Ectopic expression of *lunatic Fringe* leads to downregulation of *Serrate-1* in the developing chick neural tube; analysis using in ovo electroporation transfection technique

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Abstract ***Lunatic Fringe* (*l-Fng*) is one of the vertebrate homologues of *Drosophila Fringe*, which interacts with the *Notch* signal pathway and regulates activation of the *Notch* ligands, *Delta* and *Serrate*. To elucidate the roles of *l-Fng* in vertebrate neurogenesis, we transfected chick *l-Fng* (C-*l-Fng*) to chick neural tube using the in ovo electroporation technique and examined the subsequent changes in expression of *Notch*-related genes. We observed downregulation of C-*Serrate-1* by ectopic C-*l-Fng* expression which implied that C-*l-Fng* acts on the vertebrate *Notch* pathway to regulate the expression of its ligand.**

Key words: Chicken; *Lunatic Fringe*; *Notch* signal pathway; Neurogenesis; In ovo electroporation

1. Introduction

The *Notch* (*N*) signal pathway mediates cell fate decision in various stages of development [1–3]. In *Drosophila* wing development, *Fringe* (*Fng*) modifies the signaling between *Notch* and its ligands, *Delta* (*Dl*) and *Serrate* (*Ser*) [4,5]. Ectopic expression of *Fng* induces *Ser* expression and leads to ectopic wing outgrowth [6,7]. In vertebrates, several homologues of these genes have been identified and shown to be associated with organogenesis in various manners [8–13].

We previously reported that one of the vertebrate *Fng* homologues, chick *lunatic Fringe* (C-*l-Fng*), is involved in somitogenesis and neurogenesis [12]. In the developing neural tissues, C-*l-Fng* is expressed in limited populations of neuroblasts. In the neural tube, its expression exhibits a rostro-caudal stripe pattern [8–10,12,13]. This pattern is implicative when compared with those of the putative C-*Notch* ligands, C-*Serrate-1* (C-*Ser-1*) and C-*Delta* (C-*Dl*). C-*l-Fng* expression strikingly overlaps with C-*Dl* and is complementary to C-*Ser-1* [8,10,12,14–16]. These observations suggest a tight interaction between these genes and an essential role of C-*l-Fng* in the *Notch* signal pathway.

In this paper, we describe the technique, in ovo electroporation, that enabled us to introduce ectopic gene expression in embryos. We transfected C-*l-Fng* to chick neural tubes and analyzed the changes of expression of *Notch*-related genes.

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2. Materials and methods

2.1. Vector construction

C-*l-Fng* was cloned as we previously described [12]. For the expression construct (clfgf(−)), C-*l-Fng* cDNA containing the entire open reading frame was inserted into PBK-CMV plasmid vector (Stratagene) downstream of the CMV immediate early promoter. For the tagged construct (clfgf(+)), C-*l-Fng* cDNA was ligated to a 45 bp PCR product coding the FLAG epitope, replacing the last 50 bp of the coding region and inserted into PBK-CMV. PmiwZ was kindly provided by Dr. H. Kondoh at the Institute of Molecular and Cellular Biology of Osaka University [17].

2.2. In ovo electroporation

In ovo electroporation was performed as previously described [18,19] with the following modifications. Plasmids were dissolved at high concentration (2 μg/μl) in PBS containing 1 mM $MgCl_2$ instead of TE buffer, which improved the transfection efficiency. Nile blue was added to the plasmid solution, which enabled us to monitor the injection. Nile blue did not affect the transfection efficiency. Direct contact of electrodes with blood islands caused severe electric damage and reduced the survival rate. We found that the damage could be minimized by placing the electrodes onto the vitelline membrane.

Chicken eggs were incubated at 37°C until they reach Hamburger and Hamilton (HH) stage 10–12 [20]. In order to inject the plasmid solution into the neural tube, a pinhole was made in the neural tube at the level of somite segmentation to release the injection pressure (Fig. 1A). The injection was performed through a microcapillary into the neural tube from its most caudal part. A set of parallel electrodes, 4 mm in distance, were attached onto the vitelline membrane to sandwich the embryo and a small amount of Hanks' solution was dropped between the electrodes. Resistance between the electrodes was monitored and adjusted to 1.2–2.0 KΩ by removing excessive Hanks' solution. Square pulses (40 V, pulse length 60 ms, ×6) generated by an Electro Square Porator T820 (BTX) were immediately given. The embryos were incubated for proper periods as described in Section 3.

2.3. X-gal staining and immunofluorostaining

The embryos transfected with PmiwZ were fixed in 4% paraformaldehyde (PAF)/PBS for 30 min and subjected to X-gal staining as previously described [17].

The embryos transfected with clfgf(+) were fixed in 4% PAF/PBS for 30 min, briefly rinsed, cryoprotected and embedded in OCT compound (Miles Inc.) 12 μm cryosections were prepared. Immunofluorostaining using anti-FLAG M2 antibody (Kodak) was performed according to the manufacturer's protocol.

2.4. Probes for in situ hybridization

The following fragments were used as templates for RNA in situ hybridization. C-*l-Fng*: 1.5 kb fragment from random primed chick E4 cDNA library; C-*Ser-1*: 1.1 kb PCR product from chick E5 cDNA; C-*Dl*: 1.0 kb PCR product from chick E5 cDNA; C-*Wnt-4*: 1.0 kb fragment from oligo-dT primed chick E4 cDNA library; C-*En-1*: 0.6 kb fragment from chick E4 cDNA library. C-*Notch*-1, -2 (C-*N-1*, -2: 2.4 kb, 1.8 kb respectively) were kindly provided by H. Hamada at the Institute of Molecular and Cellular Biology of Osaka University.

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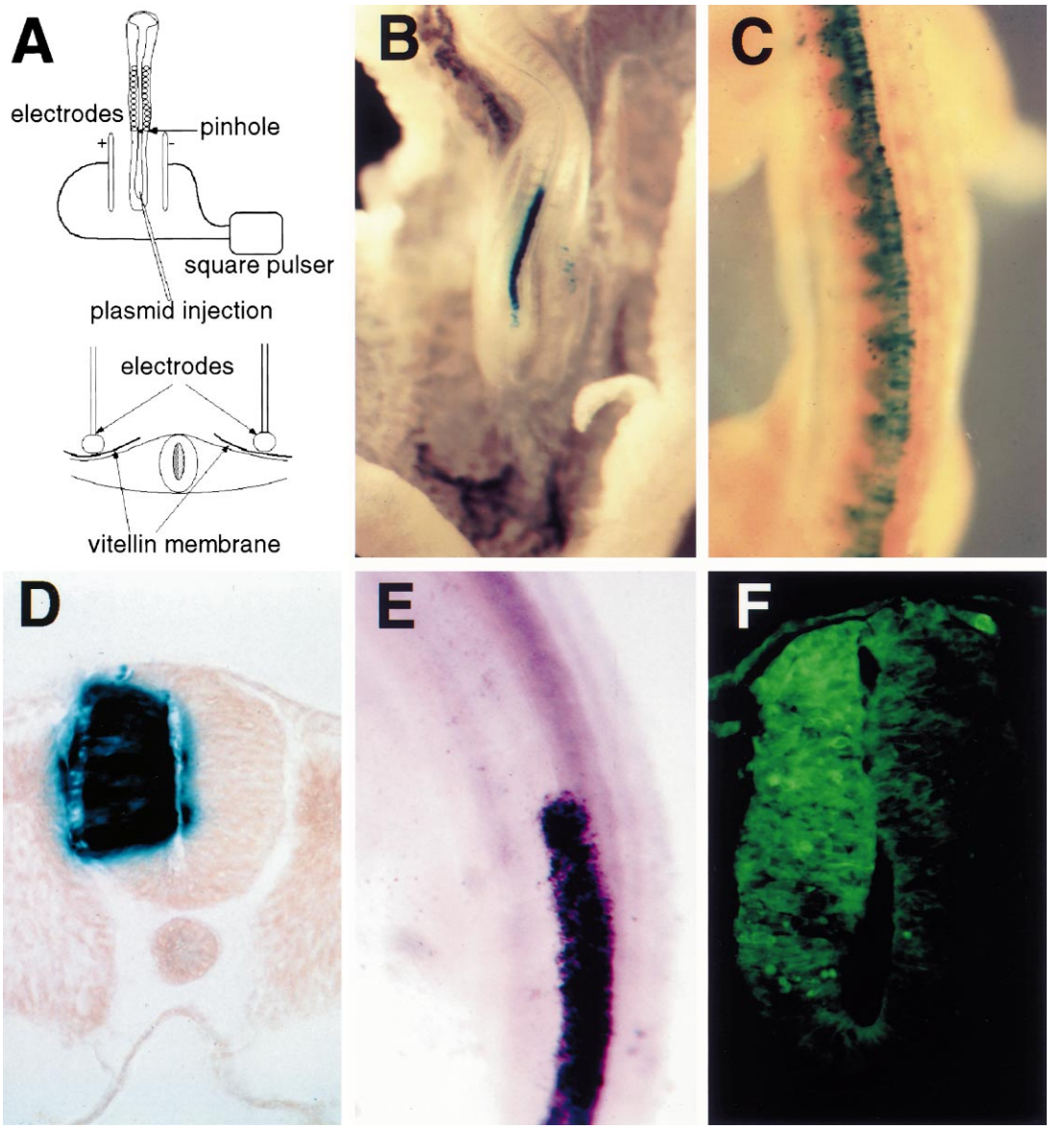

Fig. 1. In ovo electroporation to the neural tube. A: Schematic illustrations of in ovo electroporation method. B: X-gal stained embryo, transfected with PmiwZ at HH stage 11 and incubated for 12 h. Only the left side of the neural tube was transfected and positively stained. Weak ectodermal staining on the right side is due to spilt plasmid at the injection. C: X-gal stained embryo, transfected with PmiwZ at HH stage 11 and incubated for 48 h. the β-gal positive cell population became relatively sparse but was still present. The dorsal root ganglia were also positively stained. D: Transverse section of B. The neural tube was transfected hemilaterally. E: C-*l-Fng* RNA expression. The embryo at HH stage 12 was transfected with clfgf(−), incubated for 24 h and hybridized in situ with the C-*l-Fng* RNA probe. The weak stripe pattern represents the intrinsic expression. The exogenous expression was more intense than that. F: Expression of the exogenous C-*l-Fng* fusion protein. Section view of the neural tube. The embryo was transfected with clfgf(+) and incubated for 12 h. C-*l-Fng*/FLAG fusion protein was detected by immunofluorostaining using anti-FLAG-1 M2 antibody.

2.5. Whole mount in situ hybridization

Whole mount in situ hybridization was performed basically as previously described [12]. In brief, hybridization was done at 68°C with digoxigenin or/and fluorescein labeled RNA probe. After extensive washes, alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody Fab fragment (Boehringer-Mannheim) was diluted to 1/1000 and applied. The coloration was performed in NBT/BCIP substrate. For double labeled whole mount in situ hybridization, the AP of conjugated anti-digoxigenin Fab fragment was inactivated by several washes with methanol after NBT/BCIP coloration and the second coloration was performed by AP-conjugated anti-fluorescein antibody Fab fragment (Boehringer-Mannheim) and INT/BCIP substrate. The embryos were embedded in paraffin for sectioning after whole mount in situ hybridization procedures.

3. Results

3.1. In ovo electroporation to the caudal part of the neural tube

We used chicken embryos at HH stage 10–12. We could minimize the influence of intrinsic C-*l-Fng* expression as C-*l-Fng* expression in the neural tube was still undetectable at these stages. We checked the efficiency of transfection, using PmiwZ plasmid as a transfectant. Several conditions of electroporation were examined and the condition that yielded both high survival and transfection efficiency was chosen (see Section 2). Under this condition, most of the embryos developed normally with substantial transfection efficiency

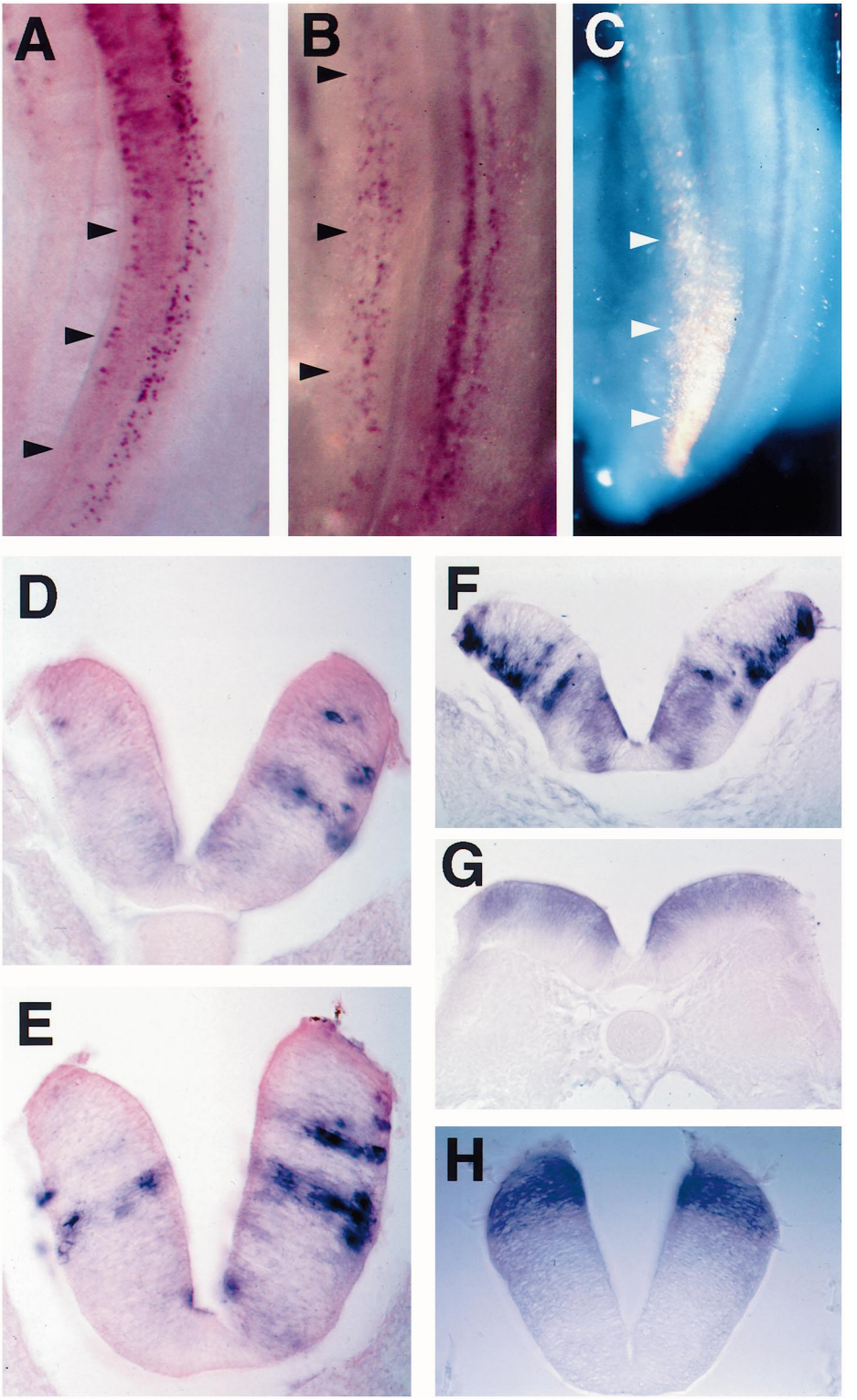

Fig. 2. Effects of forced expression of C-*l-Fng* in the neural tube. A: C-*Ser-1* expression at HH stage 15, when C-*Ser-1* started its expression. Expression on the transfected side (left) had been reduced (arrowheads). B: C-*Ser-1* expression at HH stage 19, when the stripe pattern became distinct. C-*Ser-1* was downregulated on the transfected side (arrowheads). C: Double labeled in situ hybridization of HH stage 20 embryo with C-*l-Fng* (yellow) and C-*Ser-1* (blue) probes. The region of C-*Ser-1* downregulation corresponded to the ectopic expression of C-*l-Fng* (arrowheads). D,E: Transverse section of A and B. The expression was attenuated but continuous, suggesting the cells were still maintaining their C-*Ser-1* expressing fate. F–H: Expression patterns of C-*Dl* (F), C-*N-1* (G) and C-*Wnt-4* (H) were not altered (HH stage 19).

(data not shown). Impaired development was apparently attributable to the damage on blood islands and circulation deficiency, which could be avoided by placing the electrodes onto the vitelline membrane. The best stage for transfection was HH stage 10–16. The expression was maximum at 8–24 h after transfection.

Fig. 1B,D shows the β-galactosidase expression of HH stage 17 embryos at 12 h after transfection of PmiwZ. It is characteristic with this method that the neural tube is transfected hemilaterally on the side of the positive electrode due to the negative charge of nucleic acids. On the transverse sections, it was evident that the dorsal region was transfected more efficiently than the ventral region, which was probably due to the local electrokinetics. The dorsal 1/2–1/3 region was positively stained 24 h after transfection (data not shown). The coloration was still visible 48 h after transfection. The dorsal root ganglia derived from the neural crest were also positively stained (Fig. 1C).

3.2. Effects of the ectopic C-l-Fng expression

To investigate the effects of ectopic C-*l-Fng* expression, we transfected the neural tube with clfgf(−). The expression of exogenous C-*l-Fng* was more intense than the intrinsic expression as shown in Fig. 1E. To check the translation of exogenous C-*l-Fng*, we transfected clfgf(+) and performed immunohistochemical staining using anti-FLAG antibody. It was confirmed that C-*l-Fng* fusion protein had been produced in transfected cells in vivo (Fig. 1F).

Embryos transfected with clfgf(−) developed without apparent morphological anomalies. We analyzed its effects on expression of other neurogenic genes. We transfected C-*l-Fng* to HH stage 10 embryos ($n = 50$) and investigated C-*Ser-1* expression at HH stage 13 when neither C-*l-Fng* nor C-*Ser-1* was expressed intrinsically in the transfected region. Double labeled in situ hybridization revealed intense expression of ectopic C-*l-Fng*, but C-*Ser-1* expression was not induced (data not shown). We also investigated C-*Dl* ($n = 50$) and C-*N-1* ($n = 20$) expression, but their induction due to ectopic C-*l-Fng* was not observed (data not shown).

To see whether C-*l-Fng* alters the expression patterns of *Notch*-related genes at later stages, we examined their expressions at HH stage 15 and 19. Fig. 2A shows the transfected region at HH stage 15 when C-*Ser-1* starts its intrinsic expression, exhibiting the scattered localization of the C-*Ser-1* positive cells. C-*Ser-1* signal was apparently decreased on the transfected side (Fig. 2A,D). At HH stage 19, it was evident that the stripe on the transfected side was indistinct as compared to the contralateral side (Fig. 2B). Transfection with mock DNA (PBK-CMV without insert) had no effects on C-*Ser-1* expression. Double labeled in situ hybridization confirmed that the downregulation of C-*Ser-1* corresponded to the region of the ectopic C-*l-Fng* expression (Fig. 2C). These observations indicate that C-*Ser-1* was downregulated as a consequence of ectopic C-*l-Fng* expression. The transverse sections demonstrated that C-*Ser-1* expression in the transfected region was attenuated although the stripe pattern was still visible (Fig. 2D,E).

We also examined C-*Dl*, C-*N-1*, C-*N-2*, C-*En-1* and C-*Wnt-4* expression at HH stage 19 following C-*l-Fng* transfection. The *Drosophila* equivalents of these genes interact with one another and play key roles in wing development. C-*Dl* expression exhibited the stripe pattern as reported previously, but there was no alteration of this pattern even in the transfected region ($n = 30$) (Fig. 2F). The expression patterns of C-*N-1* ($n = 15$) (Fig. 2G), C-*Wnt-4* ($n = 15$) (Fig. 2H) and C-*En-1* ($n = 15$) (data not shown) were not altered either. C-*N-2* expression was not detectable in the neural tube at this stage (data not shown).

4. Discussion

In *Drosophila*, *Notch* and *Wingless* (*Wg*) signaling are essential components for wing margin formation [21–23]. There is a positive feedback loop between *Dl* in the ventral cells and *Ser* in the dorsal cells so that they regulate each other [5,24]. Stimulation of the *Notch* signal pathway activates *Wg* and leads to wing outgrowth [21–23,25]. *Fng* mediates the interaction between *Notch* and its ligands by exerting opposing effects on *Dl-N* and *Ser-N* [4,5]. The former is potentiated by *Fng*, whereas the latter is blocked. This selective effects of *Fng* restricts the position of *Dl-Ser* feedback loop to the dorso-ventral (D-V) boundary of the wing disc, which gives rise to the wing margin [5].

In vertebrates, three homologues of *Fng* have been identified (*radical*, *manic*, and *lunatic Fringe*) [8–13]. Chick *radical Fringe* (C-*r-Fng*) regulates the apical ectodermal ridge (AER) formation and limb outgrowth. Its ectopic expression is likely to have the AER forming activity and gives rise to additional digits [10,11]. *Serrate-2* (*Ser-2*) and *Notch-1* are also expressed in the AER [10,11,26]. Germline mutation of *Ser-2* causes the arrest of limb development [27]. These results imply that *Fng*/*Ser* interaction is also involved in vertebrate limb formation.

C-*l-Fng* expression in the developing neural tube exhibits a stripe pattern which is complementary to C-*Ser-1* and overlaps with C-*Dl* [8,10,12]. This unique expression pattern indicates the possibility that C-*l-Fng* associates with the *Notch* signal pathway through interaction with C-*Dl* or C-*Ser-1*.

To understand the role of C-*l-Fng* in the vertebrate *Notch* signal pathway and early neurogenesis, we conducted gain of function experiments. Several methods are available for gain of function in chick embryos: implantation of carriers such as resin beads containing the factor, transplantation of cells or tissues expressing the gene and viral transfection. *Fng* proteins have been shown to be secretory [9,13]. But implantation of carriers or transplantation of cells did not seem to be applicable to C-*l-Fng*, because *Fng* is implicated to function in a cell-autonomous fashion [5]. Retroviral mediated transfection did not seem to be suitable for rapidly growing tissues like neural tube. Retrovirus requires one passage of cells for its gene expression so that at least one day is necessary for wide range expression in the neural tube.

The in ovo electroporation technique has been improved recently, which holds several advantages on C-*l-Fng* expression in the neural tube. First, the expression is strong and rapid. It was observed at high level at only 6 h after transfection (data not shown). Second, the transfection is hemilateral in the neural tube, which permits the untransfected side to serve as a control. Third, co- or multi-transfection is possible, which was difficult by means of viral transfection.

Intrinsic C-*l-Fng* had already shown its stripe pattern expression before the examined stage. Clfgf(−) transfection transformed it into ubiquitous expression. Only C-*Ser-1* expression was attenuated by C-*Fng-1* in the examined Notch signal-related genes, but its stripe pattern was still visible. This indicates that C-*Ser-1* positive cells still preserve their fate under C-*l-Fng* influence. A possible explanation for this observation is that C-*Ser-1* upregulation depends on the C-*l-Fng*(−) *Notch* signal pathway. Ectopic C-*l-Fng* expression modulated this *Notch* signal and prevents C-*Ser-1* upregulation. Interactive positive feedback regulation model of *Ser* and *Dl* in *Drosophila* supports this idea [5].

But it should be noted that there are fundamental differences in *Dl*, *Ser* and *Fng* expression patterns between the *Drosophila* wing and the chick neural tube. At the D-V boundary of *Drosophila* wing, *Ser* and *Fng* are expressed dorsally. *Dl* is required in the ventral region, but it is expressed in the ventral and dorsal compartments [5]. Therefore, they are not perfectly complementary to each other and it is *Ser* that overlaps with *Fng*. By these indications, we cannot deny the possibility of a vertebrate-specific mechanism although their regulatory mechanism is likely to be basically conserved. Further investigation is required in order to clarify how C-*l-Fng* associates with the *Notch* signal pathway. We are trying to perform co-transfection of C-*l-Fng*/C-*Dl* or C-*l-Fng*/C-*Ser-1* to determine if C-*l-Fng* has enhancing or silencing effects on the vertebrate *Notch* signal pathway.

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