Comprehensive analysis of molecular pathways and key genes involved in axonal guidance in the hypoglossal nerve into mouse tongue primordia

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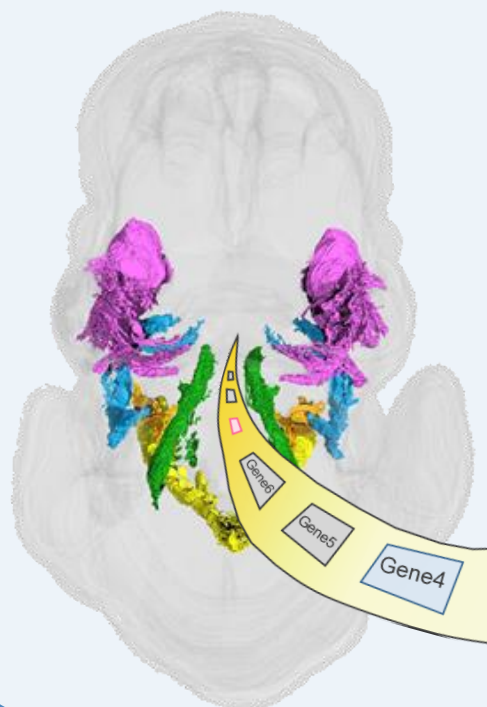
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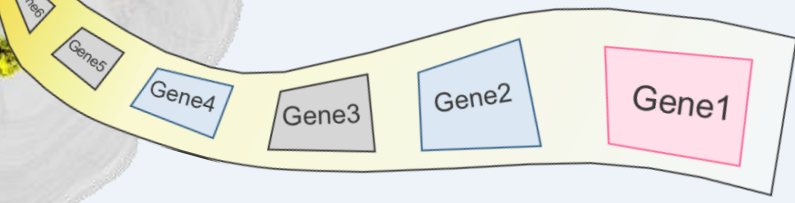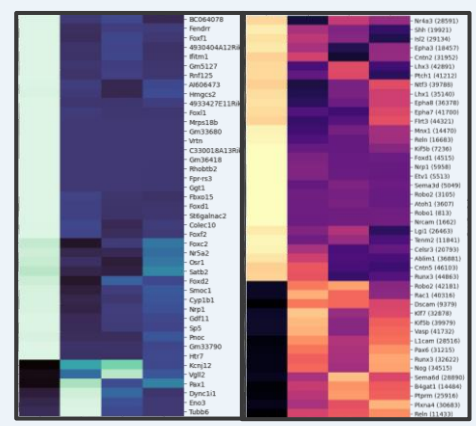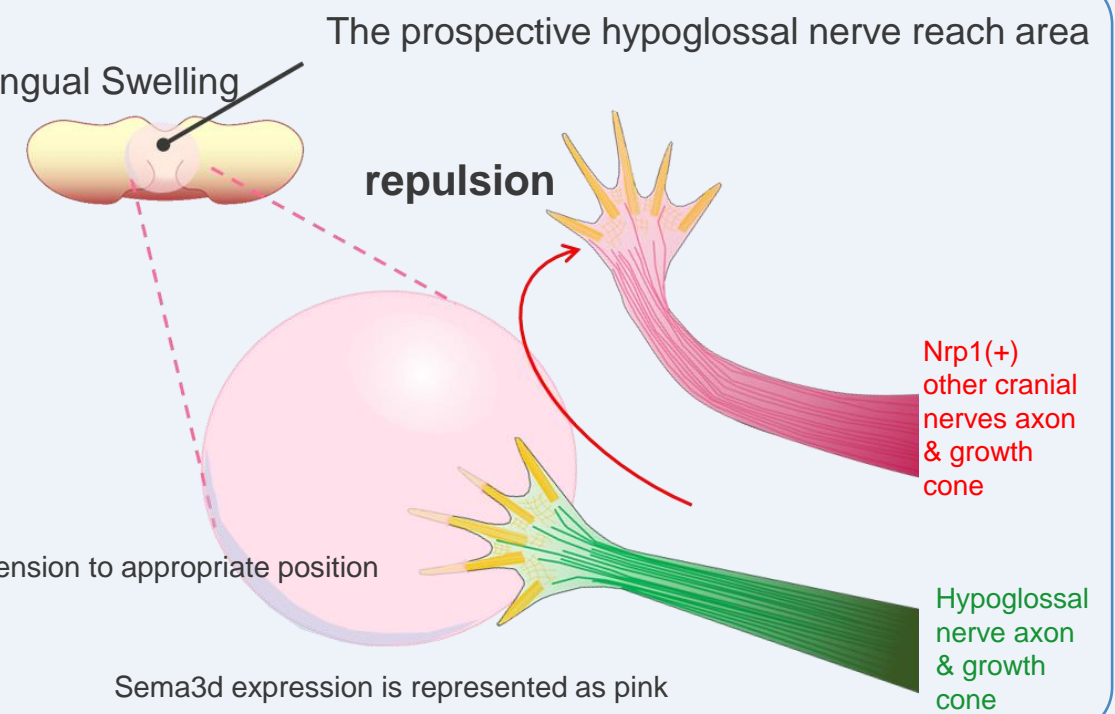
Japanese Association for Oral Biology

Conflict of Interest

Author's names: Hani T, Taya Y, Horie T, Sasaki Y, Kawamoto S, Kudo T, Sato K and Soeno Y

The authors declare no conflicts of interest associated

with this manuscript.

Background: Importance of the hypoglossal nerve and its development

Fig. 1. The hypoglossal nerve is a motor nerve that controls the movements associated with chewing, swallowing and speech. Therefore, dysfunction of this nerve can be life-threatening for the patient (a). Our previous studies have demonstrated how cranial nerves grow spatiotemporally. In particular the hypoglossal nerve travels a longer distance to reach the mouse tongue primordia.(b).
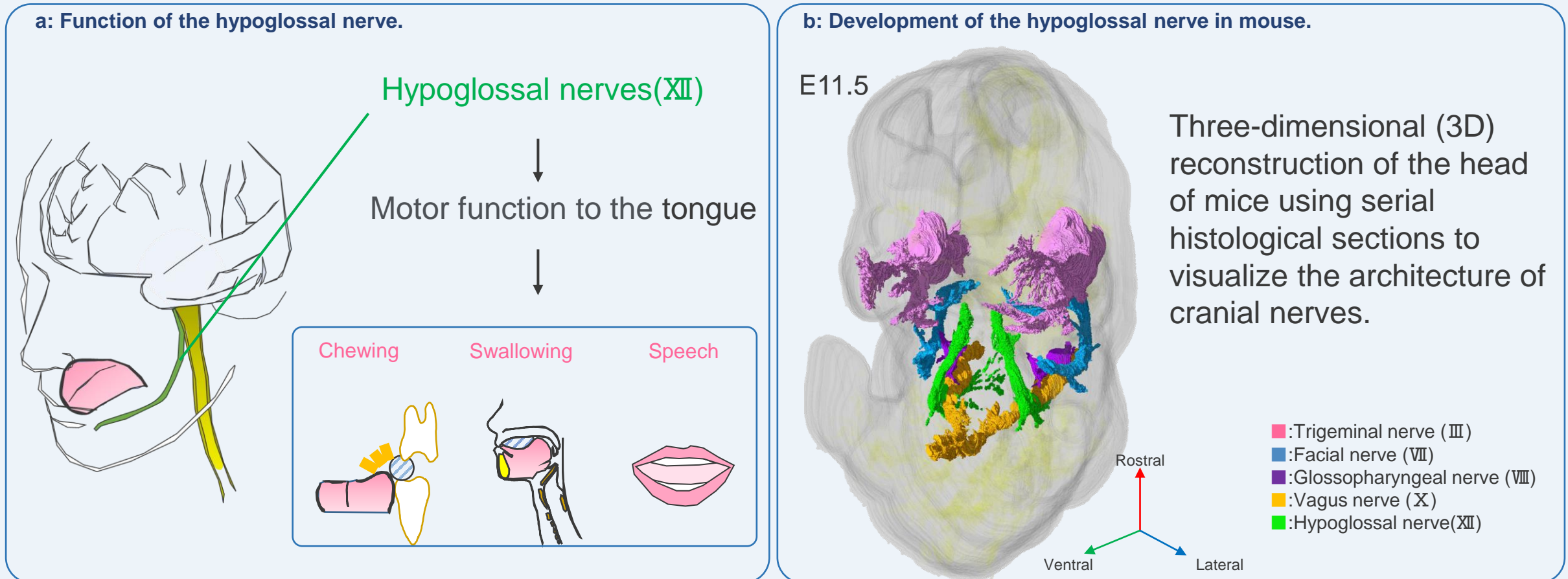
a: Function of the hypoglossal nerve.

Hypoglossal nerves(Ⅻ)

↓

Motor function to the tongue

↓

Chewing   Swallowing   Speech

b: Development of the hypoglossal nerve in mouse.

E11.5

Three-dimensional (3D) reconstruction of the head of mice using serial histological sections to visualize the architecture of cranial nerves.

Rostral

Ventral   Lateral

:Trigeminal nerve (Ⅲ)
:Facial nerve (Ⅶ)
:Glossopharyngeal nerve (Ⅷ)
:Vagus nerve (Ⅹ)
:Hypoglossal nerve(Ⅻ)

Background: Axonal guidance of the hypoglossal nerve

Fig. 2. As for the developmental mechanism, the hypoglossal neuroaxes extend from the occipital motor nuclei and reach the remote tongue primordia around E11.5 in mouse. However, the detailed molecular mechanism that drives accurate induction and innervation of the motor neuron remains unknown.

In this study, we aimed to verify the genes that guide hypoglossal nerve to intended area. To this goal, we focused on specific area of tissues where hypoglossal nerve eventually innervate.
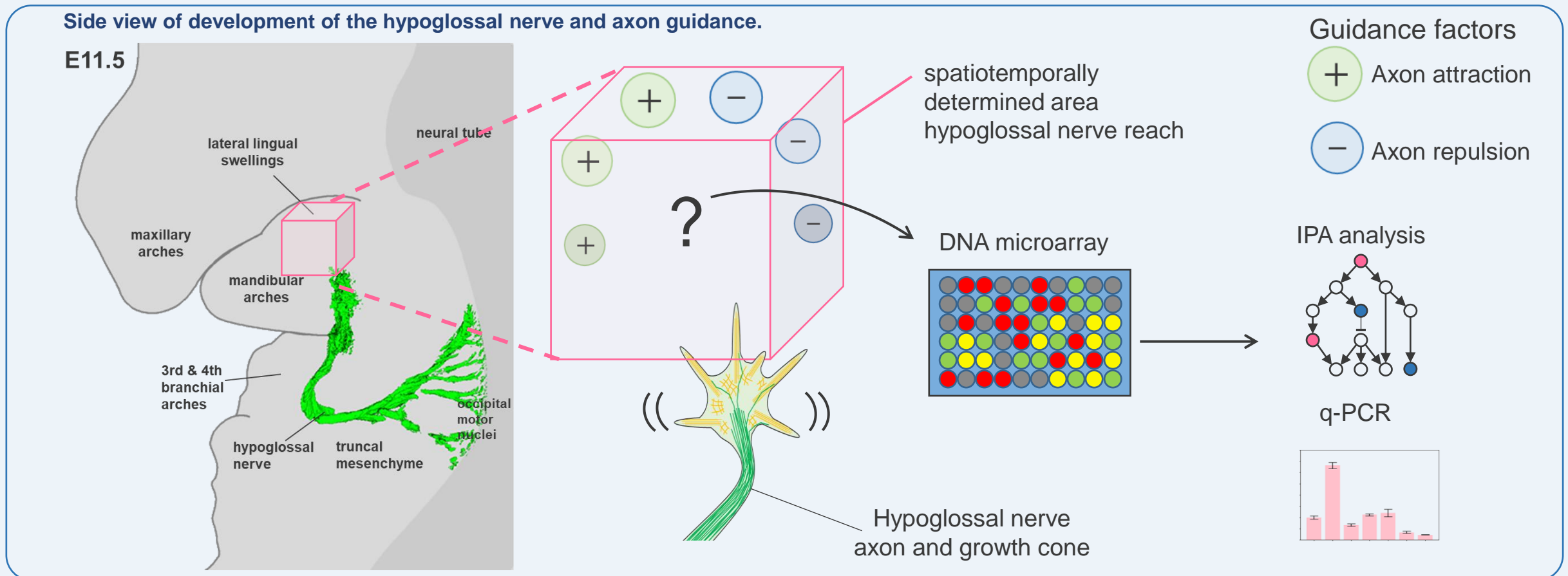
Method: Sample collection and comprehensive gene expression analysis

Fig. 3. The target area for comprehensive gene expression analysis was determined based on 3D models of the cranial nerves (a). Tissues were collected from the media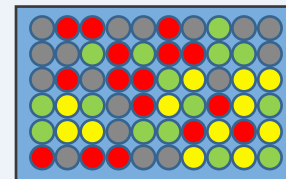n (tongue primordia) and lateral (control) portion of the mandibular arch of each ICR mouse fetus at embryonic [E] days 9.5, 11.5, and 14.5 (b).

a: 3D model of the cranial nerves and the sampling area.

E11.5

Rostral
Lateral

From 3D observation shows that the hypoglossal nerve extends to the midline of the tongue, ahead of the other cranial nerves. The square area where the hypoglossal nerve reaches, was collected in this study.

■ :Trigeminal nerve (Ⅲ)
■ :Facial nerve (Ⅶ)
■ :Glossopharyngeal nerve (Ⅷ)
■ :Vagus nerve (Ⅹ)
■ :Hypoglossal nerve(Ⅻ)

b: Tissues and their sampling sites.

E9.5

E11.5

E14.5

Median Mandibular Arches (MMA)
Lateral Mandibular Arches (LMA)

Lateral Lingual Swelling (LLS)
Lateral Mandibular Arches (LMA)

Median Tongue (T)
Lateral Tongue (LT)

・Sample collection
・RNA purified
・Reverse transcription

DNA microarray
(27309 genes)
(52145 probes)

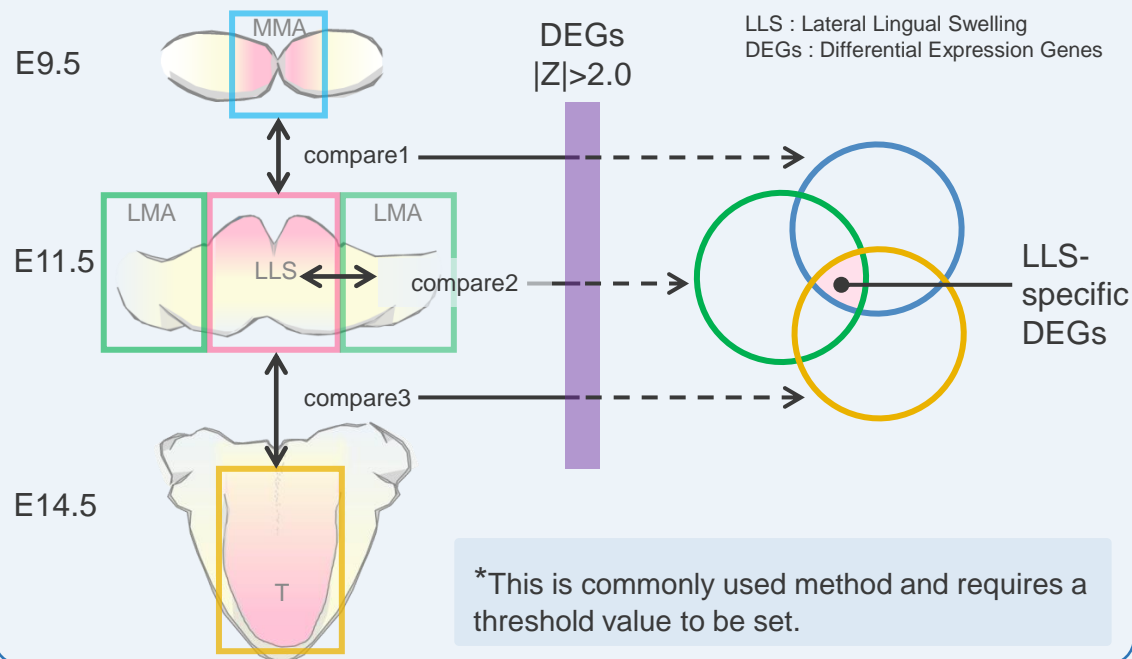Transcriptome analysis, IPA analysis and q-PCR

Method: Transcriptome analysis (Z-score based analysis and ROKU method)

Fig. 4. We conducted transcriptome analysis using Z-score based analysis and ROKU method. Owing to its algorithm, Z-score based analysis might hardly narrow down the neuron-related genes. Thus we employed ROKU method cooperated with Gene Ontology as a novel attempt. ROKU method is also advantageous to prioritize the gene by creating a ranking. The obtained DEGs were subjected to IPA and q-PCR.

Summary of the analyses in present study.

Z-score based analysis*

Z-score based analysis uses the Z-score, which is the deviation value of the ratio. We generated a list of LLS-specific candidate genes with the aid of Venn diagrams for three axes of comparisons; 1) MMA-LLS, 2) LMA-LLS, and 3) T-LLS.

E9.5

MMA

DEGs
|Z|>2.0

LLS : Lateral Lingual Swelling
DEGs : Differential Expression Genes

compare1

E11.5

LMA   LMA
LLS

compare2

LLS-specific DEGs

E14.5

T

compare3

*This is commonly used method and requires a threshold value to be set.

ROKU method**

Outlier determination (AIC) in ROKU method detected LLS-specific genes by comparing expression levels of the corresponding gene in other samples, and further created the ranking of specificity among samples by Shannon entropy.

$H(x) = 1.68$

Gene_X expression

T1   T2   T3   T4

Detection

AIC : Akaike's Information Criterion
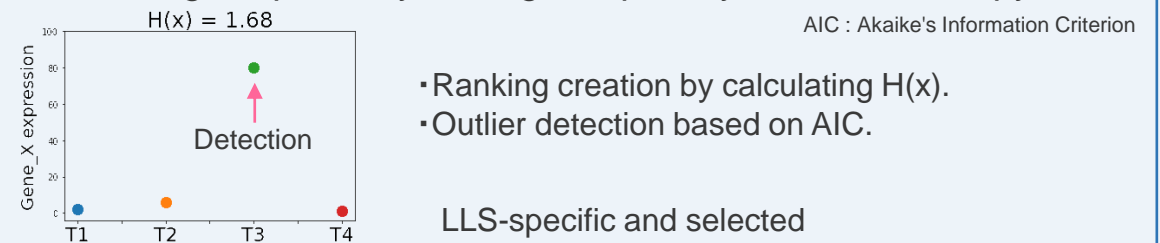
· Ranking creation by calculating $H(x)$.
· Outlier detection based on AIC.

LLS-specific and selected from GO genes.

Quick GO

The genes contained in Biological process "**axon guidance**".

Detecting the LLS-specific genes with **ROKU method**

**This calculates the ranking of gene expression and does not require a threshold value to be set.

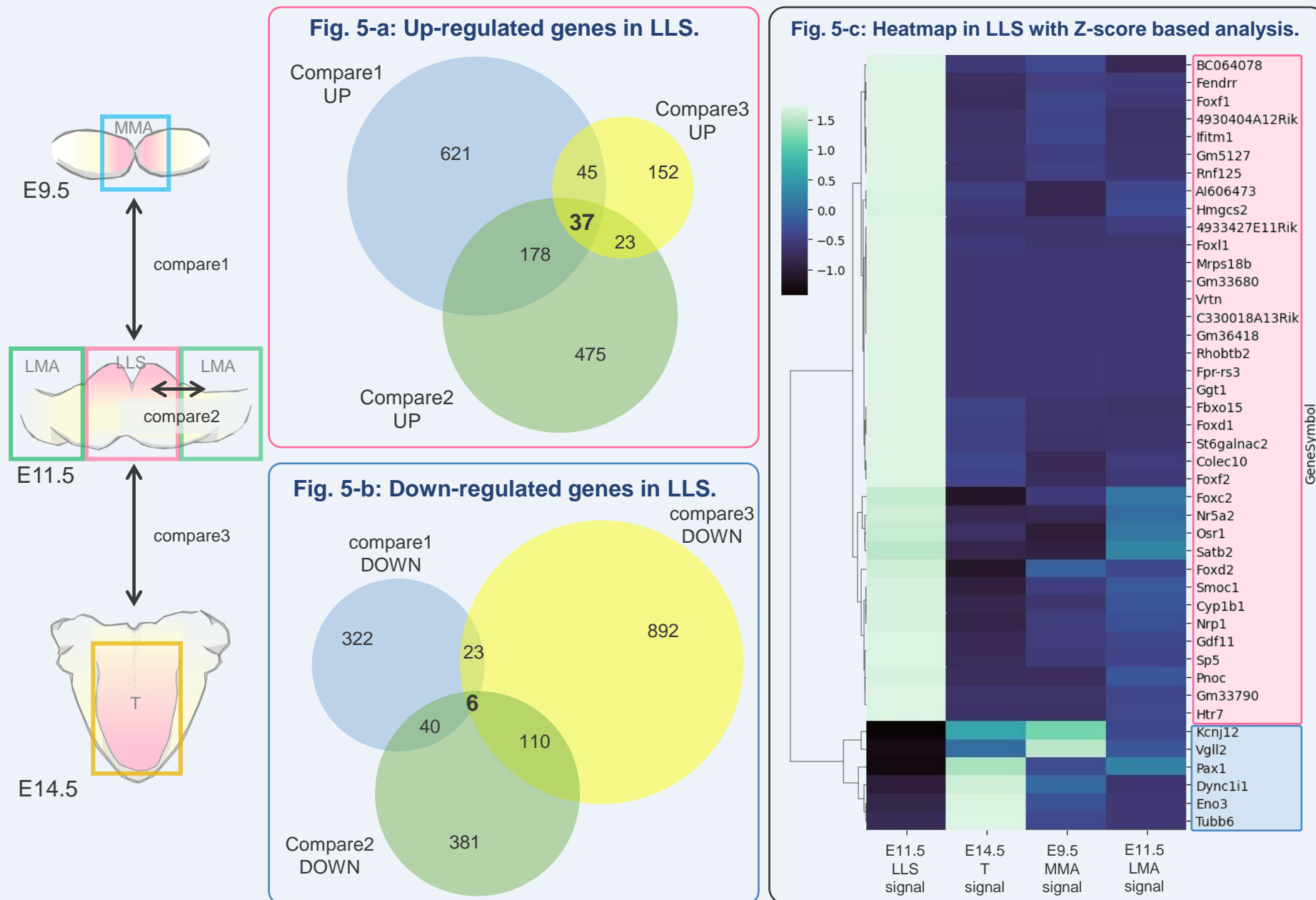# Result: Z-score based analysis narrow down genes in LLS.

Fig. 5-a: Up-regulated genes in LLS.

Fig. 5-b: Down-regulated genes in LLS.

Fig. 5-c: Heatmap in LLS with Z-score based analysis.

Fig. 5. Cross comparisons among MMA, LMA, T, and LLS revealed relative increase in the amount of DEGs as the developmental stage progressed.

Eventually, 37 up-regulated genes common in the three comparisons were depicted, while 6 down-regulated genes in LLS at E11.5 (a,b).

The heatmap discriminated those up & down-regulated genes that we determined LLS-specific genes (c).

MMA : Median Mandibular Arches
LLS : Lateral Lingual Swelling
LMA : Lateral Mandibular Arches
T : Median Tongue

Result: ROKU method narrow down genes in LLS.

Fig. 6-a: Process of ROKU method.

GO:0007411 "axon guidance" contains 246 genes

ROKU method

Up-regulated genes in Lateral Lingual Swelling (LLS): **2847** genes

Down-regulated genes in Lateral Lingual Swelling (LLS): **1093** genes

29 genes related in axon guidance
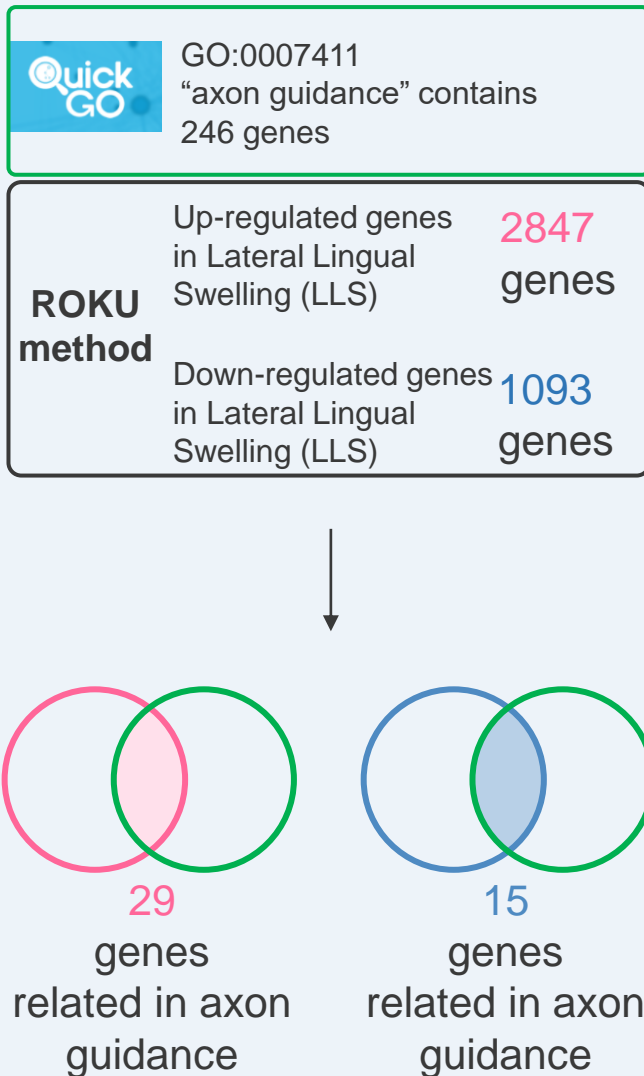
15 genes related in axon guidance

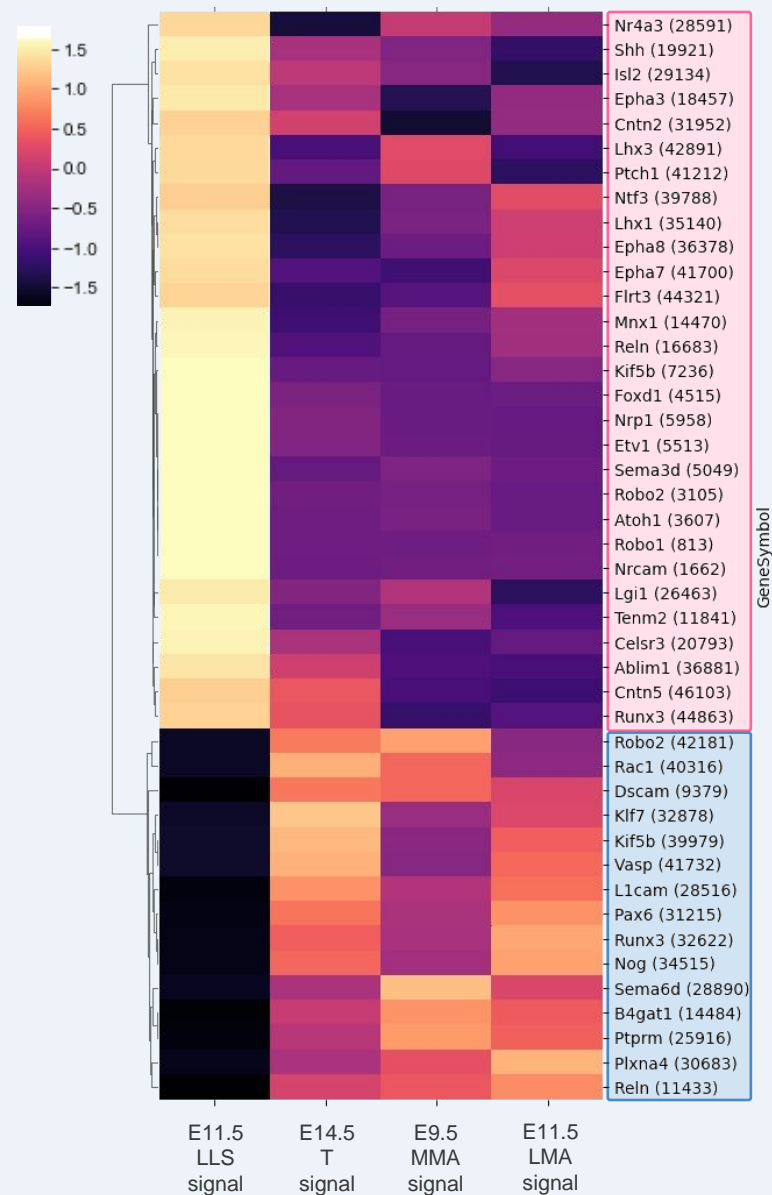## Fig. 6-b: Heatmap in LLS with ROKU method.

Fig. 6. As the base setting, we selected "axon guidance" ontology from QuickGO.

ROKU method provided 3,940 genes that were LLS-specific. In combination with GO, ROKU algorithm narrowed down the target to genes related to axon guidance, and computed 29 up-regulated and 15 down-regulated genes in LLS at E11.5.

The heatmap drawn by ROKU method branches LLS-specific up- and down-regulated genes. The numbers next to the gene symbols indicate the ranking. The higher the ranking, the more specific it is in LLS.

MMA : Median Mandibular Arches
LLS : Lateral Lingual Swelling
LMA : Lateral Mandibular Arches
T : Median Tongue

Result: Seeking pathways involved in neuronal axon guidance by IPA analysis.

Fig. 7-a: Results of IPA analysis with Z-score based analysis.
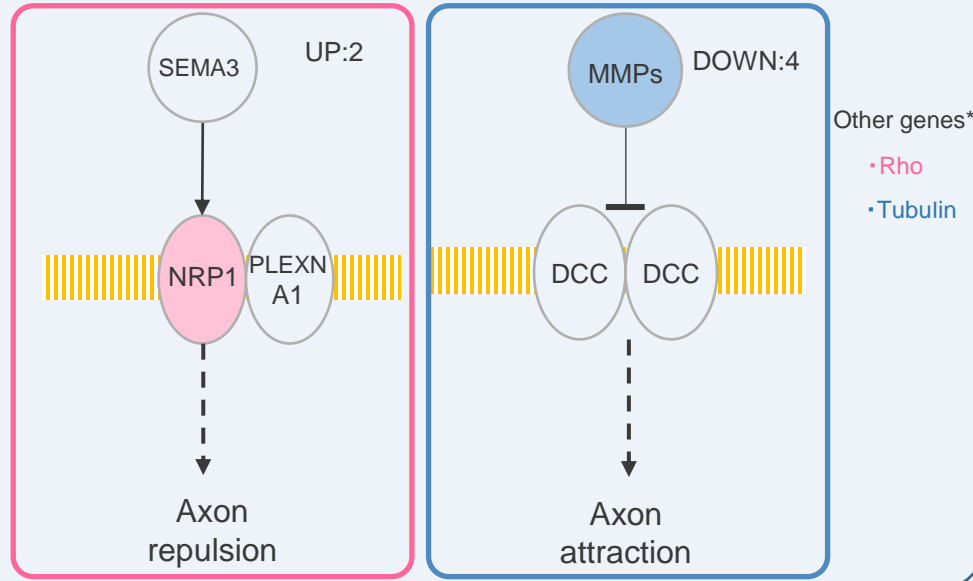

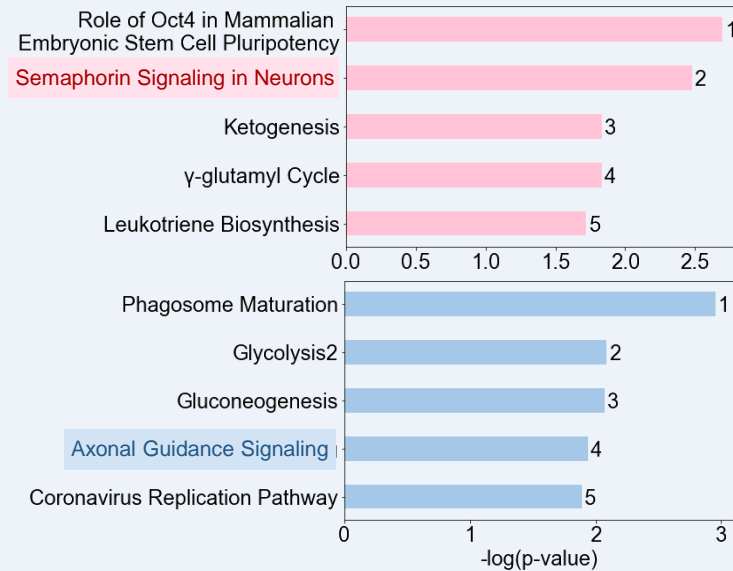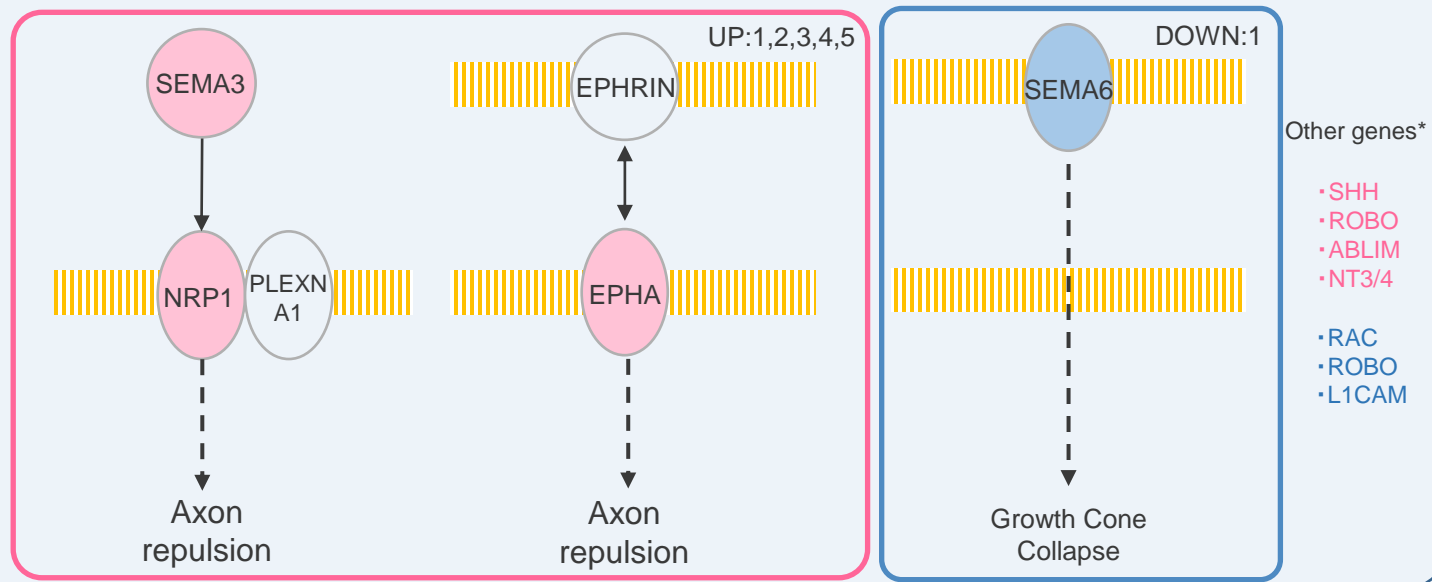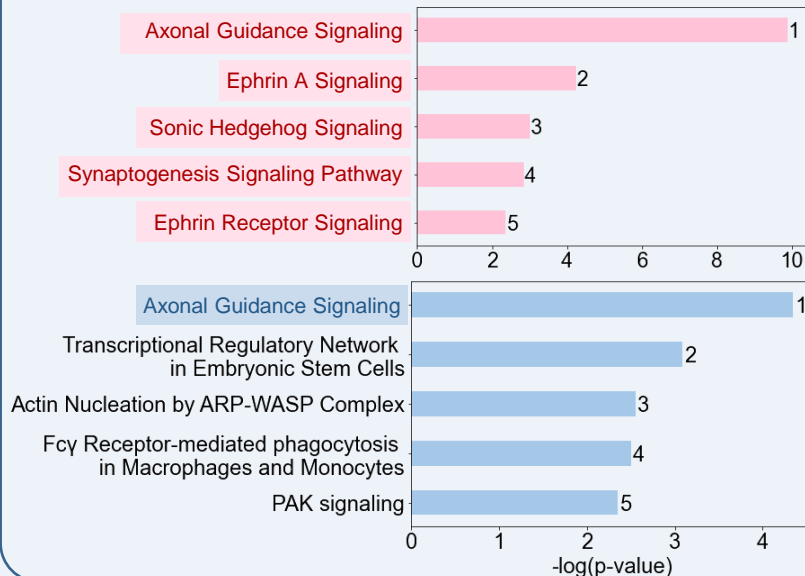## Fig. 7-b: Results of IPA analysis with ROKU method.

Fig. 7. Datasets from both Z-score based analysis and ROKU method were analyzed by IPA (Ingenuity Pathway Analysis) platform.

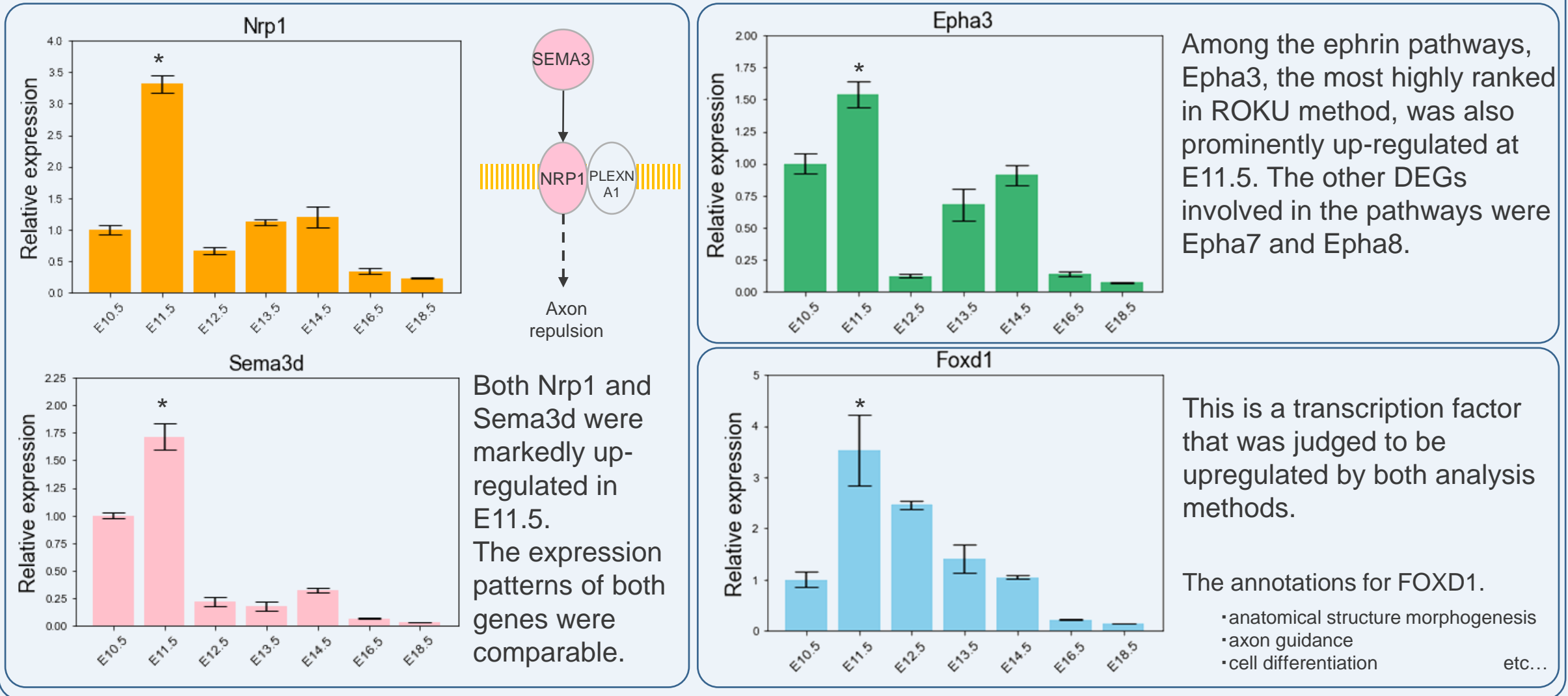Note that NRP1-mediated pathway was drawn from both approaches.

* indicates genes other than focused in this study.

Result: q-PCR of the expected reach of the hypoglossal nerve in mice

Fig. 8: The results of relative quantification by q-PCR.

Nrp1

Sema3d

SEMA3 → NRP1 PLEXN A1 ⇢ Axon repulsion

Epha3

Foxd1

Among the ephrin pathways, Epha3, the most highly ranked in ROKU method, was also prominently up-regulated at E11.5. The other DEGs involved in the pathways were Epha7 and Epha8.

Both Nrp1 and Sema3d were markedly up-regulated in E11.5.
The expression patterns of both genes were comparable.

This is a transcription factor that was judged to be upregulated by both analysis methods.

The annotations for FOXD1.
- anatomical structure morphogenesis
- axon guidance
- cell differentiation   etc…

Data in q-PCR showed that the expression of each gene was up-regulated in the region of E11.5 where the hypoglossal nerve is expected to reach area. The expression of each gene was significantly higher in the E11.5 sample than in all other samples by using Tukey-Kramer method (*$p<0.01$). Bars; mean ± SE.

Conclusion: Comparison of the analysis methods in this study used.

Table1: The difference between Z-score analysis and ROKU method.

Z-score based analysis		ROKU method
No	Creating a ranking	Yes By Shannon entropy
Detection of DEGs using the (Intensity-based) Z-Score of logFC.	Gene detection method	AIC-based detection of expression levels of a gene between tissues.
Yes In this study, |Z|>2.0 .	Setting the threshold	No Determination by AIC
Yes Filtering the comparison results by Venn diagram.	Data Preprocessing before IPA analysis	Yes Filtering by Gene Ontology

DEGs: Differential Expressed Genes   AIC: Akaike's Information Criterion

Table 1. Our survey clearly demonstrated that the genes with LLS-specific change could be practically filtered using either method. Through IPA analysis, we found that Z-score based analysis strictly narrowed down the genes in trend, while ROKU method modestly narrowed down, thus it needs to be oriented by Gene Ontology. Taken together, combinatorial use of Z-score based analysis and ROKU method largely helped us to verify gene expression dataset with multiple comparison axes.

Conclusion: Semaphorin signaling may be involved in hypoglossal nerve guidance.

Fig. 9. A series of analyses confirmed that the semaphorin signaling pathway was significantly upregulated in lateral lingual swellings. Quantitative PCR also verified that Nrp-1 and Sema3d were specifically upregulated in LLS. In particular, the Nrp-Sema3d axis is known to have a repulsive effect on neuronal axons. Therefore, we tentatively conclude that Nrp-1 and Sema3d genes may be involved as neural guidance factors in the region of the prospective hypoglossal nerve reach in the early stage of tongue development.

The hypothesis formation from this study

E11.5

The prospective hypoglossal nerve reach area

repulsion

The other Nrp1(+) cranial nerves

Extension to appropriate position

Sema3d expression is represented as pink

Hypoglossal nerve

Foxd1 may regulate Nrp1 and Sema3d genes.

FOXD1 ?

FOXD1 RNAPol Nrp1

FOXD1 RNAPol Sema3d