Analysis of Zebrafish Mutants to Model Human Genetic Defects in the *LRRC56* gene.

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Introduction

- A mutation in the *LRRC56* gene which was identified in two stillborn human fetuses is likely responsible for defects associated with motile cilia dysfunction (Bonnefoy et al., 2018).
- *lrrc56* knockout zebrafish were created to model these genetic defects.
- In zebrafish, motile cilia are involved in the establishment of the left-right axis, straightening of the body, and deposition and positioning of otoliths (Bearce & Grimes, 2021; Colatonio et al., 2009; Song et al., 2016).
- *lrrc56*$^{-/-}$ mutants possess phenotypes characteristic of ciliopathies in zebrafish such as laterality defects, and spinal curvature (Ivare, 2020). Another potential phenotype of ciliopathy in zebrafish is defects in otolith positioning in the inner ear (Colatonio et al., 2009).
- 24% of *lrrc56*$^{-/-}$ larvae had severe spinal curvatures while the adult population did not possess the same extent of spinal defects (Ivare, 2020).

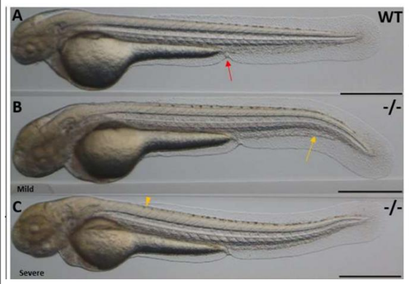

Fig. 1. Classification of spinal curvature at 2 days post fertilization (dpf). Spinal curvature occurring posterior to the urogenital pore (red arrow) is classified as mild and spinal curvature anterior to or on the urogenital pore is classified as severe. **(A)** Wildtype (WT), **(B)** *lrrc56*$^{-/-}$ larvae with mild curvature, **(C)** *lrrc56*$^{-/-}$ larvae with severe curvature. Scale bar: 0.5mm (Ivare, 2020)

Hypothesis

- *lrrc56*$^{-/-}$ mutants with severe spinal curvature have a lower probability of surviving to adulthood than those with mild spinal curvatures
- *lrrc56*$^{-/-}$ mutants have defects in otolith deposition

Objectives

1. Further analyse the spinal curvature phenotype previously observed in *lrrc56*$^{-/-}$ mutants
2. Analyse otolith deposition in *lrrc56*$^{-/-}$ mutants
3. Confirm expression of *lrrc56* using *in situ* hybridization (ISH)

Methodology

Survival Curve

Bred *lrrc56*$^{-/-}$ mutants and WT zebrafish.

↓

Separated mutants based on extent of spinal curvature at 2dpf or at 5dpf, depending on the experiment.

↓

Took observations daily until 5dpf. After 5dpf, larvae were transferred to tanks and observed twice weekly.

↓

Generated Kaplan-Meier survival curve.

↓

Determined the statistical differences between groups using the log rank test.

Otolith analysis

Counted number of otoliths under a dissection microscope at 1dpf in WT and *lrrc56*$^{-/-}$ mutants.

lrrc56 expression

Amplified *lrrc56* cDNA fragments using PCR and cloned fragments into pDrive.

↓

Synthesized sense and anti-sense dioxigenin-labelled RNA probes from cloned *lrrc56* cDNA fragments.

↓

Fixed WT embryos at various timepoints between 6hpf and 3dpf.

↓

Performed whole mount ISH.

Results

Analysis of Spinal Curvature

Fig 2. Progressive spinal straightening of *lrrc56*$^{-/-}$ larvae between 3 and 5dpf. Spinal straightening was observed in **(A)** *lrrc56*$^{-/-}$ larvae containing a severe lateral curvature and **(B)** both a severe ventral curvature and lateral curvature. By 5dpf severe ventral spinal curvature was almost completely corrected for while severe lateral curvatures were still present. Scale bars: 0.5mm.

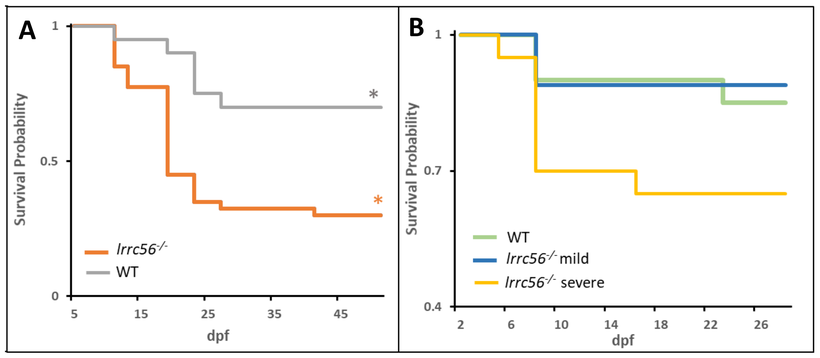

Fig. 3. Decreased survival of *lrrc56*$^{-/-}$ zebrafish in comparison to WT zebrafish. (A) Survival curve of WT (n_{5dpf} = 20) and *lrrc56*$^{-/-}$ mutant (n_{5dpf} = 40) zebrafish between 5dpf and 51dpf showed a significant difference in survival. **(B)** *lrrc56*$^{-/-}$ mutants were separated at 2dpf based on the extent of their spinal curvature according to Ivare (2020). There was no significant difference in survival between mutants with mild (n_{2dpf} = 9) and severe (n_{2dpf} = 26, n_{5dpf} = 20) phenotypes or between the mutants and WT (n_{2dpf} = 23, n_{5dpf} = 20). *indicates a statistical difference in survival as determined using the log rank test.

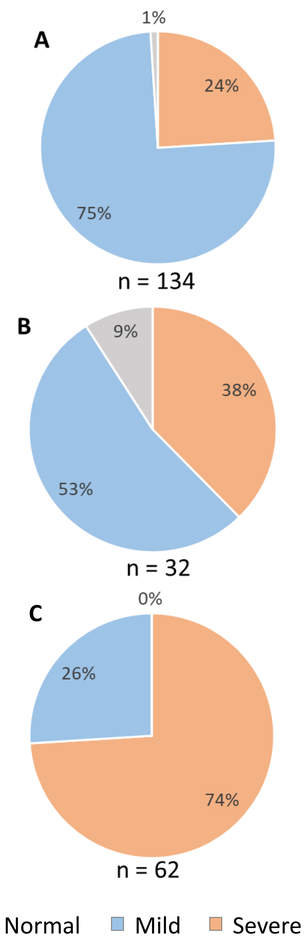

Fig. 4. Classification of spinal curvature of 2dpf *lrrc56*$^{-/-}$ larvae is subjective. Classification of spinal curvature was done using the method establish by Ivare (2020). **(A)** Data collected by Joshua Ivare (n = 134). **(B),** by Marie-Ève Proulx (n = 32). **(C),** and during this project (n = 62). Classification of spinal curvature by different people gives different proportions of mild, severe and normal *lrrc56*$^{-/-}$ mutants.

Analysis of Otolith Deposition

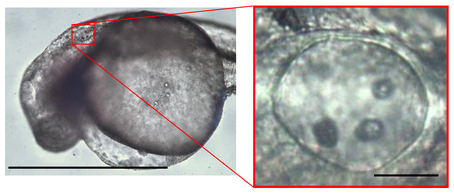

Fig. 5. Otic vesicle of *lrrc56*$^{-/-}$ mutant embryo which has 3 otoliths at 24 hpf. Scale bar: 0.0625mm

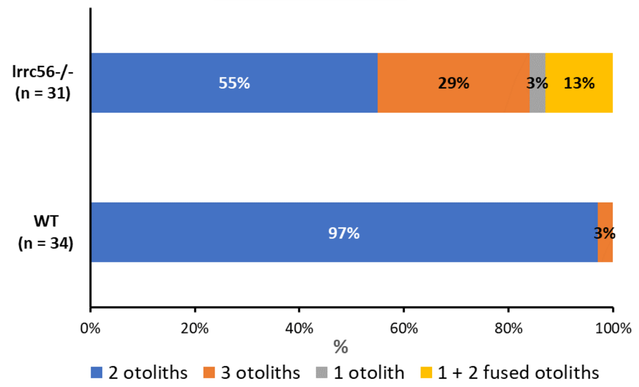

Fig. 6. *lrrc56*$^{-/-}$ mutants show abnormal otolith deposition at 1dpf. Abnormal otolith deposition was observed in 3% of WT larvae and 45% of *lrrc56*$^{-/-}$ mutant larvae, where 29% had 3 otoliths, 3% had 1 otolith, and 13% had 1 + 2 fused otoliths. These results are consistent with ciliopathy in zebrafish.

lrrc56 expression

- Three different probes were used to perform ISH, two targeting the *lrrc56* coding sequence (893nt and 453nt) and one targeting the 3'UTR (599nt).
- No ISH experiments were successful at showing *lrrc56* expression.
  - The 893nt and 599nt probes showed no true staining.
  - The 453nt probe was inconclusive due to the positive control failing.

Conclusion

Analysis of Spinal Curvature

- The *lrrc56* gene is important for survival of zebrafish in the first weeks of development.
- Number of *lrrc56*$^{-/-}$ mutants with severe spinal curvatures decreases as the population ages, potentially due to a combination of increased mortality and progressive spinal straightening.
- Classification of spinal curvature is subjective.

Analysis of Otolith Deposition

- Abnormal otolith deposition observed in *lrrc56*$^{-/-}$ mutants is consistent with ciliopathy in zebrafish.

lrrc56 expression

- Level of expression of *lrrc56* is likely low.

Future Directions

- Repeat survival curve using new system of spinal curvature classification (fig. 7) and using a larger number of zebrafish.
- Perform qRT-PCR to analyse expression of *lrrc56.*
- Repeat experiments using sibling WT and *lrrc56*$^{-/-}$ mutants.

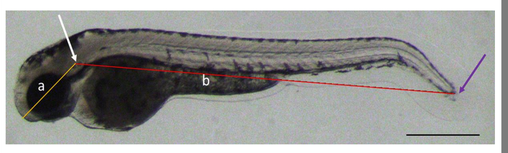

Fig 7. Classification of spinal curvature severity using ratio. Classification will be done using the ratio of a:b. The center of the otic vesicle is indicated by a white arrow and the tip of the tail is indicated by a purple arrow. Scale bar: 0.5mm.

References

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