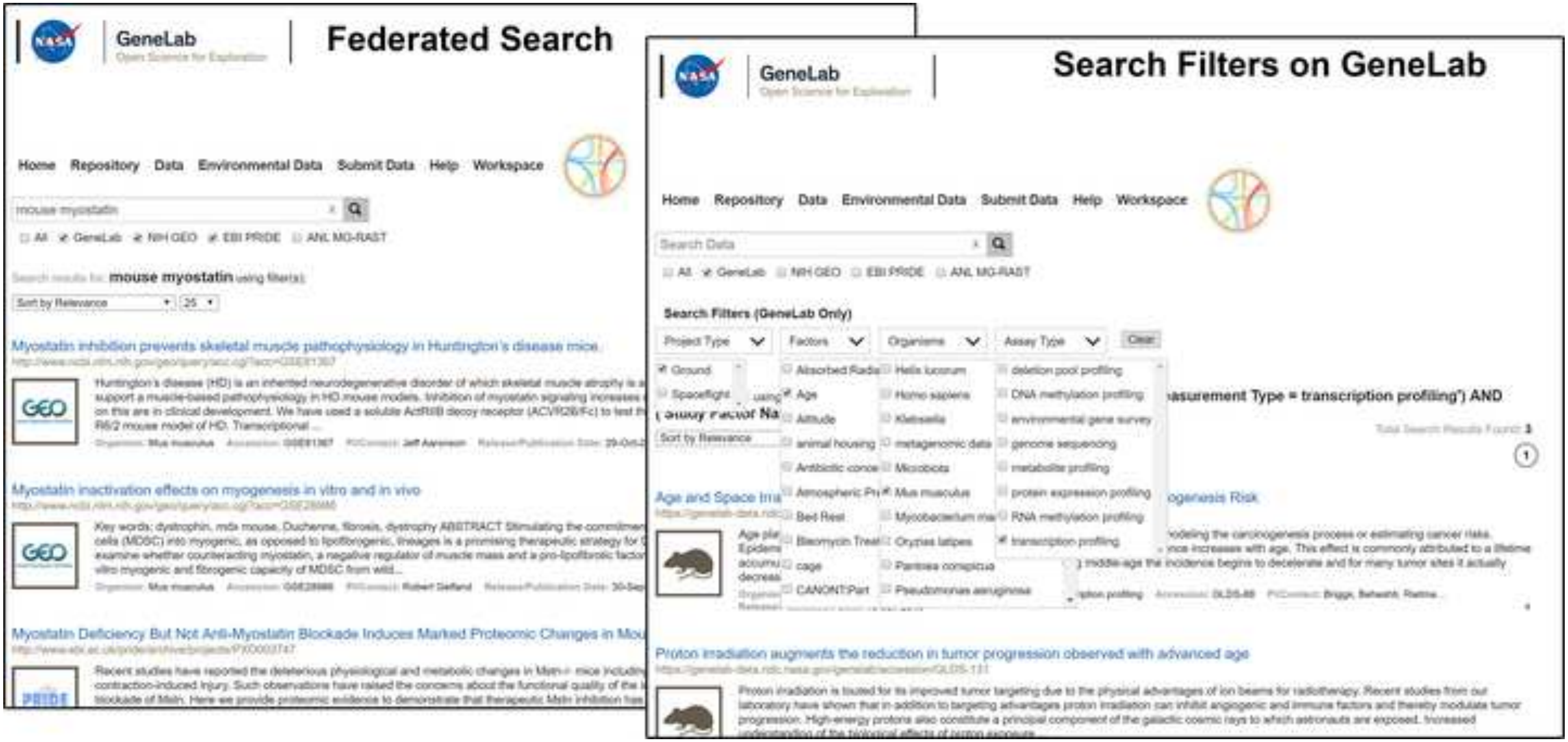

Supplemental Figure 3