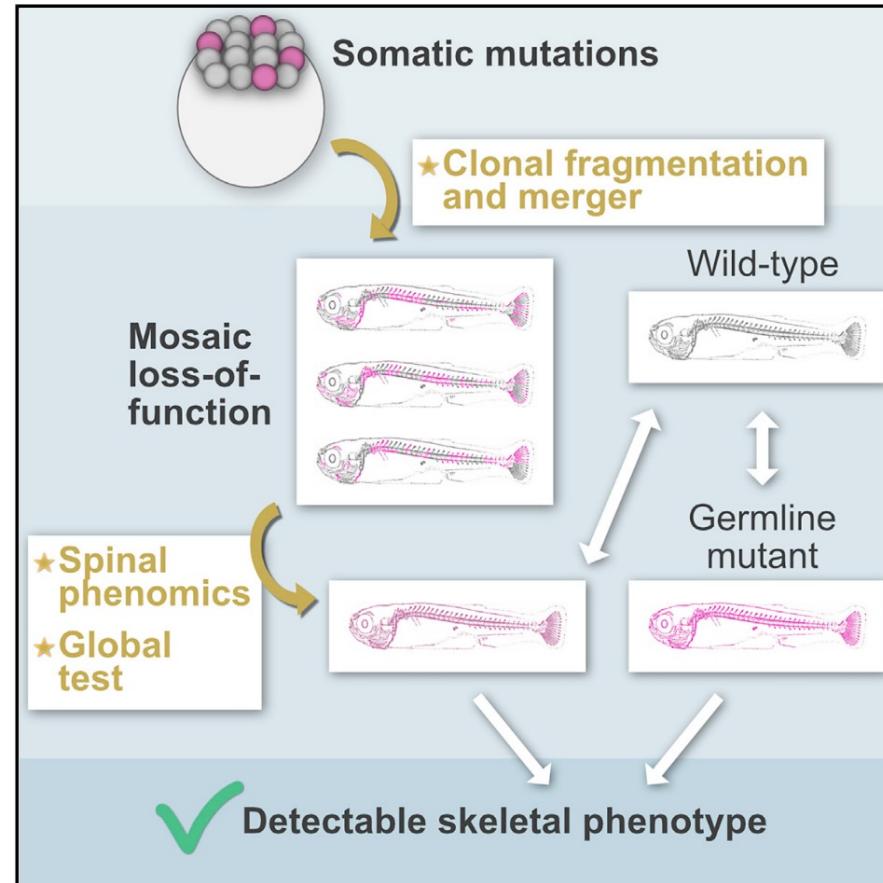




Putting it all together
Using 3D Slicer & SlicerMorph to quantify
phenotypic variation

Quantifying genotype to phenotype

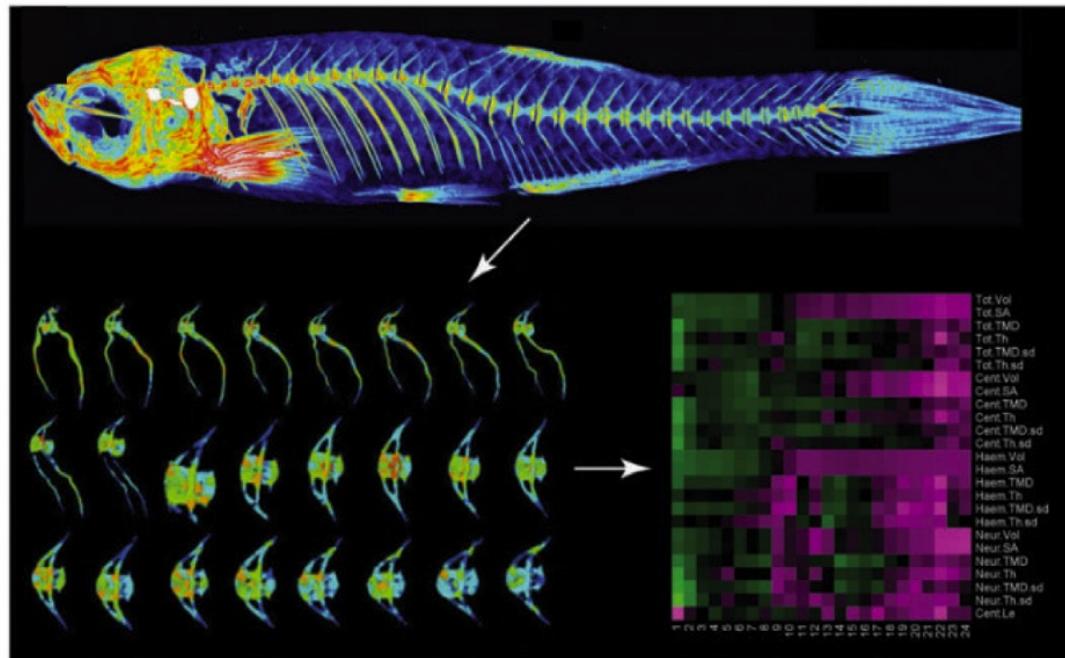
One way to quantify relationships between genotype and phenotype is to compare the phenotype of known genetic mutants to their wildtype siblings



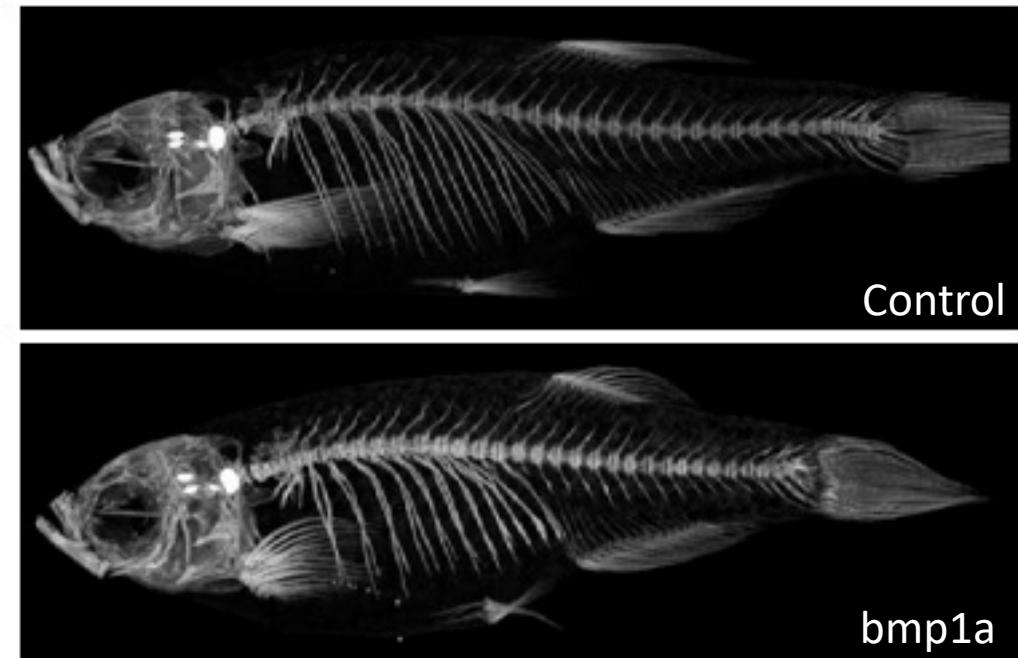
Watson et al., 2020 Cell Systems

MSB Lab developed an approach for decoding spatially variable phenotypes of the axial skeleton

Mutation in *bmp1a* results in brittle bones and shortened axial skeleton



Hur et al., 2017 eLife

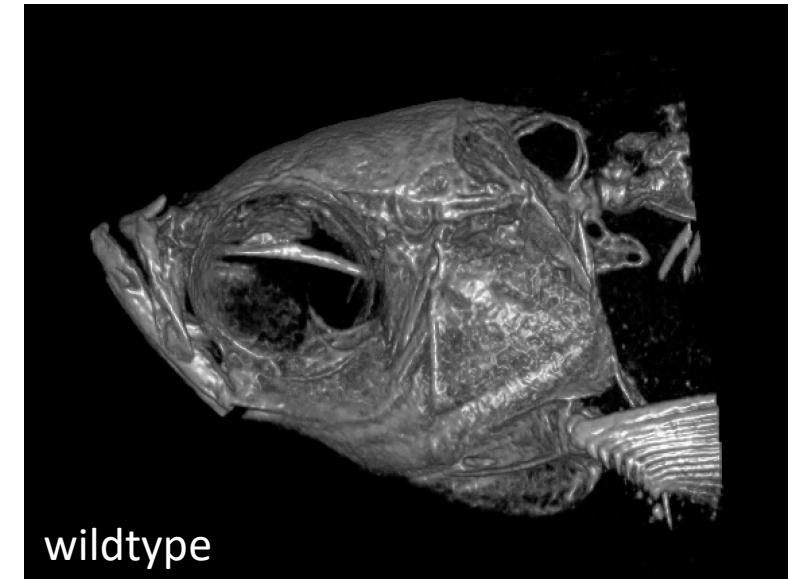
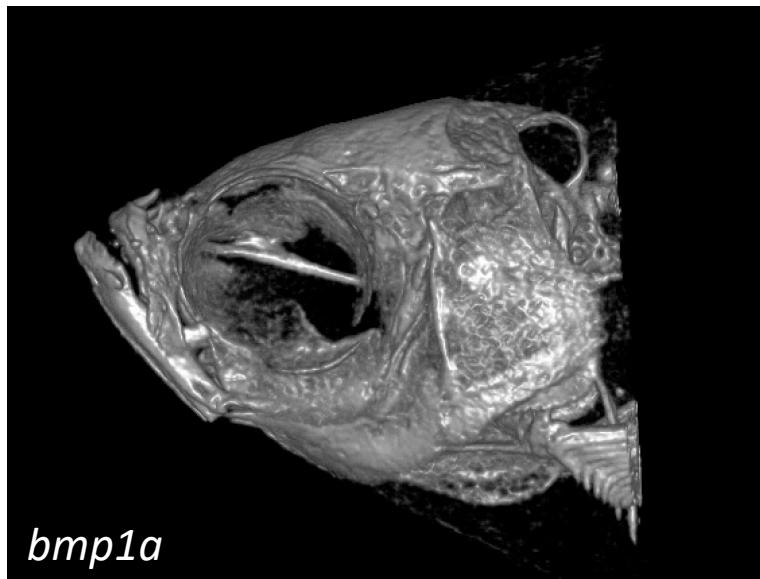


Watson et al., 2020 Cell Systems

Fish skulls are a bit more complicated

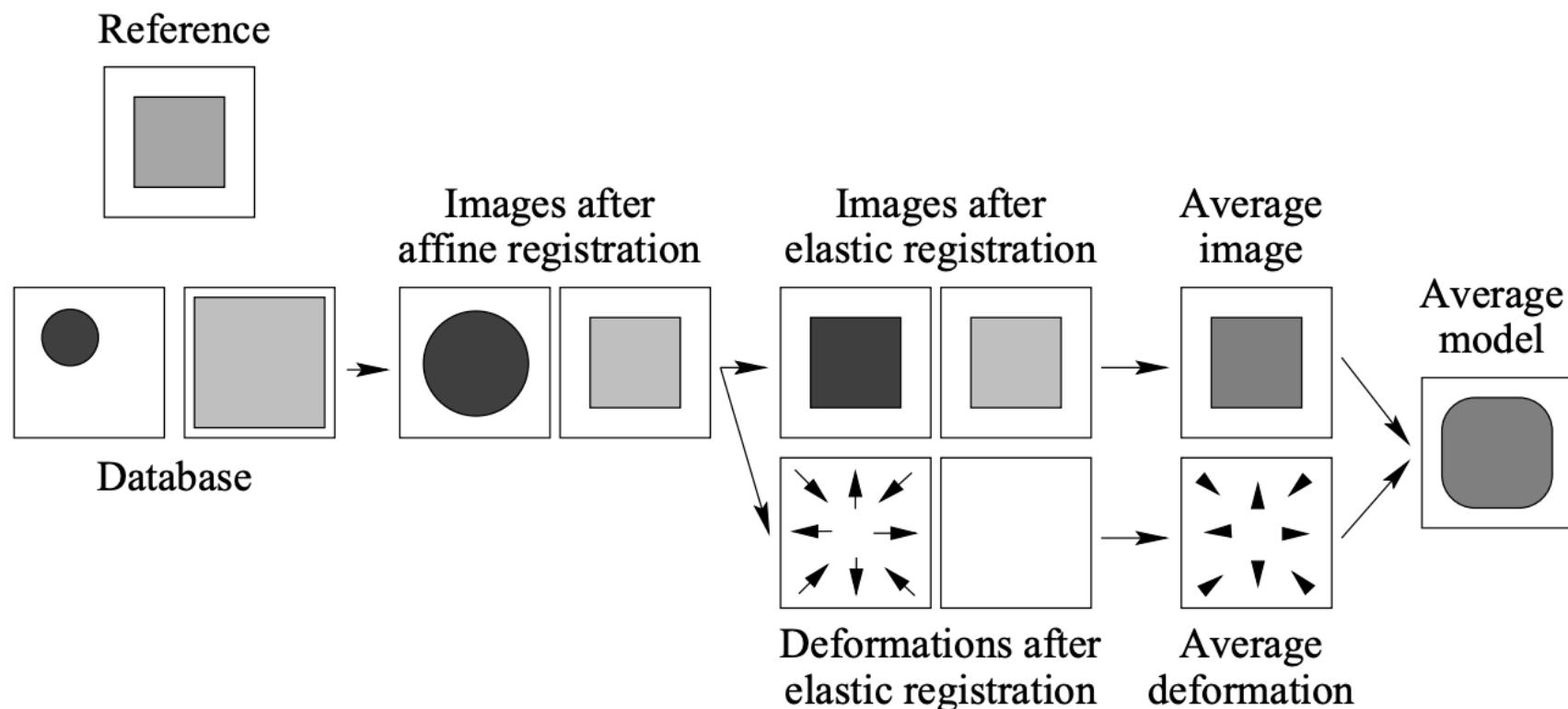
Goal: Quantify phenotype of cranial skeleton

Starting data:
microCT scans of
bmp1a and their
wildtype siblings



Atlas building

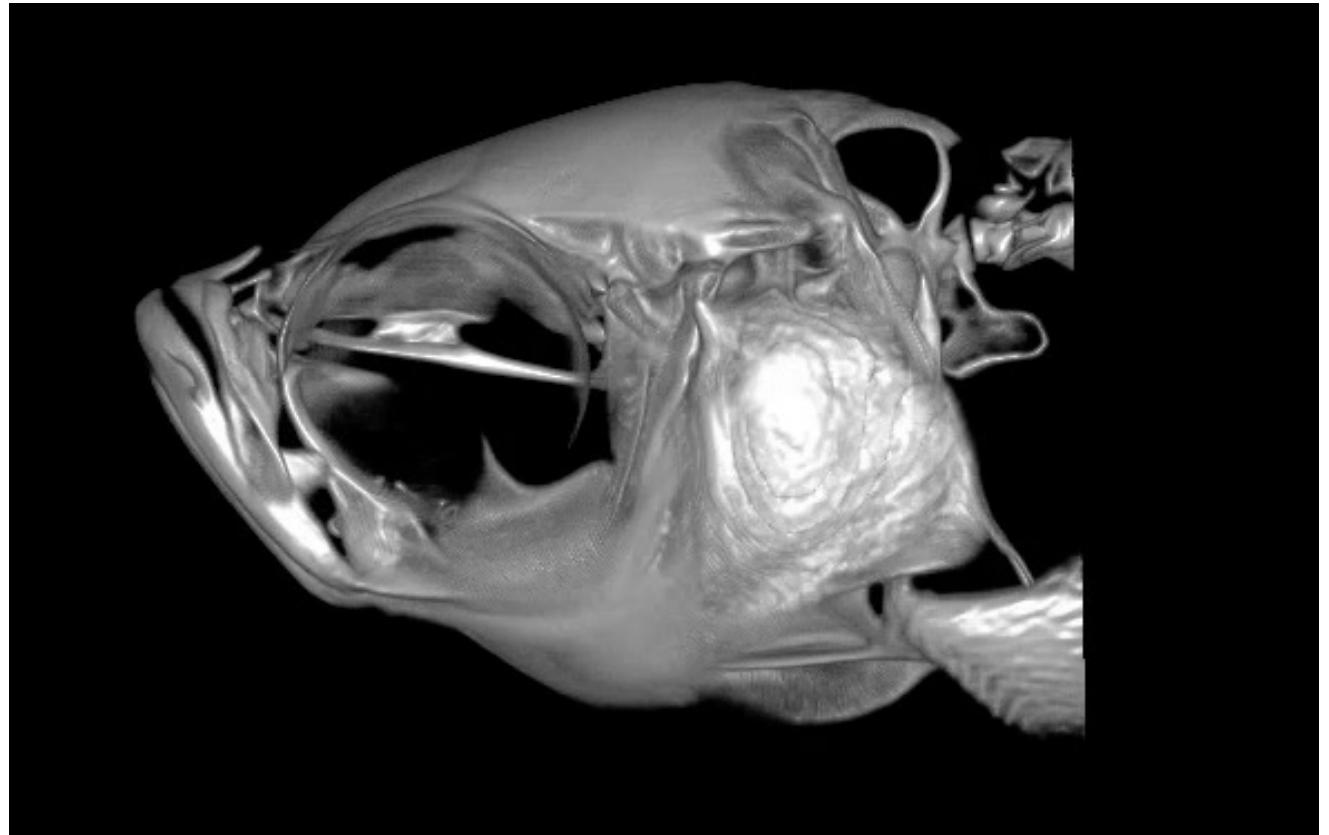
The atlas construction framework iteratively finds a virtual space which resides in the centroid of the population (i.e. deformations needed to transform all subjects into this virtual space sum to zero everywhere in this virtual space)



Atlas building

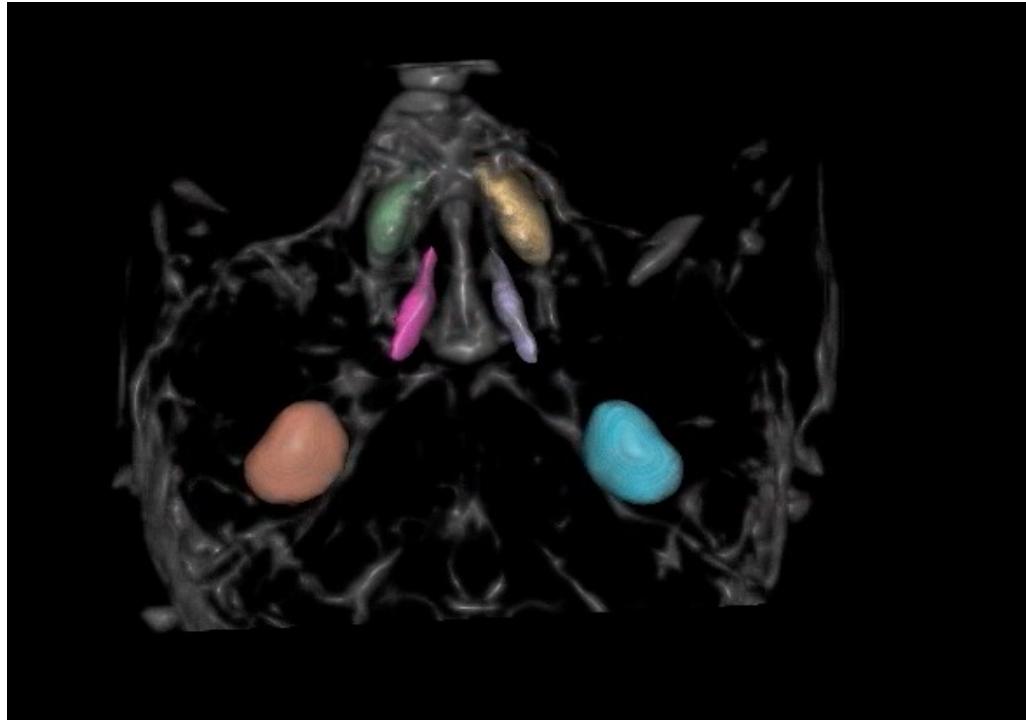
Built atlas using a process of
deformable image registration
([ANTs](#))

Only wildtype fish were used to
build our atlas

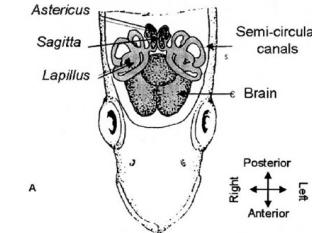
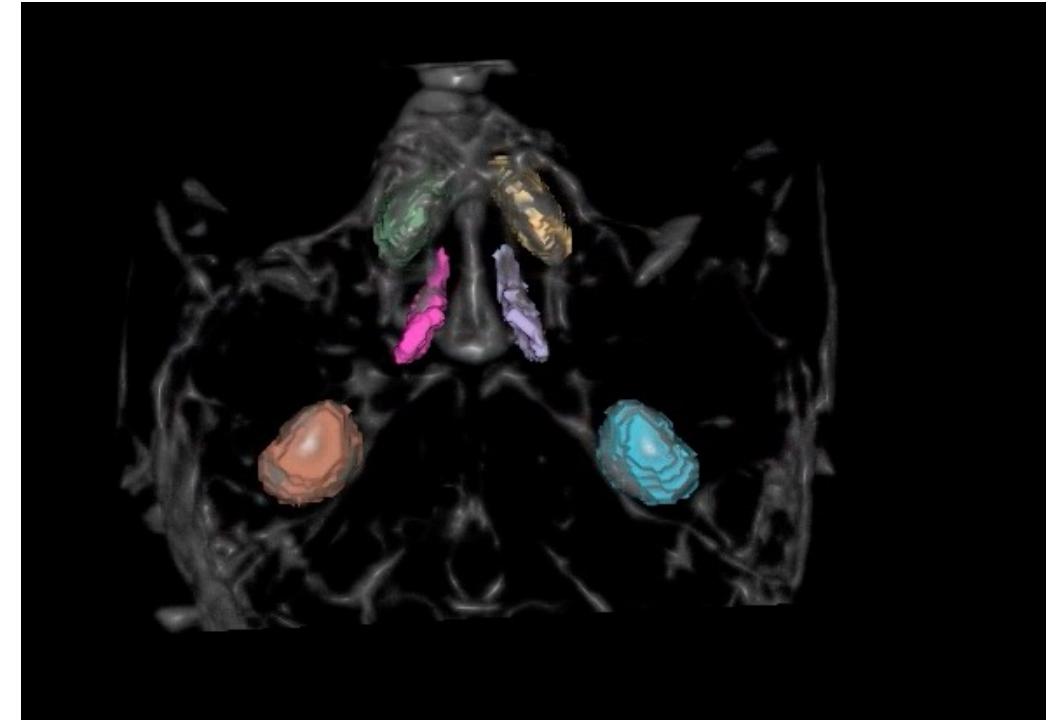


Testing the atlas

Manually segmented out the otoliths from each microCT scan using Segment Editor



Used ANTsR to segment the otoliths using the atlas as a template

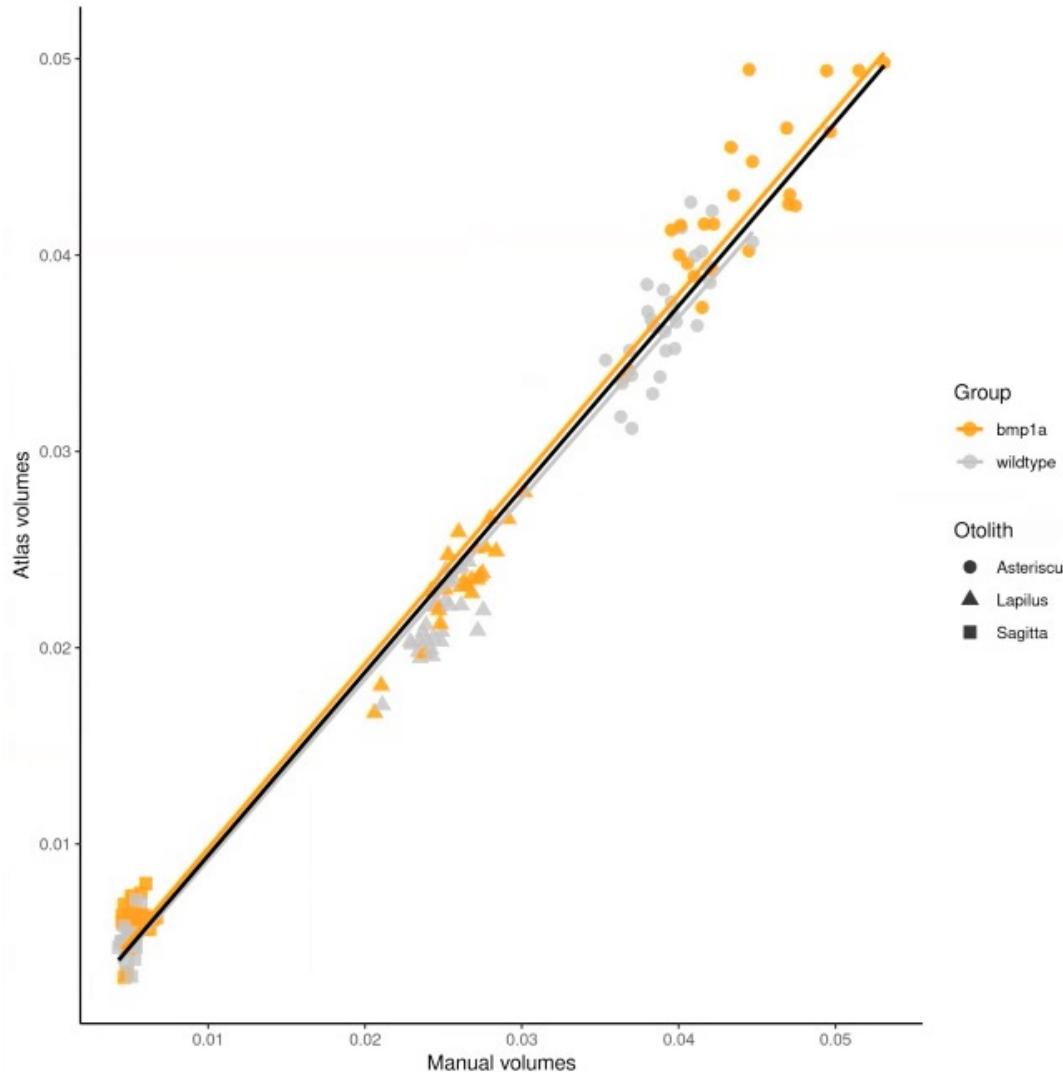


Payan et al., 2004

Testing the atlas

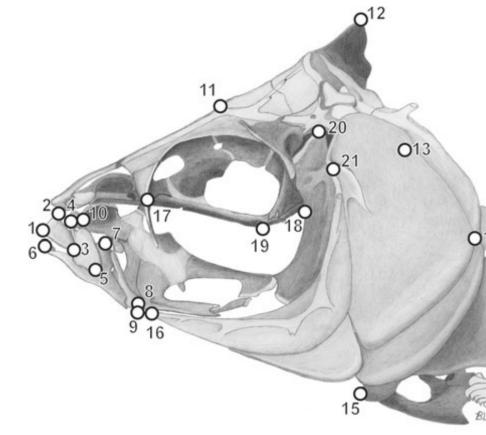
**Overall current atlas does a
decent job of segmenting
otoliths**

Model:
 $\text{Atlas volume} \sim \text{Manual volume}$
 $r = 0.991$

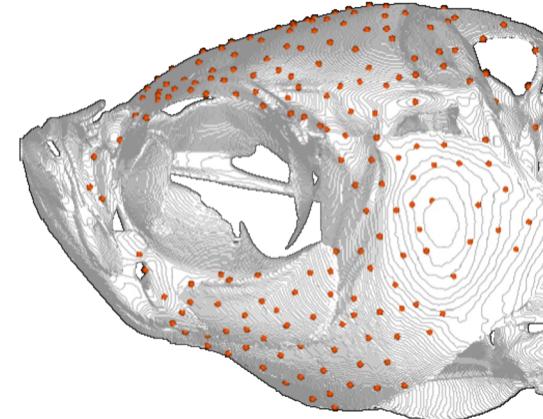


Using the atlas to quantify phenotype

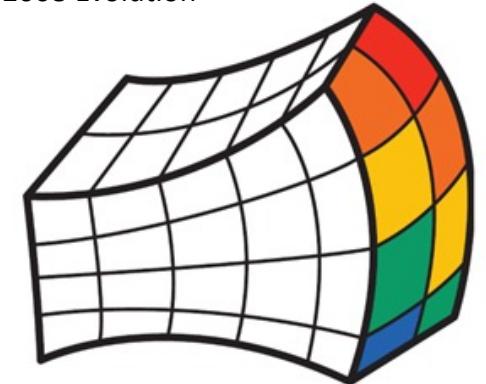
- Traditional ways to quantify cranial morphology = landmarks
 - We use this method for ground truthing our dataset
- Limitations: These mutants may have phenotypes that are too subtle to be recognized by sparse traditional landmark methods
- **Proposal: Using pseudo-landmarks to quantify skull phenotype**



Sidlauskas, 2008 Evolution

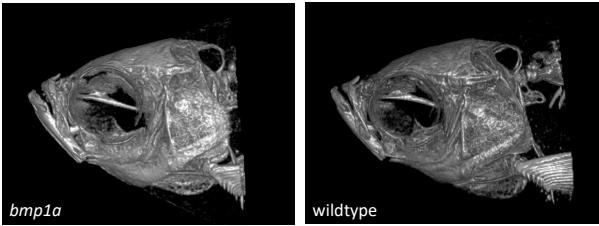


Diamond et al., 2022 Bio Open

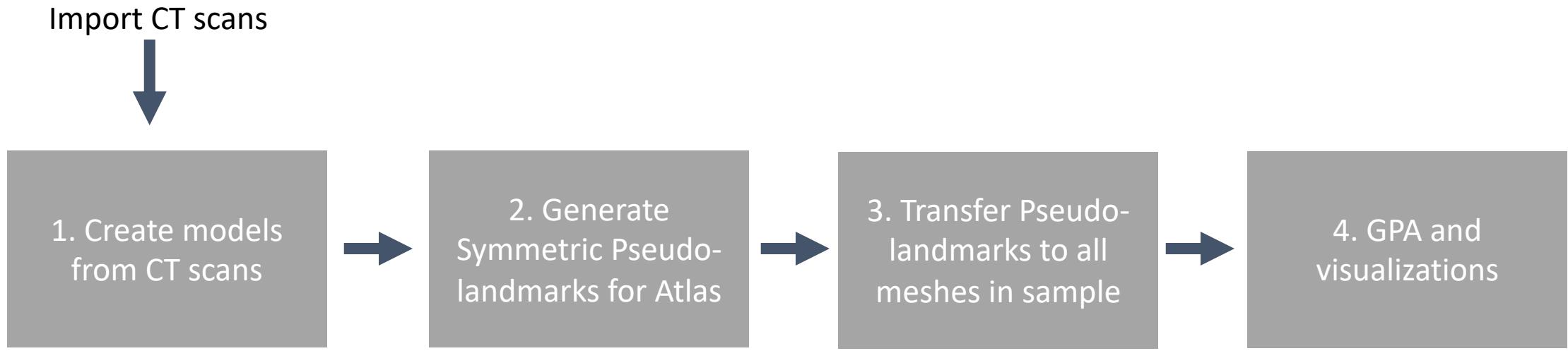


SLICERMORPH

Rolfe et al., 2021 MEE



Slicer analysis pipeline



Segment Editor

Segmentations

Surface Toolbox

Markups

PseudoLMGenerator

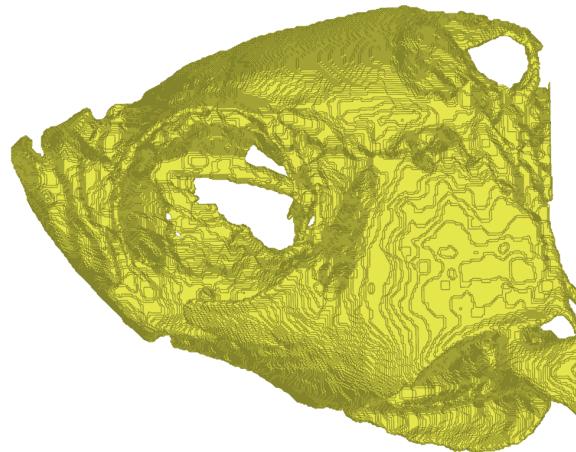
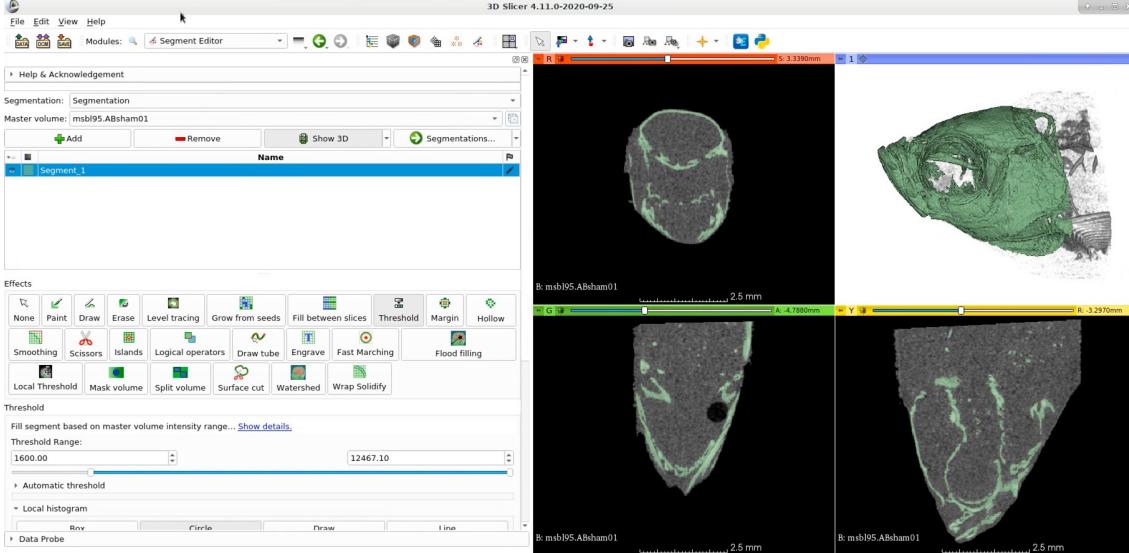
ALPACA

GPA

Output GPA for further analysis
and visualizations in R and
other 3D Slicer modules



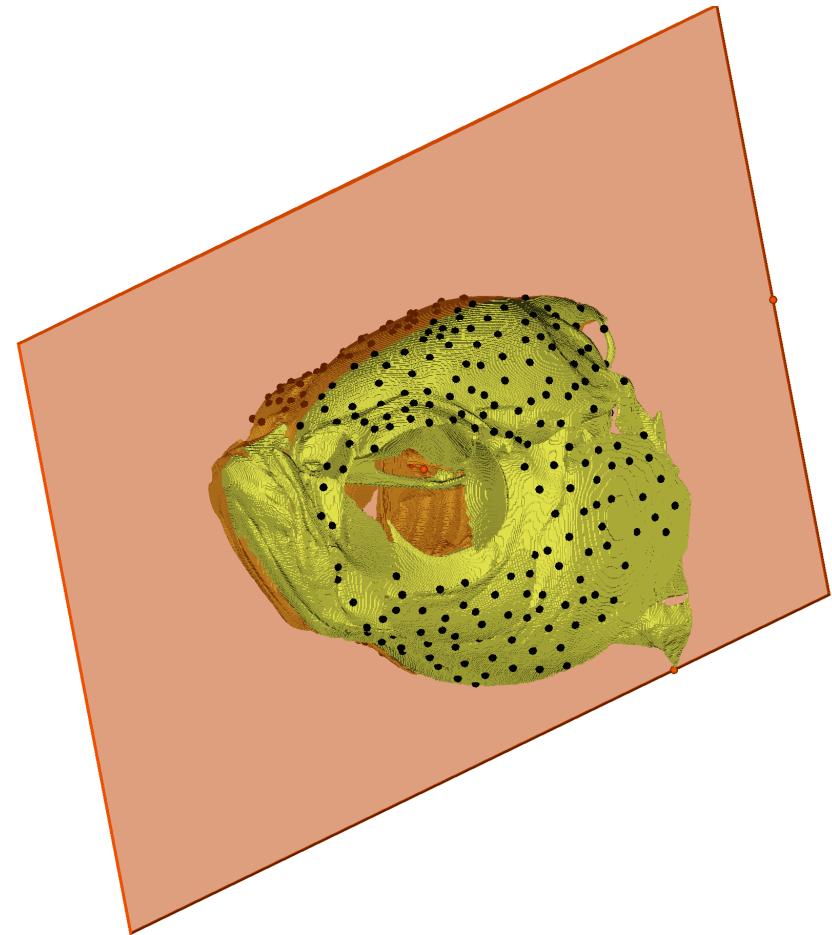
1. Create mesh models from ct volumes



- Used the **Segment Editor** module to create a segment of my ct scans that included only the bone using the tools:
 - Threshold (include only bone)
 - Islands (remove small islands)
 - Scissors (remove postcranial elements)
- Used the **Segmentations** module to create a model from my segmentation
 - Used the **Surface Toolbox** for model smoothing and decimation

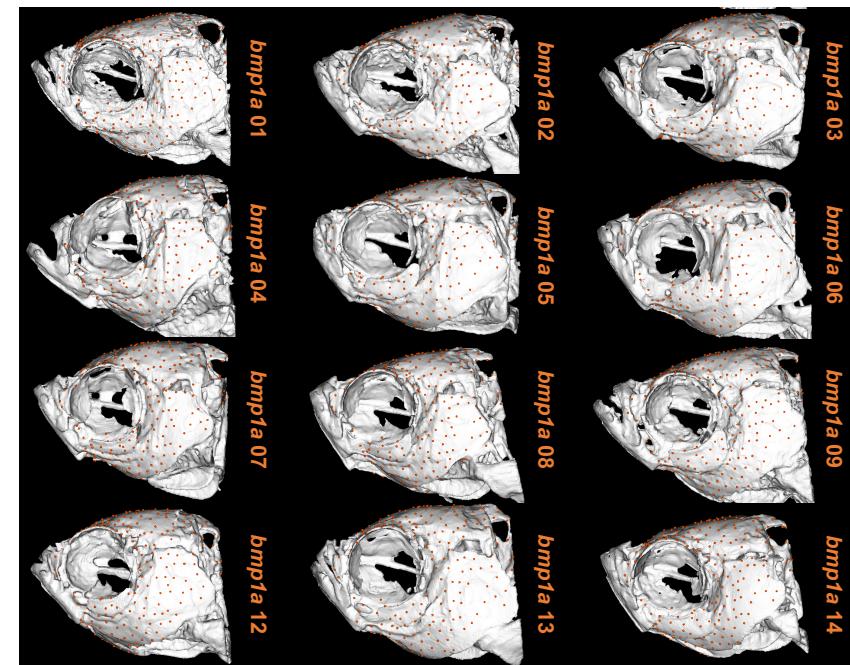
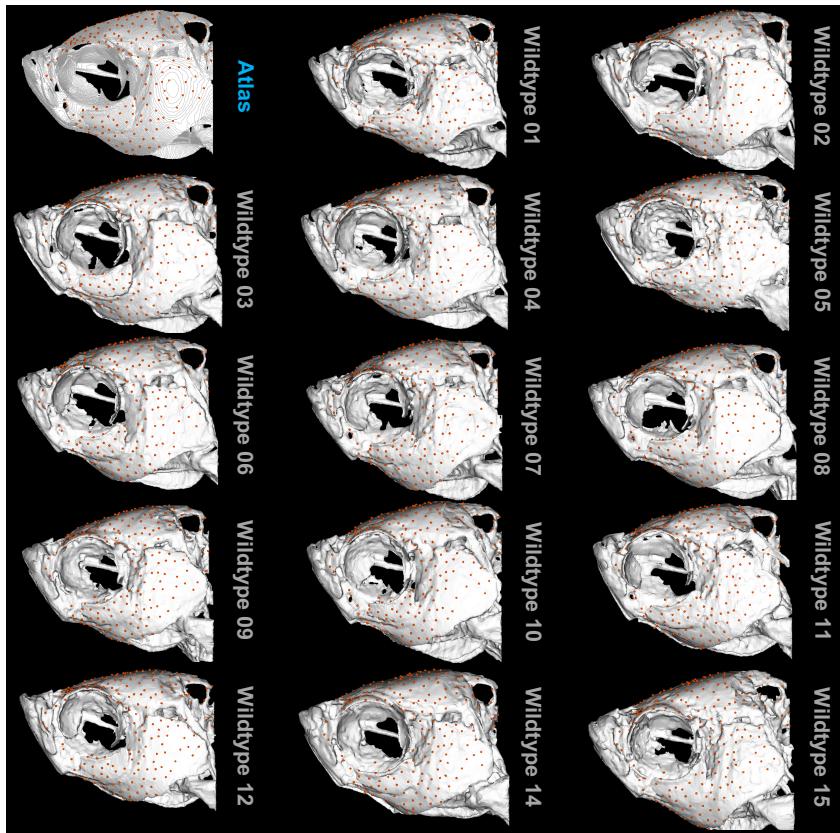
2. Generate symmetrical pseudo-landmarks

- First, I created a plane in the **Markups** module that separated the skull into left and right halves
- Next, I created a set of symmetric pseudo-landmarks using the **PseudoLMGenerator** module
- Finally, I used the **Markups** module to remove points from part of the atlas that I didn't want to include
 - After cleaning up the points there are 372 points on the atlas that I used for analysis



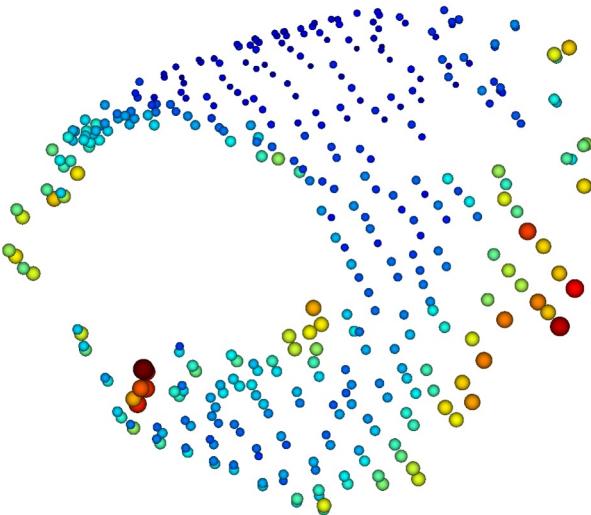
3. Transfer pseudolandmarks to samples

Used the ALPACA module to transfer the 372 pseudo-landmark points from the atlas to every fish in our sample

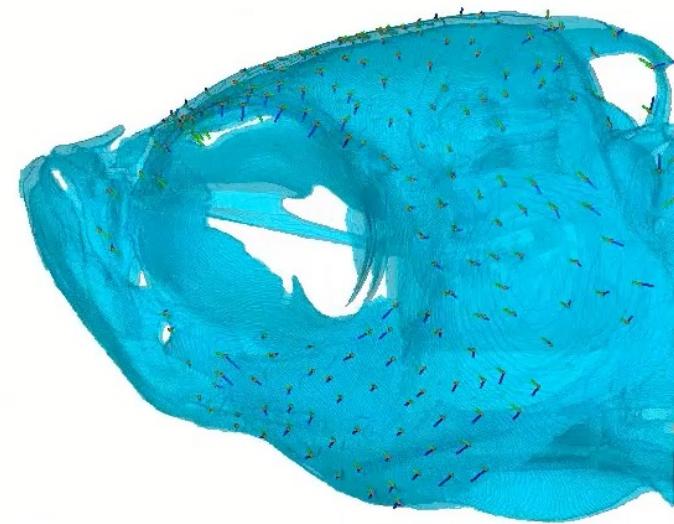


4. Analyzing pseudo-landmark points

Ran a General Procrustes Analysis on pseudo-landmarks and visualized results using the **GPA** module in slicer



Visualizing variation in pLMs
(brighter colors = more variation)



Lollypop vector plot for pLMs



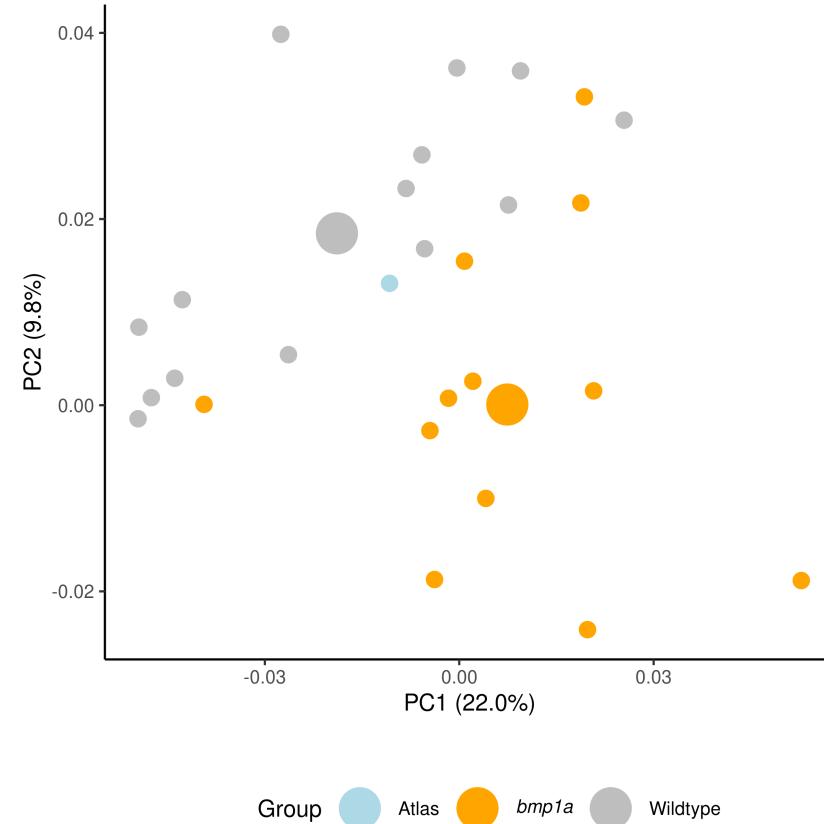
PC plot from GPA analysis
Rolfe et al., 2021 MEE

5. Further data analysis in R

Using the output from the GPA analysis I can do more visualizations and analysis in R

Including:

- Making better plots with *ggplot*
- Testing if PCs statistically vary between *bmp1a* and wildtype fish with *geomorph*
- Symmetry analysis with *morpho*

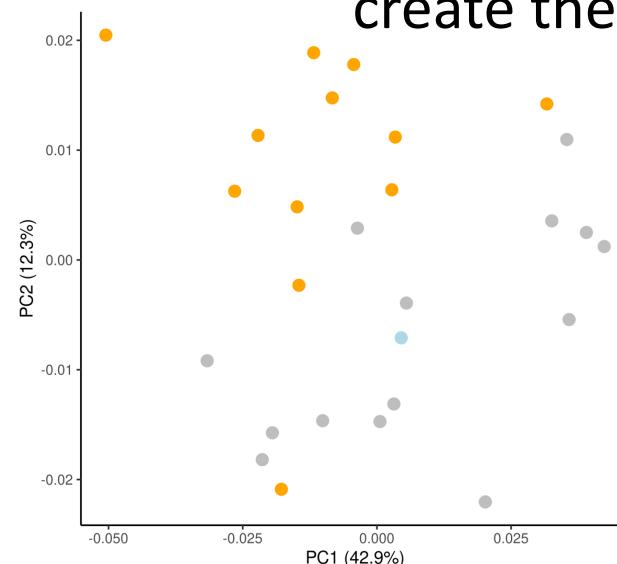
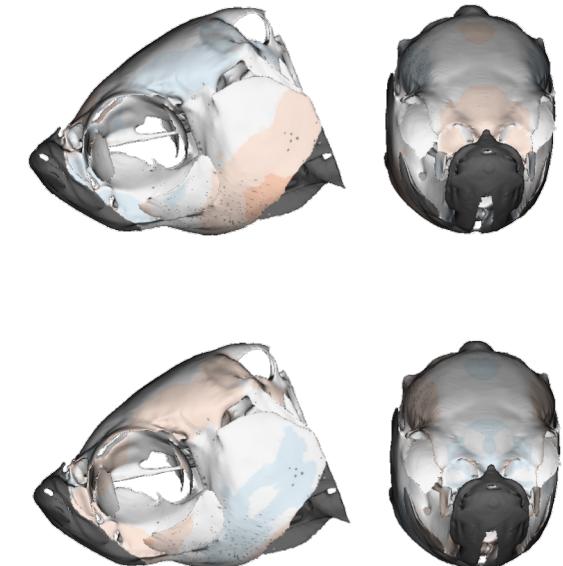


Larger shapes indicate average for each group and method

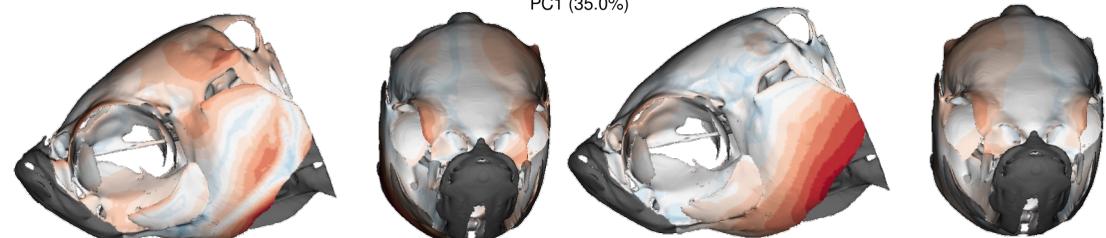
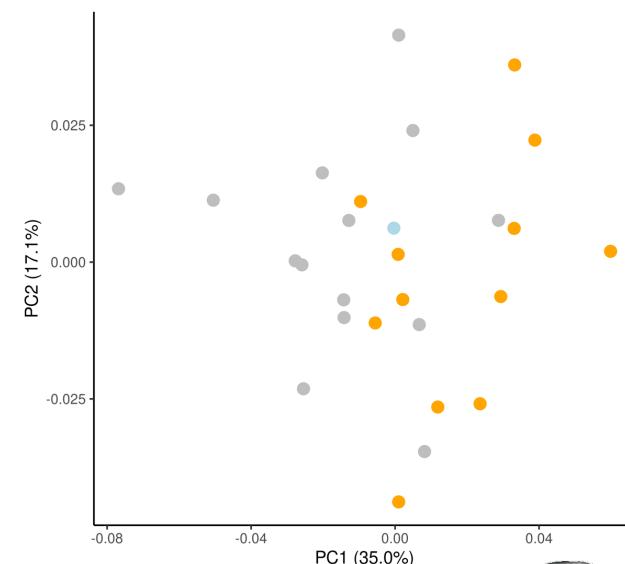
Symmetry visualizations

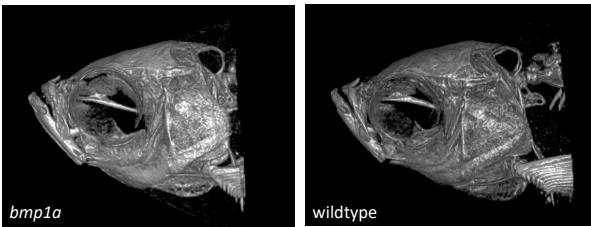
Using morpho in R I created average images of the symmetric and asymmetric components of shape variation. Then used the **Model to Model Distance** module to visualize where the different components of symmetry occur in our morphospace.

Then Loaded all meshes into the same view and used the **Transforms** module to create the visuals below.



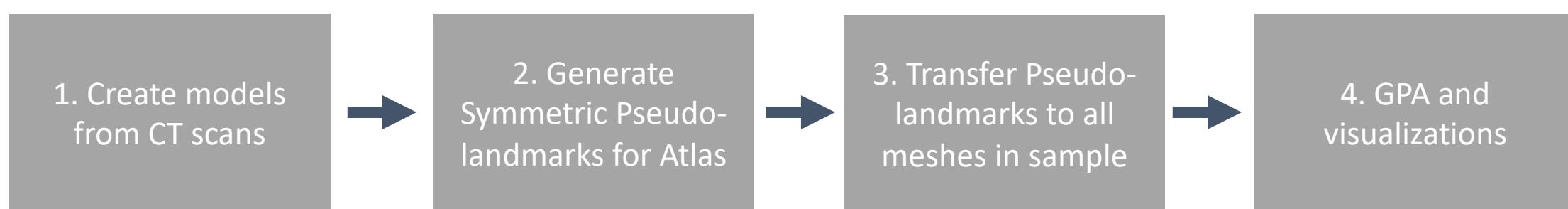
Group ● Atlas ● *bmp1a* ● Wildtype





Slicer analysis pipeline

Import CT scans



Segment Editor
 Segmentations
 Surface Toolbox

Markups
 PseudoLMGenerator

ALPACA

GPA

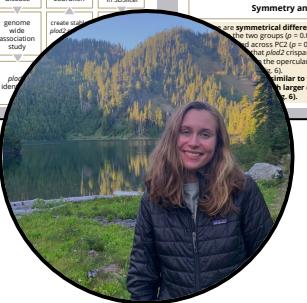
Output GPA for further analysis
and visualizations in R and
other 3D Slicer modules



Scan for link to our
paper!



Applying this pipeline more broadly



Abby



Daanya



Kurtis



Sanford

The effects of *plod2* on zebrafish 3D craniofacial phenotype

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²University of Washington, ³Department of Biology, ⁴Department of Orthopaedics and Sports Medicine, ⁵Institute for Stem Cell and Regenerative Medicine, Department of Pediatrics, University of Washington, Seattle, WA

Background

- Brick syndrome is a rare form of osteogenesis imperfecta, or brittle bone disease, and has been associated with the *PLOD2* gene in human genome-wide association studies.
- Due to advances in 3D phenotyping and CRISPR gene editing, zebrafish have become a great model system to assess gene function relevant to skeletal disease.
- Previous studies have elucidated the phenotypic impact of *plod2* on the axial skeleton (Fig. 1), but not yet in the craniofacial skeleton [2].

Objective

We aimed to identify shape differences between *plod2* mutants and wildtype fish.

Manual landmarking data validates semi-automatic landmarking approach

Discussion

Both manual and semi-automated landmark data sets show that *plod2* crispant fish have shorter, more robust skulls than control fish, providing support for our previous findings and the validity of our pseudolandmark approach.

The shortened skulls observed in *plod2* mutants match qualitative clinical observations of shortened body axes in humans with *plod2* mutations [3].

Shape differences are concentrated in areas of the skull that have neural crest-derived compact cartilage, such as the vertebrae (Fig. 3).

These results agree with our pseudolandmark approach (below), lending support to our semi-automated approach.

Symmetrical shape differences exist between *plod2* mutants and wildtype fish

Pseudolandmark shape analysis

Overall shape also differs between *plod2* crispant and wildtype fish ($p=0.04$) for the 308 pseudolandmarks (Fig. 4).

The first 10 PCs explain 80.0% of the total variation in the dataset.

plod2 crispant fish show the greatest shape variation compared to their wildtype siblings (Fig. 5).

These differences agree with the shape differences observed in our manual landmarks as well as the symmetry analysis (below).

Overview of methodology

genetic disease association study → micro-CT scan of zebrafish → 21 manual landmarks in 3D → create point cloud → *plod2* injection → similar to the pseudolandmark → similar differences in shape in both groups

Symmetry analysis

There are symmetrical differences in skull shape between the two groups ($p=0.02$; Fig. 6).

plod2 crispant fish show the greatest shape differences compared to their wildtype siblings (Fig. 6).

Similar to the pseudolandmark analysis, similar differences in shape in both groups

Using a semi-automated screening tool to quantify craniofacial variation in *meox1* crispant zebrafish

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²University of Washington, Department of Biology, ³Department of Orthopaedics and Sports Medicine, ⁴Seattle Children's Hospital, Seattle, WA
⁵University of Washington, Institute for Stem Cell and Regenerative Medicine, Department of Pediatrics, Seattle, WA

Objective

The goal of this study is to determine if *meox1* crispant zebrafish have homologous skull phenotypes to Klippel-Fell syndrome in humans.

Methods

microCT scans of the cranium of 22 zebrafish (11 control, and 11 *meox1* crispant) were collected.

To establish a baseline of shape difference between groups, 21 manual landmarks were placed on major anatomical landmarks of the zebrafish cranium (Fig. 1).

Next, a set of 308 pseudolandmarks were generated and transferred onto the surface of each zebrafish cranium using a reference atlas model built from wildtype individuals [4]. This was performed using the Pseudolandmark Generator and APALICA modules of the SlicerMorph extension in Slicer 4 (Figure 2).

A Generalized Procrustes Analysis was performed on each data set to remove the effects of size and orientation.

Aligned coordinates were then used in Principal Components Analyses. Procrustes ANOVAs were used to determine if there were differences between control and control groups.

The pseudolandmarks were also used to perform a symmetry analysis to determine if the change was present on both sides of the bilaterally symmetrical cranium of the zebrafish. Average shape models for each principal component (PC) using the morphoJ package in R.

Heatmaps were created by taking the difference between the max, the minimum and the average for the respective PC.

All statistical analyses were performed in R using the geomorph package.

Implications

We did not find craniofacial asymmetries as predicted.

- Differences in musculature between the axial and cranial skeletons could explain the differences in asymmetry between the two groups.
- In contrast to humans and zebrafish, *meox1* mice neither display craniofacial asymmetries nor do they exhibit scoliosis [1], suggesting zebrafish may be a better model for Klippel-Fell Syndrome.
- This study provides a semi-automated screening tool to quickly report the use of our method to fast-track the genotype to phenotype association and minimize human biases in quantifying craniofacial structures.

Acknowledgements

We would like to thank the Musculoskeletal Systems Biology Lab for providing the CT scans of zebrafish used in this study, and the Maga Lab for providing feedback on this project.

Literature Cited

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- Devar et al., 2019. J. Clin. Orthop. Res. 10: 459–465.
- Tracy et al., 2004. Clin. Orthop. 424: 183–190.
- Diamond et al., 2022. Biol. Open. 11: bio55948

Results

Manual Landmarks

Pseudolandmarks

Symmetry Analysis

Discussion

Manual landmarking results showed that craniofacial phenotype differs between *meox1* crispant and control fish (Figure 4).

PC1, which explained 24.4 percent of the variation, differed between groups ($p=0.001$).

Anatomical variation across PC3 shows that *meox1* crispant fish are stouter in length and have wider posterior craniums.

The pseudolandmark points showed variation in overall shape between *meox1* and control groups (Fig. 5).

Groups vary along PC1 ($p<0.001$) and PC3 ($p=0.012$).

As shown in the heatmap, PC1 and PC3 are most in the subocular and intracranial regions (Figure 3).

Shape differences are smaller across PC3, with shape differences concentrated in the opercular and occipital regions (Figure 5).

The symmetry analysis revealed significant variation between groups.

Quantifying craniofacial phenotype of zebrafish with mutations in the *sost* gene, known homolog for human *SOST* associated with hyperostosis

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²University of Washington, Department of Biology, ³Department of Orthopaedics and Sports Medicine, ⁴Seattle Children's Hospital, Seattle, WA
⁵University of Washington, Institute for Stem Cell and Regenerative Medicine, Department of Pediatrics, Seattle, WA

Objective

In this study, we aimed to identify shape differences between *sost* mutants and wildtype zebrafish skulls.

Methods

- We used microCT scans from 24 zebrafish (7 mutants, 9 heterozygotes, and 8 controls) to compare craniofacial phenotypes.
- 21 manual landmarks and pseudolandmarks (308 points) points were placed onto the skull using 3D Slicer software (Figure 2).
- We used a Generalized Procrustes Analysis to remove the effects of size and orientation from the data and then performed a symmetry analysis on the size-corrected coordinates for both manual, pseudolandmarks, and symmetrically aligned landmarks.
- For the pseudolandmark points we also conducted a symmetry analysis to examine how shape differed on the right and left sides of the skull.

Background

- SOST* gene encodes腋天蛋白, an osteocyte-specific glycoprotein that negatively regulates bone formation through the inhibition of canonical Wnt signaling pathways in osteocytes.
- In humans, a nonfunctional *SOST* gene causes sclerosteosis [2, 3]. Van Buchem et al. [4] found that sclerosteosis is characterized by hyperostosis (excessive bone) in the skull and elongated axial skeleton.
- In addition to initial bone formation, *SOST* is also important for bone remodeling, growth, and maintenance [5].
- In humans [5] and zebrafish [6], sclerostin levels decreased in response to increased mechanical loading.
- Overall, the skeletal phenotype of *sost* mutants is still significantly different than in mammals due to differences in bone formation and development [6].

Results

- We found no differences in overall shape among the three groups (mutant, heterozygotes, and control) for the PC1 ($F=0.094$, $Z=0.486$, $P=0.314$) or pseudolandmark ($F=0.344$, $Z=0.49$, $P=0.373$) datasets.
- In the pseudolandmark dataset, we find a trend between control and mutant groups ($F=1.898$, $Z=1.437$, $p=0.087$).
- Groups** separate along the PC2 axis, with control and heterozygote groups clustered on the left side of the plot and mutant group clustered on the right side.
- Groups varied across PC1, with control individuals occupied negative PC1 space, mutants occupied positive PC1 space, and heterozygotes were intermediate (Figure 3).
- Groups varied across PC2, with control individuals occupied positive PC2 space, and heterozygotes and mutants occupied negative PC2 space, and heterozygotes were intermediate (Figure 3).
- Overall shape changes in skull morphology are clearly localized in the opercular regions of the skull (Figures 2,4).

Pseudolandmarks

Symmetry Analysis

Discussion

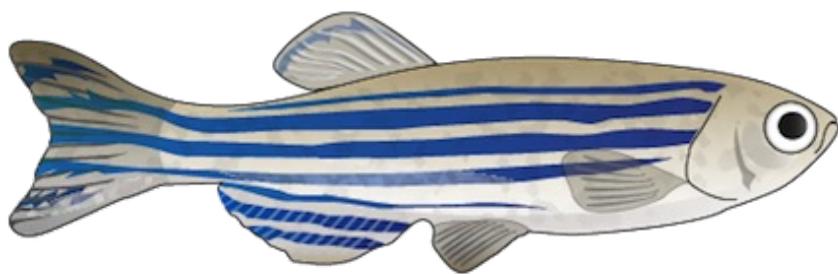
- Craniofacial shape changes resulting from nonfunctional copies of the *sost* gene in occur in a symmetric manner.
- The lack of divergence in overall shape in the heterozygous group (except for the symmetry analysis) relates to the fact that the gene is dominant.
- This implies that the gene would display similar trends in humans, with symmetrical phenotypes prevalent with both copies of the *SOST* gene render non-functional.
- We find that the heterozygote group has a more robust skull than both mutants and controls that possess osteoclasts and zebratines for bone development.
- This is consistent with the fact that more parallel cell targets in bone therapeutic.
- In addition to our results regarding zebrafish *sost* phenotypes, our pseudolandmark offering a promising new screening tool for other zebrafish models of skeletal disease.

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- Van Buchem et al., 2008. J. Clin. Endocrinol. 150: 587–593.
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- Wang et al., 2011. J. Clin. Endocrinol. Metab. 152: 209–215.
- Weigle, L, and Franco-Odorik, T.A. 2016. Anat. Rec. 299: 93–103.

Learn more here:

- Rolfe et al. SlicerMorph: An open and extensible platform to retrieve, visualize and analyze 3D morphology. MEE. <https://doi.org/10.1111/2041-210X.13669>
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- Porto et al. ALPACA: a fast and accurate approach for automated landmarking of three-dimensional biological structures. MEE. <https://doi.org/10.1111/2041-210X.13689>
- Diamond et al. Computational anatomy and geometric shape analysis enables analysis of complex craniofacial phenotypes in zebrafish mutants. Bio Open. <https://doi.org/10.1242/bio.058948>



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