Lmx1b regulation and function during limb development and evolution

P49

Sofía Zdral[1], Alejandro Castilla-Ibeas[1], Silvia Naranjo Duran[2], Irene Mate[1], Miguel Juliá[1], Laura Galán[1], Kerby C. Oberg[3], Juan J. Tena[2], and Marian Ros[1]

[1]Instituto de Biomedicina y Biotecnología de Cantabria (CSIC-UC-SODERCAN). Santander, Spain; [2]Centro Andaluz de Biología del Desarrollo (CSIC-UPO-Junta de Andalucía). Sevilla, Spain; [3] Department of Pathology and Human Anatomy, Loma Linda University. Loma Linda, CA, USA.

INTRODUCTION

Lmx1b is the key regulator of dorso-ventral (DV) patterning in the limb

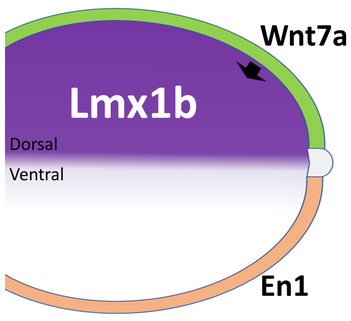


Fig.1: Limb bud schema showing the expression pattern of genes involved in DV limb patterning.

DV asymmetry is crucial for limb function and, during development, is determined by three crucial factors: Wnt7a, En1 and Lmx1b (Fig.1).

Targeted disruption of *Lmx1b* in mice (Chen et al., 1998) results in **double-ventral limbs** (Fig.2), but mice die perinatally. In humans, mutations in *LMX1B* cause the rare disease **Nail-Patella Syndrome** (OMIM:161200).

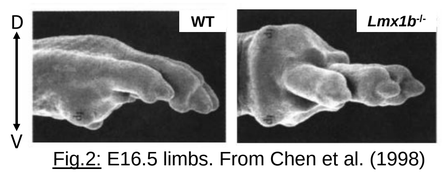
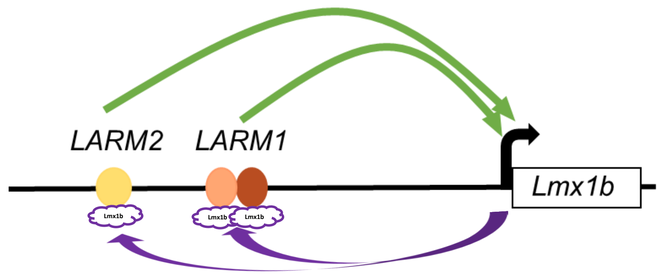


Fig.2: E16.5 limbs. From Chen et al. (1998)

Two limb-specific and autoregulatory enhancers control *Lmx1b* expression with antero-posterior (AP) specificity



The deletion of both enhancers (***ΔLARM1/2***) recapitulates the ***Lmx1b*-null phenotype exclusively in the limb** (Fig.3B). **T**he removal of each enhancer reveals that ***LARM1*** controls *Lmx1b* expression in the **posterior** (Fig.3C) and ***LARM2*** in the **anterior** part of the limb (Fig.3D) (Haro et al. 2021).

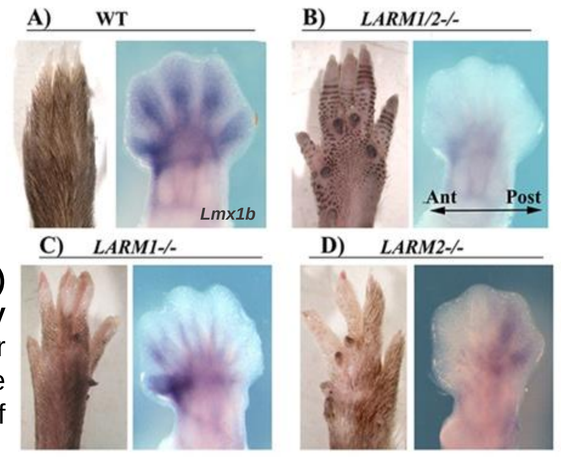
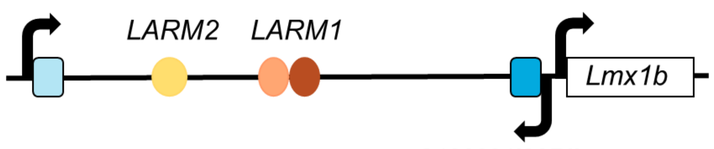


Fig.3: Spatial modularity of *LARM* sequences.

RESULTS

[1] The *Lmx1b* regulatory domain includes two lncRNAs



The ***C130021I20Rik*** is expressed in an identical pattern to *Lmx1b* (Fig.4).

The ***AK020435*** is expressed distally and with a ventral domain in the limb (Fig. 4, red arrow).

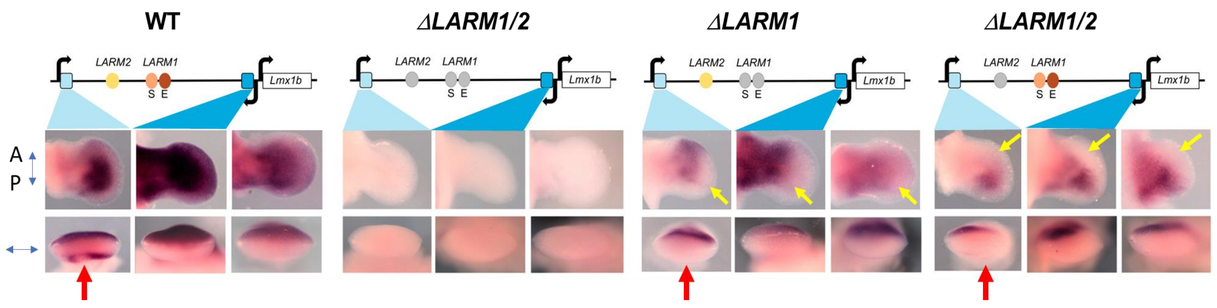
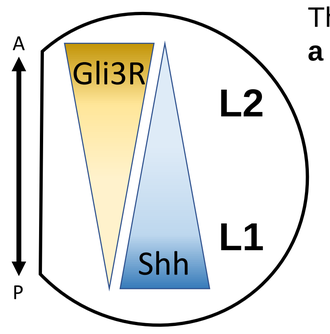


Fig. 4. Expression pattern and *LARM*-dependent regulation of the *Lmx1b* associated lncRNAs. WMISH of E11.5 forelimbs for *AK020435*, *C130021I20Rik* and *Lmx1b* in WT and *LARM* mutants.

Both lncRNAs are regulated by the *LARM* enhancers with the same **AP specificity** (Fig. 4, yellow arrows).

[3] The *LARM* sequences connect the AP and DV axes



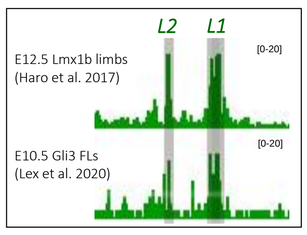
The **modular phenotype of *ΔLARM1* and *ΔLARM2*** mutants **suggest a connection between AP and DV axes.**

Hypothesis:

Gli3R → *LARM2*

Gli3R ⊣ *LARM1*

Accordingly, **Gli3** binds to *LARM* sequences



ChIP-seq

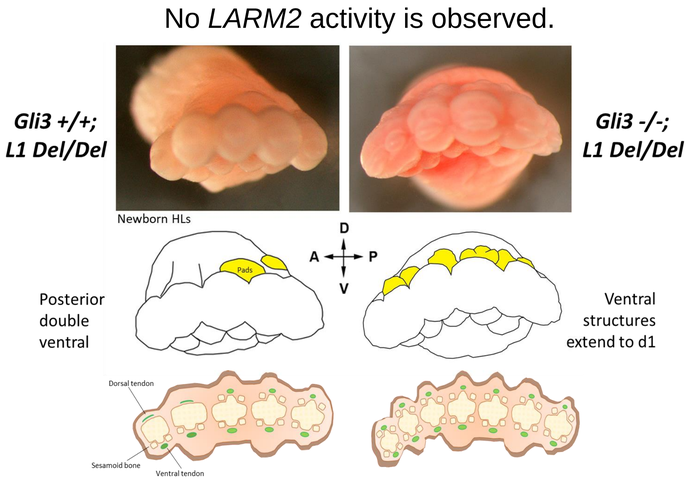
Removal of:

COMPOUND MUTANTS

Gli3;LARM1

Exacerbates the *ΔLARM1* phenotype, complete double-ventral limbs.

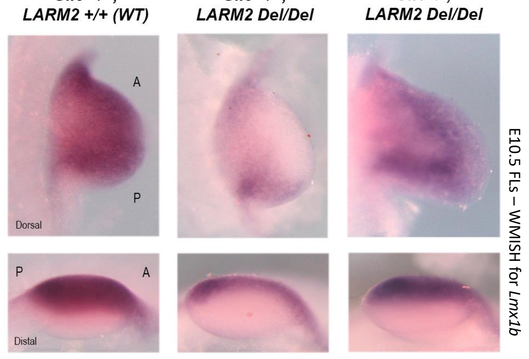
No *LARM2* activity is observed.



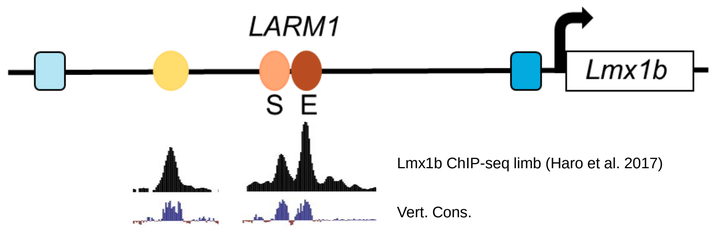
Gli3;LARM2

Rescues the *ΔLARM2* phenotype: anterior *Lmx1b* expression is recovered

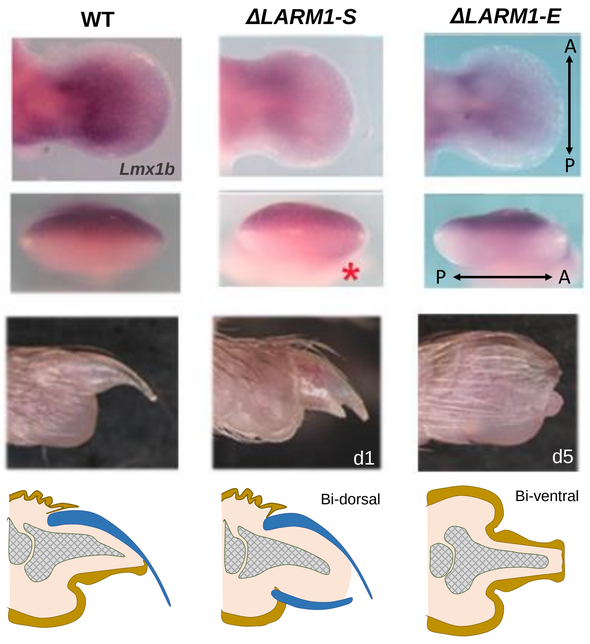
LARM1 activity all along the AP axis.



[2] *LARM1* contains a functional silencer sequence



Based on conservation and chicken reporter assays (Haro et al., 2021), ***LARM1* can be divided in two segments**, a 5' element that functions as a **silencer (S)** and 3´ that functions as an **enhancer (E).**



The CRISPR/Cas9 removal of ***LARM1S*** leads to the **ectopic ventral activation of *Lmx1b* in the anterior mesoderm** (Fig.5, asterisk) and a **double dorsal digit 1.**

The removal of ***LARM1E* reproduces the posterior double-ventral phenotype** of ***ΔLARM1,*** but restricted to digit 5 instead of d2-d5 (Fig. 5, right).

The milder phenotype of ***Δ****LARM1E* (d5) compared with ***Δ****LARM1* (d2-d5) suggest that, in the absence of the E, the S displays enhancer activity, or the S activity over *LARM2* is decreased.

Fig.5: In the *Lmx1b* landscape coexist positive and negative regulatory sequences.

The positional specificity of the *LARM* sequences supports a model in which **different regulatory schemes are used in different positions** (Fig.6, left).

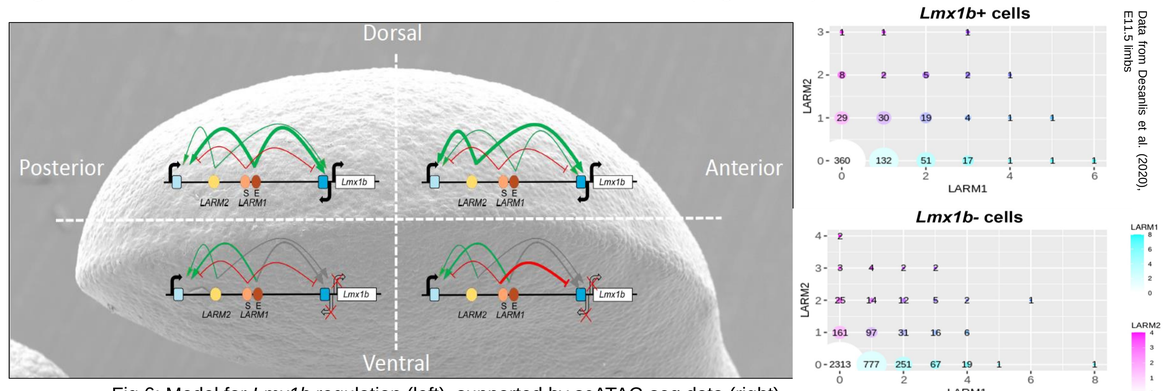


Fig.6: Model for *Lmx1b* regulation (left), supported by scATAC-seq data (right).

The accessibility of only one enhancer in most cells support this model (Fig.6, right). A small percentage have both accessible. Also, their accessibility is similar in dorsal and ventral cells.

[4] *Lmx1b* regulation is highly conserved during evolution

Considering that the **double-ventral limbs of *ΔLARM1/2* mutants fail to support locomotion,** we hypothesized that the elaboration of the DV asymmetry by modification of *Lmx1b* regulation, or its functional targets, had to accompany the fin to limb transition.

We **have identified orthologs of the *LARM* sequences in fishes** (Fig.7)**,** all of them show activity in the pectoral fin in zebrafish reporter essays (Fig. 7, right).

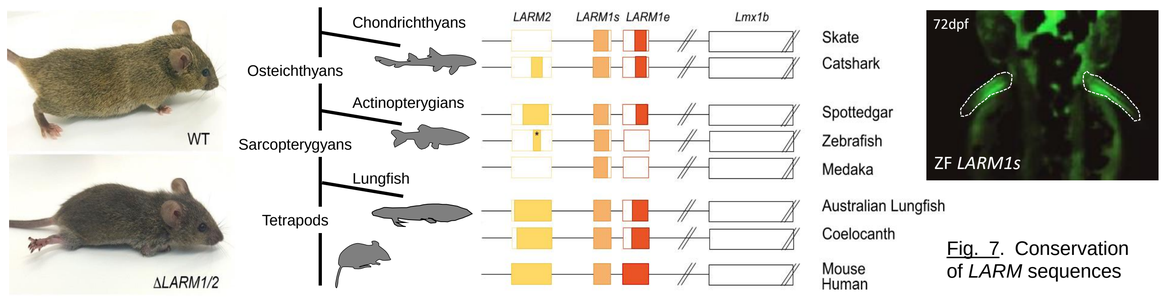


Fig. 7. Conservation of *LARM* sequences

CONCLUSIONS

- ❑ *LARM1* and *LARM2* control *Lmx1b* expression and its associated lncRNAs with AP specificity in the limb.
- ❑ *LARM1* contains a functional silencer sequence.
- ❑ Gli3 modulates *LARM1* and *LARM2* activity in opposite direction.
- ❑ An ancestral *Lmx1b* regulatory landscape is already present in chondrichthyans.

FUTURE WORK

- Functional analysis of the lncRNAs using CRISPR/Cas9 system.
- Spatial quantification of LARM activity (eRNA and chromatin marks).
- To elucidate the *LARM* trans-acting regulatory factors and their function.
- To explore the *Lmx1b* mode of action by identifying its direct targets.

Bibliography: **Chen**, H. et al. *Nat. Genet*. 18, 231–236 (1998); **Desanlis**, I. et al. *Nat. Commun*. 11, 2491 (2020); **Haro**, E. et al. *Dev*. 144, 2009–2020 (2017); **Haro**, E. et al. *Nat. Commun*. 12, 5533 (2021); **Lex**, R. K. et al. *Elife* 9, e50670 (2020)

Acknowledgments: Spanish Ministry of Science and Innovation Grant PID2020-114525GB-I00 to Marian Ros. Sofía Zdral is supported by a PhD fellowship of the University of Cantabria and Alejandro Castilla Ibeas by a PhD fellowship of the Ministry of Science and Innovation (FSE/AEI/PRE2018-083421).