# LLN3: Identification and characterization of a potential regulator of axonal transport

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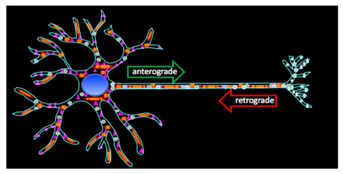
## Introduction

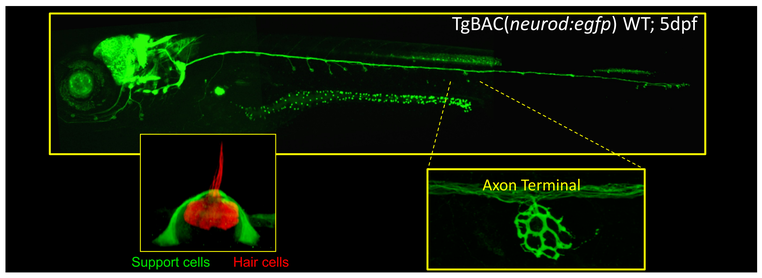
Transport of cellular cargos along axons is critical for neuronal health and function. Disruptions in cargo transport are causal in many neurodegenerative diseases. To identify modulators of cargo transport, we conducted a forward genetic screen using the zebrafish lateral line as a model system. Through our screen, we identified a novel mutant line, *lln3,* which contains axon terminal swellings, a phenotype indicative of cargo accumulation and defective transport. Further investigation into the *lln3* phenotype and underlying genetic cause will provide insight into regulatory factors that are vital to ensure properly functioning axonal transport.

### Axonal Transport of Cargos in Neurons



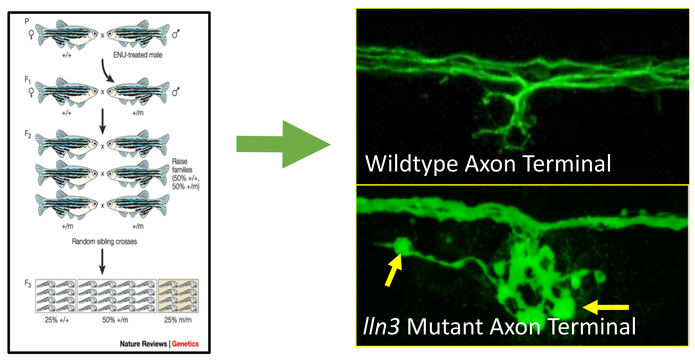

*Axonal transport occurs in two directions: anterograde (away from the cell body) and retrograde (towards the cell body). Dynein is responsible for retrograde movement while a family of kinesin motors are responsible for anterograde movement.*

### Zebrafish Posterior Lateral Line: A Model to Study Axonal Transport




*Zebrafish embryos and larvae are ideal for imaging due to their translucence during development.*

### ENU Screen to Identify Novel Regulators of Retrograde Axonal Transport: *lln3*

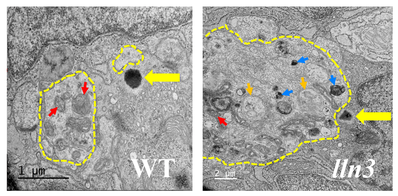



## Methods

- Mutant characterization using fixed and live imaging of zebrafish larvae at 4 days post fertilization (dpf).
- Transmission electron microscopy (TEM) analysis of axon terminal ultrastructure.
- RNA-seq based single nucleotide polymorphism (SNP) mapping combined with CRISPR/Cas9 G0 screening to determine the causative gene underlying the *lln3* mutant phenotype.

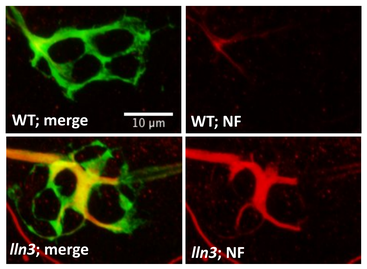
## Characterization

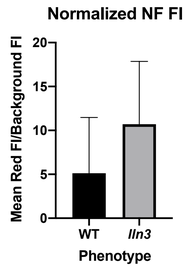
### 1. TEM Imaging of Axon Terminals Reveals Morphological Differences Between Wild Type and *lln3* Mutants




*TEM image of an afferent axon terminal from a WT and lln3 mutant. TEM allows for high resolution imaging of axon terminals to identify and quantify specific structures and examine general morphology . Yellow arrows indicate ribbon synapses, red arrows point to mitochondria, blue arrows indicate lysosomes, orange arrows point to autophagosomes, and dashed lines outline afferent nerve endings.*

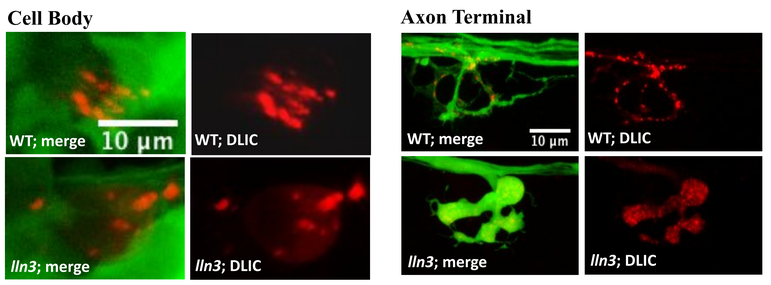
### 2. Neurofilament Accumulates in Axon Terminals of *lln3* Mutants

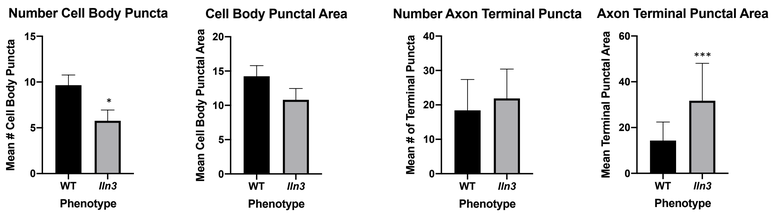



*Immunolabeling of neurofilaments (a cytoskeleton component) reveals an accumulation in lln3 terminals as indicated by an increase in mean normalized fluorescent intensity.*

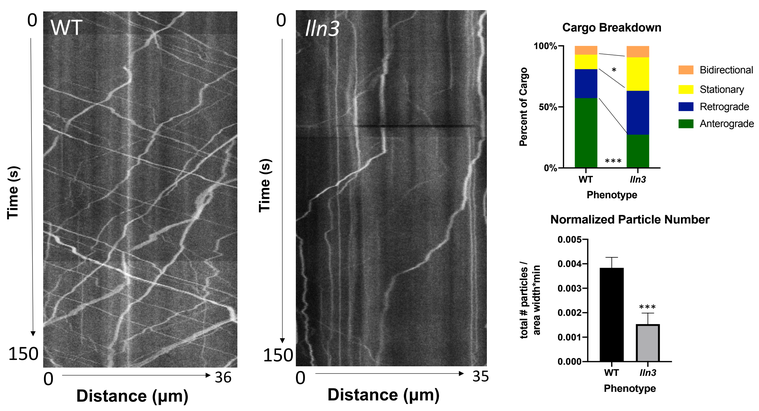
### 3. Dynein Light Intermediate Chain is Abnormally Distributed in *lln3* Mutants




*Live imaging of the cell body and axon terminals of zebrafish overexpressing mRFP labeled dynein light intermediate chain allows for analysis of punctal number, volume, and area. The number of cell body dynein puncta was significantly decreased in the lln3 mutants ( * $p < 0.05$) while axon terminal punctual area was increased (*** $p < 0.001$).*

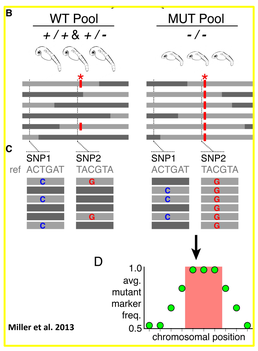
### 4. Dynein Motility is Disrupted in *lln3* Mutants




*Kymograph analysis of dynein motility allows for analysis of particle number, particle direction, and particle velocity. The total number of particles is reduced in lln3 mutants (*** $P < 0.001$). lln3 mutants also show a lower ratio of anterograde particles and higher ratio of stationary particles (*** $p < 0.001$, * $p < 0.05$).*

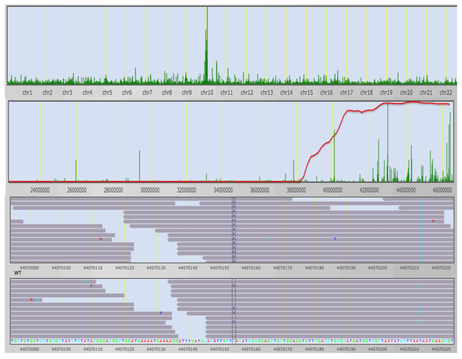
## Gene Identification

### 1. Analysis of SNPs in Mutant vs Wildtype Pools Allows for Mapping of Gene Location




*SNPs that are homozygous in the mutant pool and heterozygous in the wildtype may reveal causative mutations.*

### 2. RNA-seq data maps the *lln3* mutation to the distal arm of chromosome 10



*Alignments from WT and lln3 mutant pools map the location of the mutation to the distal arm of chromosome 10. Analysis of the reads reveal a number of missense SNPs that may be causative of the mutant phenotype.*

### 3. A Short List of Gene Candidates Is Generated By Analyzing SNP's in the Region of Interest

| Gene Name | Base/Amino Acid Change | | Position | Predicted Gene Role/Function |
|---|---|---|---|---|
| *Rhobtb2b* | C/T | His/Tyr | 44070154 | GTPase activity and protein kinase activity. Predicted to have a role in actin cytoskeleton development and Rho protein signaling. |
| *Stc1l* | C/T | Val/Iso | 44046785 | Predicted to have hormone activities. May have roles in calcium ion homeostasis and transcription. |
| *VcanB* | C/A | Ala/Glu | 44710641 | Calcium ion and hyaluronic acid binding activity. Involved in intramembranous ossification and skeletal system development. |
| *ACOT12* | C/A | Val/Leu | 44275599 | Acetyl Coenzyme A thioesterase. Regulates levels of acyl-CoA, fatty acids, CoASH. |
| *Rab11fip1b* | C/T | Gly/Glu | 42681785 | Rab GTPase binding activity. Plays a role in rab11 mediated recycling of vesicles. Predicted to be involved in regulated exocytosis. |
| *GPR64* | A/C | Thr/Pro | 36362594 | G protein coupled receptor 64. A member of the adhesion GPCR family. Known to be expressed in human and mouse epididymis and may have roles in fertility. |
| *Pora* | G/A | Val/Iso | 36315550 | P450 cytochrome oxidoreductase. Predicted to be involved in response to hormones and have FMN binding activity, NADPH hemoprotein reductase activity, and flavin adenine dinucleotide binding activity. |

*Candidate genes can be investigated in number of ways including through CRISPR/Cas9 G0 screens in which genes are individually knocked out at the one cell stage of development. The resulting phenotype of knockout fish can give insight into the gene's function and whether or not downregulation of the gene contributes to the lln3 phenotype.*

## Conclusions

- *lln3* mutants show axon terminal swellings, a phenotype indicative of defective axonal transport.
- TEM imaging reveals disruptions in axon terminal morphology as well as accumulation of various cargos such as autophagosomes.
- Neurofilament is seen to accumulate in axon terminals as is dynein light intermediate chain. Gross disruptions in dynein localization and transport are seen in *lln3* mutants.
- RNA mapping reveals that the mutation is most likely to be a missense mutation located on a telomere of chromosome 10.

## Future Directions

- Future studies will focus on employing additional methods to identify the mutation underlying the *lln3* phenotype. These methods include gene overexpression and *in situ* hybridization.
- Additional characterization of *lln3*, such as quantification of structures seen in the TEMs and examining microtubule dynamics, will potentially give insight into which specific components of axonal transport are responsible for the phenotype.

## Acknowledgments

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