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The Hedgehog effector Netrin controls optic fissure and stalk morphogenesis

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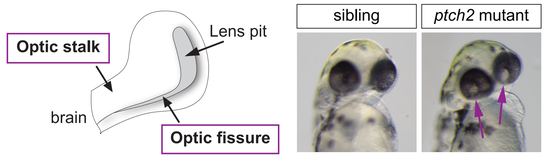
Developmental defects in eye structure commonly account for visual impairment in newborns. One such defect, uveal coloboma, is caused by failed development of the optic fissure, a transient yet critical structure through which retinal ganglion cell axons exit the eye and vasculature enters. In humans, this structural defect is a significant cause of pediatric blindness worldwide. Although the genetic underpinnings of coloboma are heterogeneous, one pathway central to optic fissure development is the Hedgehog (Hh) signaling pathway: loss-of-function mutations in the Hh receptor *ptch2* lead to overactive Hh signaling, resulting in coloboma. Previously, using zebrafish multidimensional imaging and quantitative analysis of cell behaviors, we determined the basis of optic fissure formation, and pinpointed morphogenetic defects in *ptch2* mutants (Gordon and Lusk et al., 2018). Overactive Hh signaling disrupts optic fissure development in a Gli-dependent manner, through both cell- and non-cell-autonomous mechanisms. Our model is that a specific level of Hh signaling is crucial for optic fissure formation; overactive Hh signaling transcriptionally upregulates multiple factors that are directly responsible for disrupting cell movements and morphologies.

To identify the downstream transcriptional targets of Hh signaling that control optic fissure morphogenesis, I have initially taken a candidate approach and am examining the Netrin family of secreted ligands: multiple Netrin ligands are upregulated in the *ptch2* mutant at the right time and place to be influencing optic fissure development. To test if upregulation of Netrin is sufficient to cause coloboma, I have taken a gain-of-function approach to overexpress *netrin1* in a spatiotemporally specific manner. To determine whether Netrin is necessary for the *ptch2* mutant coloboma phenotype, I have utilized loss-of-function alleles for *netrin1* to test if loss of Netrin in the *ptch2* mutant background rescues the coloboma phenotype. Additionally, our model suggests that multiple Hh effectors are responsible for disrupting optic fissure development in the *ptch2* mutant, thus I am carrying out single-cell RNA-sequencing to identify additional Hh target genes, to be tested using similar gain- and loss-of-function approaches. Together, this work will uncover molecular mechanisms controlling optic fissure morphogenesis, and in turn, coloboma.

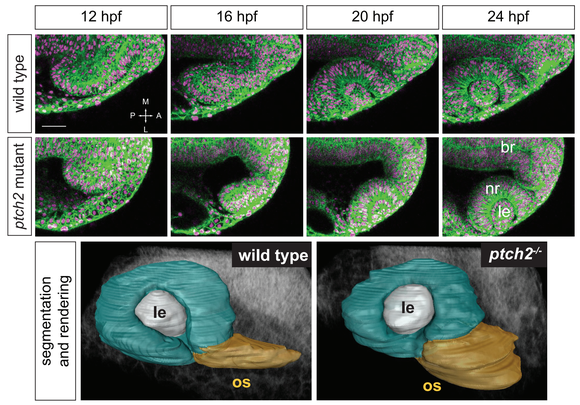
Overactive Hedgehog signaling can cause uveal coloboma

- Uveal coloboma, a birth defect and significant cause of pediatric blindness, is caused by disruptions to optic fissure development
- The Hh signaling pathway is crucial: mutations in the Hh receptor *patched2 (ptch2)* lead to overactive Hh signaling and coloboma in humans and zebrafish
- What are the underlying mechanisms?



Early steps of optic fissure development are disrupted in the *ptch2* mutant

membranes (EGFPCAAX, green) and chromatin (H2A.F/Z-mCherry, magenta) dorsal view, average projection, 12-24 hours post fertilization (hpf)

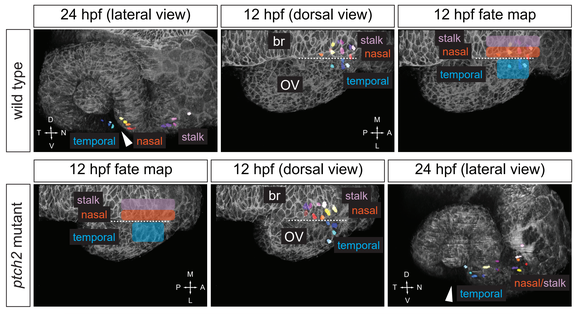


At 24 hpf the optic fissure fails to form and the optic stalk is ectopically large

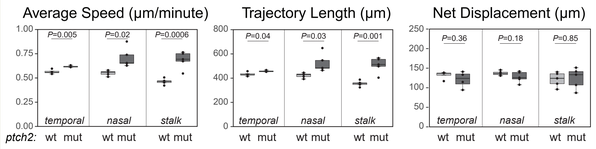
How does overactive Hh signaling disrupt cell movements underlying optic fissure and stalk formation?

4-dimensional cell tracking reveals movement of optic fissure cells and defects in the *ptch2* mutant

Retrospective cell tracking shows origins and trajectories of cells that make up the optic fissure and the optic stalk



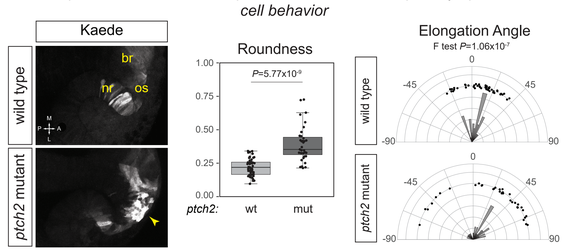
Cells in the enlarged optic stalk originate from a similar position as normal nasal optic fissure cells



Mutant cells move significantly faster and over a longer total trajectory length

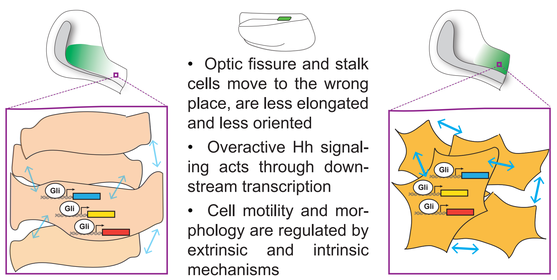
Migratory behaviors of cells contributing to the optic fissure are altered in the *ptch2* mutant

Use of photoactivatable Kaede protein to visualize and quantify optic fissure cell behavior



Stalk cells in mutant embryos are less elongated and oriented more variability than wild type counterparts

Summary



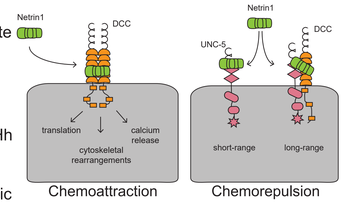
- Optic fissure and stalk cells move to the wrong place, are less elongated and less oriented
- Overactive Hh signaling acts through downstream transcription
- Cell motility and morphology are regulated by extrinsic and intrinsic mechanisms

Model: intrinsic and extrinsic signaling factors disrupt cell behavior and are upregulated in the *ptch2* mutant

What are the extrinsic factors downstream of Hh signaling that are responsible for the morphogenetic defects?

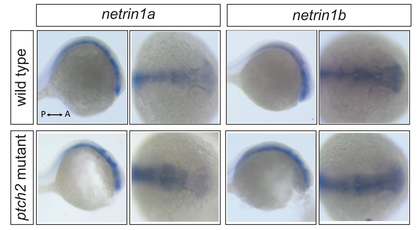
Dissecting the genetic interaction between Netrin and Hh signaling during optic fissure and stalk formation

- Netrin family of secreted ligands are compelling candidate effectors
- Some Netrin ligands are expressed in the midline and optic vesicle in zebrafish and some have been shown to be Hh responsive
- Downstream signaling is complex, a role in zebrafish optic cup morphogenesis is unknown

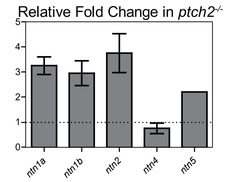


Netrin ligands are upregulated in the *ptch2* mutant

In situ hybridization to visualize expression of each netrin gene and determine if expression is increased in response to Hh signaling



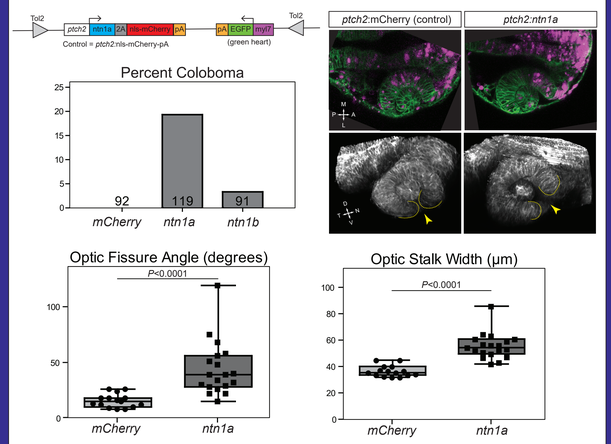
RT-qPCR shows that expression of multiple netrins are upregulated in the ptch2 mutant
Analysis was done using the ΔΔCt method, normalized to eef1a1l1, compared mutant to WT



Multiple netrin genes are expressed in the developing CNS and expression is increased in the *ptch2* mutant

Netrin1a overexpression in a spatiotemporally specific manner is sufficient to cause coloboma

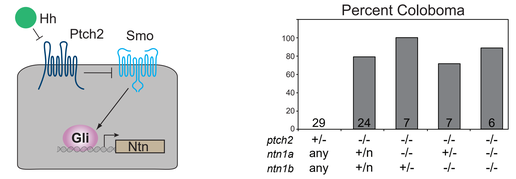
Using the Tol2kit system, each netrin gene can be transiently overexpressed under the Hh-responsive promotor



Overexpression of *ntn1a* in cells responding to Hh disrupts optic fissure and stalk development to result in coloboma

Is Netrin required for the *ptch2* mutant phenotype?

Compound mutants have been made using loss-of-function alleles for ntn1a and ntn1b in the ptch2 mutant background

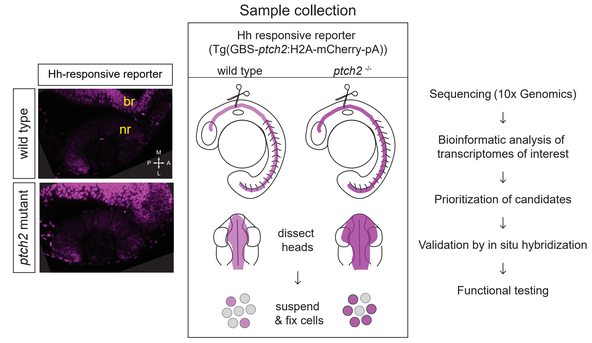


Preliminary results suggest that both *ntn1a* and *ntn1b* are not required for the *ptch2* coloboma phenotype;
Additional netrins are in the process of being tested

How can we identify and functionally test other Hh signaling effectors?

Identify differentially expressed transcripts in Hh-responsive cells using single-cell RNA-Sequencing

Using a transgenic line carrying a Hh-responsive reporter, cells from ptch2 mutant and wild type heads will be fixed for scRNA-seq



Conclusions and Future Questions

- Identified cell movements underlying optic fissure formation and have pinpointed cell populations with disrupted movement and morphology in the *ptch2* mutant.
- Overactive Hh signaling acts through a canonical, Gli-dependent transcription mechanism and through exrinsic and intrinsic factors
- What are the critical factors downstream of overactive Hh signaling? Netrin has been shown to disrupt optic fissure and stalk morphogenesis when overexpressed in a spatiotemporally specific manner, and scRNA-seq will allow identification of others.
- Are additional Netrin ligands required for the *ptch2* mutant coloboma?
- What aspects of cell motility and morphology are controlled by Netrin?

Acknowledgments

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