A Drosophila larval screen of postnatal growth retardation related genes reveals lozenge as a regulator of growth in response to hypoxia

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Abstract

Postnatal growth is important for all organisms; therefore, many essential gene interactions and regulatory pathways are highly conserved. Understanding such pathways provides insight into disorders represented by human postnatal growth retardation. The Hypoxia Inducible Factor (HIF)-mediated hypoxia pathway regulates growth during periods of low oxygen availability, a condition that delays or suppresses development in many animals. Hypoxic conditions for mammals and the fruit fly ,*Drosophila melanogaster*, result in gene expression changes that are controlled by HIF. Larval growth and adaptation to hypoxic conditions provides a model for human prenatal and postnatal growth retardation diseases. We have previously proposed that specific signaling pathways in the larval fat body, the functional equivalent of the mammalian liver, including the Insulin receptor (InR) signaling pathway and Warts (I would define Warts here), are mediators of the adaptive response to hypoxia. We screened 134 *Drosophila* transgenic lines with homologs of human postnatal growth retardation disorders related genes . Overexpression or downregulation of candidates in the *Drosophila* fat body identified five genes that increase or reduce larval size under normoxia, phenocopying the larval response to hypoxia. Overexpression of one of those genes, *lozenge (lz)*, the homolog of human RUNX genes, decreases larval size, increases larval translucency, and promotes lipid droplet aggregation in the fat body, similar to hypoxic conditions. Rearing *lz*r^{15} mutants under hypoxic conditions rescues larval size but is associated with significant lethality, while *lz* downregulation in the fat body rescues the hypoxic growth restriction and increases larval survival. Experiments in mice have suggested an interaction between vertebrate RUNX and the HIF-α homolog, further supporting the conservation of this adaptive mechanism. These findings expand our understanding of the hypoxia-related growth regulatory pathways and postnatal growth retardation in both humans and *Drosophila*.

Methodology

Homolog Search**:* Human genes were extracted from Online Mendelian Inheritance in Man using the keywords “Postnatal and growth and retardation”. An algorithm was be developed with Python and Selenium WebDriver to search each of the genes in MARRVEL for the most related Drosophila homolog. They were ranked by their established role in growth retardation in human syndromes. ***Drosophila Stocks: The dsRNAi and overexpression lines of the searched homolog were ordered from Bloomington Stock Center. Lozenge-GFP line was provided by the Martinez lab. *lz*r^{15} mutation line was provided by Banerjee Lab, UCLA. The w^{1118} line was used as a wildtype control. ***Larval Growth Measurements**:* Larval growth was measured at the third-instar growth stage by immobilizing the larvae on ice, obtaining pictures with a light microscope and Leica software, and measuring larval length with Photoshop. Statistical analyses was performed using a Student's t-test. ***Oil-Red-O staining**:* Larval fat body lipid analysis was performed by dissecting in 3.7% formaldehyde and fixing in ice-cold for 60 minutes. Tissues were be rinsed twice with cold distilled water, incubated for 60 minutes in Oil-Red-O stain, and rinsed twice with distilled water. Tissues were mounted in Vectashield. Bright-field micrographs of Oil-Red-O-stained fat bodies and oenocytes were obtained using a Zeiss AX10 microscope. ***Starvation and Hypoxia Experiments**:* Similar to the previous experiments in Martinez lab, Drosophila larvae were collected within 3 hours after hatching and transferred to their standard media. For starvation, after 48 hours, they were placed on agar plates with PBS/1% sucrose and dissected after 24 hours. Hypoxia experiments was done by growing the larvae in normal conditions for 24 hours, and transferring to the hypoxia chamber from Coy Laboratory Products by mixing nitrogen with ambient air at 3.6% of oxygen, and wait until dissection after 48 hours. The control experiments was to be kept on standard Drosophila media and grow in ambient air. ***GFP Fluorescence Analysis**:* Fluorescence micrographs were obtained using Lexica MZ10F microscope. Larvae were dissected in 3.7% formaldehyde and fixed in ice-cold for 60 minutes. Tissues were rinsed twice with cold distilled water.

Screen for fat body mediated regulators of larval growth

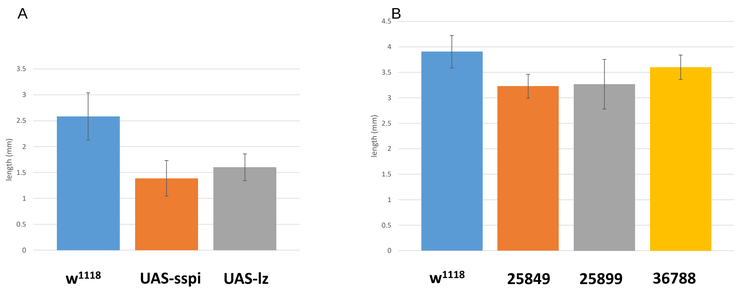

Figure 1. We screened 136 transgenic lines that represent *Drosophila* homologs of human genes associated with human growth retardation. We identified 5 lines associated with larval growth retardation when overexpressed in the larval fat body. **A.** Crossing UAS-lz and UAS-Sspi lines with R4-Gal4 causes a very significant decrease in larval size at 48 hours. (p-value = 8.7486e-14, p-value = 5.5143e-12) The UAS-Sspi x R4 cross is lethal for all the larvae at 72h. **B.** Crossing BL25849, BL25899 and BL36788 lines with R4-Gal4 causes a statistically significant decrease in larval size at 72 hours. (p-value = 0.0018, p-value = 0.0003, p-value = 6.76E-06; the numbers are the average of two independent replicates.)

Lozenge functions in the fat body to regulate larval growth

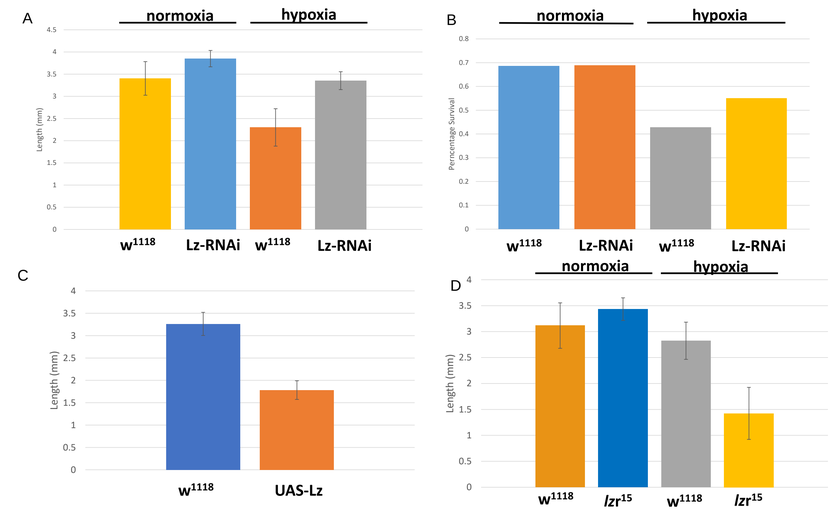

Figure 2. Larval size responses to Lz-UAS overexpression, Lz-RNAi and *lz*r^{15} at 72 hours. **A.** Lz-RNAi x R4-Gal4 increases the larval size in hypoxia and normoxia. (p-value = 3.5842E-10, p-value = 0.0003) **B.** Lz-RNAi x R4-Gal4 increases survival under hypoxia conditions. **C, E.** Lz-UAS x R4-Gal4 decreases larval size in normoxia. (p-value = 1.1102E-16) **D, F.** *lz*r^{15} mutants are resistant to hypoxia-induced larval growth restriction, but associated with a low survival rate (~20%).

Lozenge expression in the fat body causes lipid droplet aggregation

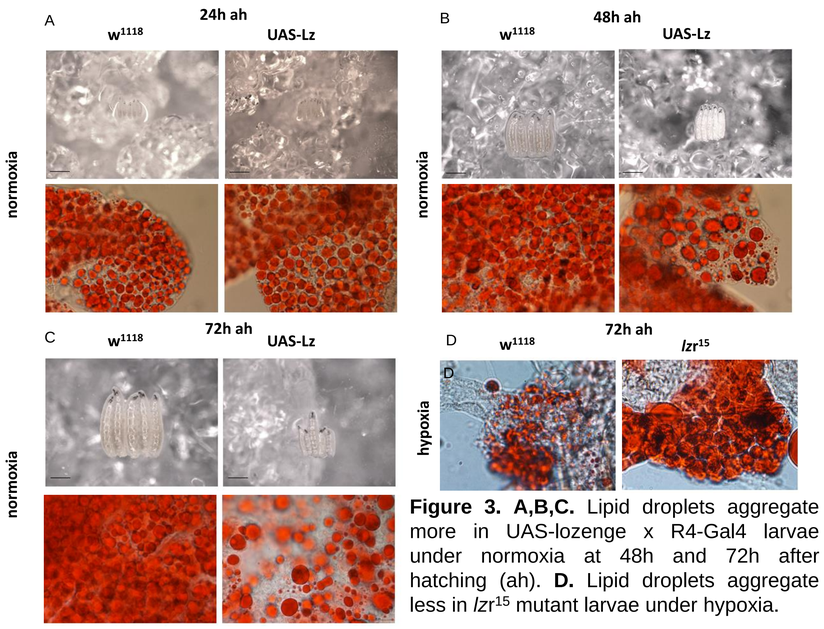

Figure 3. A,B,C. Lipid droplets aggregate more in UAS-lozenge x R4-Gal4 larvae under normoxia at 48h and 72h after hatching (ah). **D.** Lipid droplets aggregate less in *lz*r^{15} mutant larvae under hypoxia.

Down-regulation of lozenge in the fat body under hypoxia conditions rescues larval growth

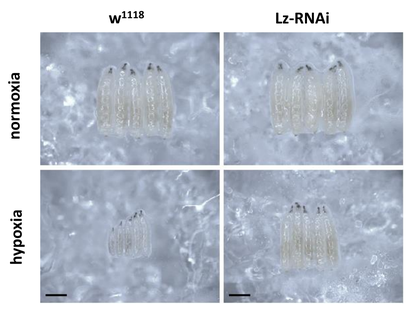

Figure 4. The hypoxic phenotype of growth restriction is rescued in Lz-RNAi x R4-Gal4 cross.

Hypoxia induces *lozenge* expression in developing larvae

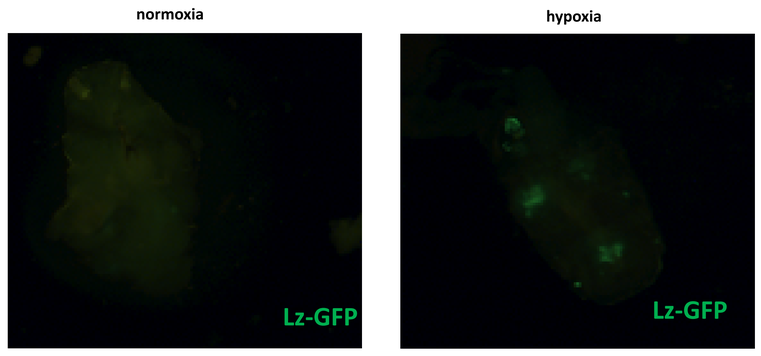

Figure 5. Lozenge-GFP levels are increased under hypoxic conditions compared to normoxic conditions.

Lozenge immunostaining reveals the presence of Lozenge protein in the hypoxic fat body

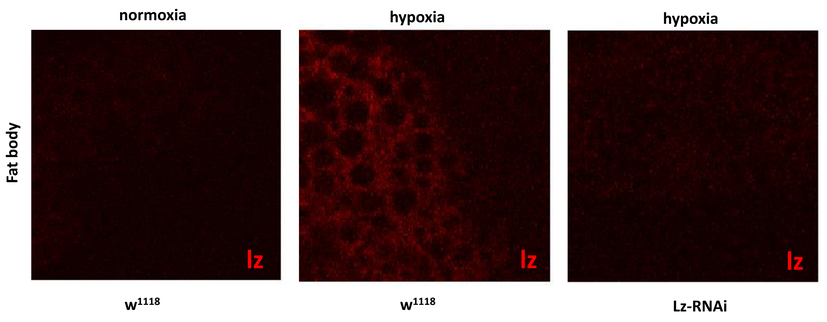

Figure 6. Immunostaining for Lozenge under normoxia and hypoxia reveals an increase in Lozenge expression in fat body under hypoxia. have been removed by Lz-RNAi.

Overexpression of sspi or lozenge in the fat body causes similar lipid droplet aggregation

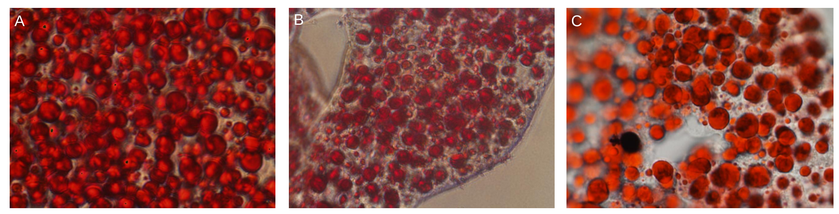

Figure 7. Overexpression of sspi in the fat body (B) phenocopies the lipid droplet aggregation observed upon lozenge overexpression (C, at 48 hours), compared to normoxia control (A). This suggests, along with the effect of both on larval growth, that sspi and lozenge may work together in mediating the effects of hypoxia.

Conclusions

- *spitz*, *lozenge*, *ppk21, Pi3K21B* and *chico* were identified in a screen for regulators of larval growth among 136 transgenic lines
- Lozenge regulates larval growth and fat body metabolism
- Down-regulation of lozenge in the fat body under hypoxia conditions rescues larval growth restrictions
- Hypoxia induces lozenge expression in developing larvae
- Lozenge protein levels increase in the fat body under hypoxia
- Spitz and Lozenge regulate larval growth and lipid metabolism in a similar manner, suggesting potential interaction in hypoxia

Future Directions

- Investigate the interaction between Lozenge and proteins in the hypoxia-induced signaling pathway (HIF/sima)
- Establish the relationship between Lozenge and previously identified regulators of larval growth

Acknowledgments

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