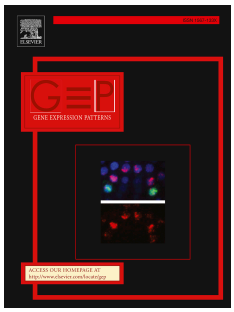

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Expression Analysis of Chick Frizzled Receptors during Spinal Cord Development

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Abstract:

Frizzleds (Fzds) are transmembrane receptors that can transduce signals dependent upon binding of Wnts, a large family of secreted glycoproteins homologous to the Drosophila wingless gene. FZDs are critical for a wide variety of normal and pathological developmental processes. In the nervous system, Wnts and Frizzleds play an important role in anterior-posterior patterning, cell fate decisions, proliferation, and synaptogenesis. Here, we preformed a comprehensive expression profile of Wnt receptors (FZD) by using situ hybridization to identify FZDs that are expressed in dorsal-ventral regions of the neural tube development. Our data show specific expression for FZD1,2,3,7,9 and 10 in the chick developing spinal cord. This expression profile of cFZD receptors offers the basis for functional studies in the future to determine roles for the different FZD receptors and their interactions with Wnts during dorsal-ventral neural tube development *in vivo*. Furthermore, we also show that co-overexpression of Wnt1/3a by *in vivo* electroporation affects FZD7/10 expression in the neural tube. This illustrates an example of Wnts-FZDs interactions during spinal cord neurogenesis.

1. Introduction

In the late 1990's Frizzleds were identified as the elusive Wnt receptor. They have been shown to be involved in regulation of several embryogenesis events such as, cell proliferation, neural patterning, and neural synapse development (Clevers, 2006; Medina et al., 2000). The mis-regulation of FZDs has been reported to be involved in many diseases including cancer and neural tube defects (Luo et al., 2007; Malaterre et al., 2007; Ueno et al., 2013; Wang et al., 2006).

In chicken, ten Frizzled receptor genes have been identified and numbered from 1 to 10. Expression of cFzds is dynamic during chick embryogenesis and is found in different developing tissues (Chapman et al., 2004; Fuhrmann et al., 2003; Quinlan et al., 2009; Stark et al., 2000; Theodosiou and Tabin, 2003). Most of the cFzds are expressed in early stages of development during pattering and neural specification. Six cFZD receptors show overlapping expression in the primitive streak and Hensen's node, including cFzd 1, 2, 4, 7 and cFzd9, except cFzd8 is exclusively expressed in Hensen's node (Chapman et al., 2004).

All cFzds, with the exception of cFzd10, are expressed in the developing eye and their expression is specific in different parts of the eye (Fuhrmann et al., 2003). In addition, it was found that, cFzd1, 2 and 7 are present in the cranial placodes and in the somite during development (Stark et al., 2000). Their expression patterns are shown in early forming somites and overlap with myogenic markers such as MyoD (Linker et al., 2003).

Blockade of FZD signalling by cFZD7-CRD electroporation reduces MyoD expression, indicating the importance of FZDs during myogenesis. Also, it was shown that, cFzd7 mediates Wnt6 function through the canonical Wnt signalling in the dorsal part of the somite (Linker et al., 2005). Gros and colleagues, 2009 documented that cFzd7 inhibition *in vivo* affects cell polarity and elongation of myocytes in the myotome of chick somites. This was not dependent on canonical mechanisms and suggested that cFzd7 might act as Wnt11 receptor through non-canonical Wnt signalling (PCP).

A number of cFzds, including cFzd1, 2, 4 and 7, are expressed strongly in the limb bud at different stages of development (Kengaku et al., 1997; Nohno et al., 1999; Stark et al., 2000). cFzd2 is found in the proximal limb mesenchyme and cFzd4 exhibits a specific expression pattern in the apical ectodermal ridge of the limb (Nohno et al., 1999). By stage HH30, cFzd4 shows high expression in the cartilage and in the interdigital spaces (Stark et al., 2000). cFzd1 and 7 expression is dynamic and distinct; cFzd1 expression is located on the ventral side of ectoderm and mesenchyme, whilst cFzd7 is expressed in the proximal-distal axis of the limb bud (Kengaku et al., 1997). cFzd1 and cFzd7 are expressed during chondrogenesis in the limb and their misexpression in chick wing reduces chondrogenesis (Hartmann and Tabin, 2000). This study suggested that cFzd7 mediates Wnt4 signal in the articular chondrocytes. Another study showed that cFzd7 misexpression reduced chondrogenesis in chick limb culture (micromass), but cFzd1 misexpression did not have any effects (Tufan et al., 2002).

Various cFzds receptors have been shown to be expressed in kidney, liver and the gut during the development of chick embryo (Theodosiou and Tabin 2003; Matsumoto et al., 2008; Stark et al., 2000). cFzd4 shows a striking expression pattern in chick kidney and

colocalizes with Wnt4 and other kidney markers (Stark et al., 2000). cFzd1, 4, 7 and 9 are also detected in the chick liver, and cFzd7 and cFzd9 expression are overlapping with Wnt9a expression (Matsumoto et al., 2008).

CFzds are also expressed during the development of the central nervous system (Chesnutt et al., 2004; Galli et al., 2014; Kawakami et al., 2000; Quinlan et al., 2009). It has been reported that cFzds exhibit complex and dynamic expression patterns in different regions of the developing brain (Quinlan et al., 2009). For example, cFzd1, 2 and 7 show strong expression in the ventral midbrain, while cFzd4 and 8 are expressed in the forebrain. CFzds expression patterns overlap with many Wnt ligands in the mid and forebrain including Wnt1, Wnt3a, Wnt5A, and Wnt8B. This illustrates that Wnts and their receptors are present during the development of the brain. Also, several Wnt genes are expressed in the neural tube, namely, Wnt1, Wnt3, Wnt3a, Wnt4, Wnt7a and Wnt7b in deferent species including chicken (Alvarez-Medina et al., 2008; Megason and McMahon, 2002; Parr et al., 1993). Studying cFzds expression and intractions with Wnts during neural tube neurogenesis is important to understand their roles in this biological event.

In this study, we investigated the expression of all cFzds during development of neural tube to identify cFzds that are expressed in dorsal-ventral part of neural tube. In situ hybrisdation was used to observe and analyse cFzds expression and to perform a complete expression prolife of cFzds in dorsal-Ventral neural tube at the onset of neurogenesis. Moreover, we used *in vivo* electroporation to study the effect of Wnt1/3a on FZD7/10 expression in the neural tube.

2. Results

2.1. Characterisation of cFzds expression in the chick developing spinal cord:

Many Wnts are expressed in neural tube (Alvarez-Medina et al., 2008; Megason and McMahon, 2002; Parr et al., 1993) but their specific receptors for each Wnt are still to be investigated. To identify which Fzds that may be involved in the development of the spinal cord, a detailed analysis of the expression patterns of these receptors was performed in chick embryos. Specific antisense RNA probes were generated for each cFzd then whole mount in situ hybridization was used followed by cryo-sections (Table 1). Figure 1 shows the expression of each cFzd in the HH14/15 neural tube. CFzd1 expression was seen in the dorsal spinal cord covering the roof plate and was graded dorsal to ventral at stage HH14-15 (Fig.1B). CFzd2 transcripts were detected in the intermediate ventral spinal cord and high level of expression was observed in the floor plate region (Fig.1C). CFzd3 was strikingly expressed throughout the dorsal ventral spinal cord and was restricted in the ventricular zone (Fig.1D). CFzd4, 5 and 6 were not present in the developing spinal cord at stage HH14-15 (Fig. 1E, F, G). CFzd7 showed specific and strong expression in the intermediate and ventral spinal cord (Fig.1H). CFzd8 did not appear to be expressed in the posterior neural tube (Fig.1I). CFzd9 was ubiquitously expressed and seemed to be strongly expressed around the lumen of the chick spinal cord exluded the roof plate (Fig.1J). CFzd10 transcripts were specifically expressed in the dorsal spinal cord and strong signal was seen in the dorsal neural progenitors (Fig. 1K). As a result, we identified that six out of ten cFzds were expressed in the spinal cord and showed specific and different expression patterns. CFzd1,2,3,7,9 and 10 are expressed during neurogenesis of neural tube, but cFzd4, 5, 6 and 8 are not.

These results are summarized in supplementary Table S1.

Fig.1 Expression patterns of cFzds in the developing spinal cord were detected by in situ hybridization at stage HH14-15. (A) Cross section of spinal cord with three domains of neural progenitors indicated. (B) Expression of cFzd1in the dorsal spinal cord. (C) cFzd2 expression in the ventral spinal cord. (D) cFzd3 expression throughout the spinal cord. (E,F,G and I) cFzd4, 5, 6 and 8 are not expressed in the spinal cord. (H) Expression of cFzd7 in the ventral spinal cord. (J) cFzd9 expression around the lumen of the spinal cord. (K) cFzd10 expression in the dorsal spinal cord.

2.2. CFzd7 and cFzd10 are expressed during spinal cord neurogenesis:

CFzd7 and cFzd10 are expressed during early neural specification and their expression continues throughout embryogenesis (Chapman et al., 2004). We showed that they are strongly expressed in specific domains in the spinal cord at stage HH14-15 during the initiation of neurogenesis (Fig. 1H, K and Table S1).

It has been reported that almost half of all neurons are generated after 4days of development in chick spinal cord (HH24) (Le Dréau et al., 2012). Thus, we investigated the spatial and temporal expression of cFzd7 and cFzd10 in this stage of development (Fig.2A). CFzd7 transcripts were found to be highly expressed in the intermediate and ventral spinal cord where the intermediate and ventral progenitors are located and its expression pattern correlated with Pax6 and Nkx2.2 expression (Fig. 2C, D, E). Whereas cFzd10 expression was detected in the dorsal domains of neural progenitors and its

expression correlated with Pax7 and partly with Pax6 (Fig. 2B, C, F). Both, cFzd7 and cFzd10 expression seems to be restricted to the proliferative progenitors whilst neurogenesis takes place and their expression was not detected in differentiating neurons (Fig. 2A, E, F).

Fig.2 Expression patterns of cFzd7 and cFzd10 correlate with neural progenitors markers in the spinal cord at stage HH24. (A) Cross section of the developing spinal cord at stage HH24 stained with DAPI; red circles mark six dorsal progenitor domains while green circles represent five ventral progenitor domains. VZ, the ventricular zone; MZ, the mantle zone. (B) Pax7 stained 6 dorsal progenitors. (C) Pax6 marked intermediate progenitors. (D) Nkx2.2 is a marker for p3 ventral progenitors. (E) cFzd7 expression in the intermediate ventral progenitors and excluded from MZ. (F) cFzd10 expression in the dorsal domain of the spinal cord during neurogenesis.

2.3. CFzd7 and cFzd10 expression is altered by dorsally expressed Wnts (Wnt1/3a) in the spinal cord:

We showed previously cFdz7 expression is found in the intermediate-ventral domain of the developing spinal cord and cFzd10 is expressed in the dorsal domain (Fig. 2E ,F).

Here, we investigated how Wnt1 and Wnt3a regulate the expression of both cFzds in the developing spinal cord, thus we used *in ovo* electroporation, immunohistochemistry and in situ hybridization. Electroporation allowed us to overexpress Wnt1 and Wnt3a ectopically, along the dorsal-ventral axis of the chick spinal cord. We electroporated Wnt1 or Wnt3a construct individually in chick neural tube at stage HH11-12 (Fig. 3A, B). Embryos were dissected after 24 hours post electroporation and then screened for GFP, with GFP expressed from the same plasmid. Both constructs resulted in GFP expression after electroporation as seen in dorsal view of whole mount pictures of injected embryos (Fig. 3A, B). Then, we co-electroporated Wnt1 and Wnt3a in chick neural tube at stage HH11-12. After 24 hours embryos were dissected, fixed and cryosectioned to visualize GFP in the transfected side of the neural tube before immunostaining (Fig. 3C).

To analyse the effects of Wnt1/3a co-overexpression in neural tube we used neural markers such as Pax7 and Nkx2.2. Pax7 is a marker for six dorsal progenitors and it was ventrally expanded on the transfected side after Wnt1/3a misexpression compared to the control side (n=8/9) (Fig. 3C). Nkx2.2 is expressed in the ventral neural tube and it was severely repressed in the transfected side compared to control side (n=8/9) (Fig. 3C).

We next investigated cFzd7 and cFzd10 expression following Wnt1/3a overexpression. Embryos showed strong GFP expression after co-electroporation were processed for whole mount in situ to analyze changes in the expression patterns. We found cFzd7 expression was lost in the intermediate domain and ventrally restricted on the electroporated side of neural tube compared to the control side (n=13/15) (Fig. 3E and

Fig. S1). This may indicate that expression of ventrally expressed cFzds (cFzd7) is repressed by dorsally expressed Wnts (Wnt1/3a) in the developing spinal cord. Conversely, cFzd10 expression was strongly upreglated and ventrally expanded on the electroporated side of neural tube (n=12/15) (Fig. 3D and Fig. S2).

Fig. 3 Over-expressed Wnt1 and Wn3a affect cFzd10 and cFzd7 expression in chick spinal cord (24hours PE). (A,B) Dorsal views of whole mount pictures of electroporated embryos show GFP expression in chick neural tube. (A',B') Green colour shows GFP expression after Wnt1 and Wnt3a electroporation indicating successful transfection. (C) Cross section of the neural tube shows GFP expression (green) in the electroporated side, Pax7 expression is ventrally expanded (red), Nkx2.2 expression was repressed in the ventral (red) (n;). (D) cFzd10 expression is ventrally expanded. (D) cFzd7 expression is lost in the intermediate region and repressed in the ventral neural tube. EP side is shown on the right in these experiment.

3. Discussion

FZDs are expressed during the development of the central nervous system both in chick (Chesnutt et al., 2004; Galli et al., 2014; Kawakami et al., 2000; Quinlan et al., 2009) and in other species (Garcia-Morales et al., 2009; Wheeler and Hoppler., 1999). In chicks, all cFzds are expressed during the early stage of development and they are detected in different tissues, including the developing brain (Chapman et al., 2004; Fuhrmann et al., 2003; Quinlan et al., 2009; Stark et al., 2000; Theodosiou and Tabin, 2003). Here, We

were particularly interested in FZD expression patterns during chick dorsal-ventral spinal cord development and neurogenesis. Therefore, We established a complete expression profile of cFzds receptors in chick spinal cord and identified cFzds that could be involved in the dorsal-ventral patterning of the spinal cord (Fig.1 and Table S1). Through in situ hybridization, we identified that six out of ten cFzds were expressed in the spinal cord and showed specific and different expression patterns at stage HH14-15. CFzd1 and cFzd10 expression was seen in the dorsal neural tube and cFzd10 was strongly and specifically detected in the dorsal part of the neural tube (Fig. 1B, K). Whereas cFzd2 and cFzd7 were found in the intermediate ventral spinal cord and their expression patterns seemed to overlap, cFzd7 showed stronger expression in the neural progenitors (Fig. 1C, H). CFzd3 and cFzd9 transcripts were found throughout the dorsal ventral spinal cord (Fig.1D, J). CFzd3 expression was specific in the ventricular zone where proliferation occurs, while cFzd9 expression was ubiquitous. Whereas cFzd4, 5, 6 and 8 expresions were not detected during neural tube neurogenesis (Fig. **1**E, F, G and I). We also observed that cFzd7 and cFzd10 were expressed during spinal cord neurogenesis (Fig. 2). The expression profile of cFzds could suggest potential interactions of these receptors with Wnt ligands that are expressed during spinal cord development. Moreover, it could be proposed that dorsal cFzds, such as cFzd1 and cFzd10 may mediate signalling that is activated by Wnt ligands expressed in the dorsal spinal cord, whereas ventral cFzds, such as cFzd2 and cFzd7 may transduce the signals of Wnt ligands in the ventral spinal cord. Our data show that specific expression for cFzd1,2,3,7,9,10 in the dorsal-ventral of spinal cord during neurogenesis correlate with Wnts that are also expressed in the spinal cord (Hollyday et al., 1995).

Wnt1 and Wnt3a are expressed in the dorsal spinal cord and play important roles during its development (Alvarez-Medina et al., 2008). Therefore, we investigated the effects of dorsally expressed Wnts (Wnt1/3a) on Pax7, Nkx2.2 and cFzds (cFzd7/cFzd10) regulation in spinal cord. We found that Pax7 expression was expanded but Nkx2.2 expression was repressed in elctoroporated side of spinal cord. These results are consistent with a study showing that Wnt1 and Wnt3a co-electroporation in neural tube affect neural progenitors patterning (Alvarez-Medina et al., 2008). Also, we showed that co-misexpession of Wnt1 and Wnt3a led to expansion of cFzd10 expression into the ventral regions of the spinal cord indicating that cFzd10 expression is positively regulated by Wnt signaling, potentially enhancing the ability of cells to respond in a positive feed-back loop. This result might also suggest that cFzd10 may be involved in mediating canonical Wnt signalling in the chick spinal cord. (Fig.3.D). Conversely, cFzd7 expression was decreased in the intermediate-ventral spinal cord (Fig.3.E). This could indcate that the expression of cFzd7 and potentially other ventrally expressed cFzd receptors is inhibited by secreted Wnts from the dorsal spinal cord. Thus, dorsally over-expressed Wnts (Wnt1/3a) affect cFzd7 and cFzd10 expressions in vivo in the developing spinal cord. Indeed, more investgation is required to study how Wnt1/3a regulate FZD7/10 expression.

Moreover, other cFzd receptors need to be studied in detail to understand their functions and interactions with Wnts during dorsal-ventral neural tube development in vivo.

4. conclusion

we preformed a complete expression profile of Wnt receptors (cFzd) in order to determine cFzds expression in Dorsal-ventral neural tube. Functional studies in the future

are required to determine roles for the different cFzd receptors and their interactions with Wnts during chick neural tube development in vivo. Furthermore, we show that co-overexpression of Wnt1/3a affect cFZD10/7 expression in vivo by electroporation in neural tube which illustrates an example of possible Wnts-FZDs interactions during spinal cord neurogenesis.

5. Materials and Methods

5.1. In situ hybridization, cryosections and photography

Embryos were treated with DEPC/PBS, washed and fixed in 4% paraformaldehyde overnight at 4°C. Whole mount in situ hybridization was done as described previously in (Schmidt et al., 2004). For cryosectioning, embryos were embedded in OCT (Tissuetec) and 20 um sections were collected on TESPA coated slides, washed with PTW, coverslipped with Entellan (Merck, Germany) and examined using an Axioplan microscope (Zeiss). Sections were photographed on an Axiovert (Zeiss) using Axiovision software. Montages of images were created and labeled using Adobe Photoshop.

In situ probes list:

Table 1

N	Name	Vector	Sense	Antisense	Source
1	FZD1	pBS-KS	T3	T7	C. Tabin
2	FZD2	pGEM-T	Sp6	T7	PCR
3	FZD3	pGEM-T	T7	Sp6	PCR
4	FZD4	pBS	T7	T3	C. Tabin
5	FZD5	pBS-Sk+	T7	T3	Sabine Fuhrmann
6	FZD6	pGEM-T	T7	Sp6	PCR
7	FZD7	pGEM-T	T7	Sp6	PCR
8	FZD8	pBS	T3	T7	C. Tabin
9	FZD9	pBS	T7	T3	C. Tabin
10	FZD10	pGEM-T	T7	Sp6	PCR

5.2. Injection and electroporation into neural tube

Fertilized eggs were incubated at 37^{o}C until the desired stage of development was reached (Hamburger and Hamilton, 1992). Expression constructs were injected into the lumen of neural tubes of HH11-12 embryos and embryos were electroporated using 24V, five 50msec pulses with 100msec intervals. Embryos were harvested after 24 or 48 hours for analysis, at least 3 embryos and 15 sections were examined per experimental condition and marker gene.

5.3. DNA constructs

Plasmids encoding mouse Wnt1 and Wnt3a (pCIG) were kindly provided by Elisa Marti (Alvarez-Medina et al., 2008).

5.4. Immunohistochemistry

Immunohistochemistry was performed as described previously (Abou-Elhamd et al., 2015). Sections were incubated overnight at 4^{o}C with primary antibodies at the following concentrations: Pax7, Pax6 and Nkx2.2 (74.5A5),) (1:100, all from Developmental Studies Hybridoma Bank, University of Iowa). Secondary antibodies were anti-mouse Alexa Fluor 568 at 1 mg/ml in 10% goat serum/PBS. DAPI was used at a concentration of 0.1 mg/ml in PBS. After staining, cryosections were mounted and visualized using an Axioscope microscope using Axiovision software (Zeiss, Germany). Images were imported into Adobe Photoshop for analysis and labeling.

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Author contributions

A.F.A. performed experiments. AF.A., G.N.W. and A.M. designed experiments, discussed and analyzed data. AF.A. and A.M. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests.

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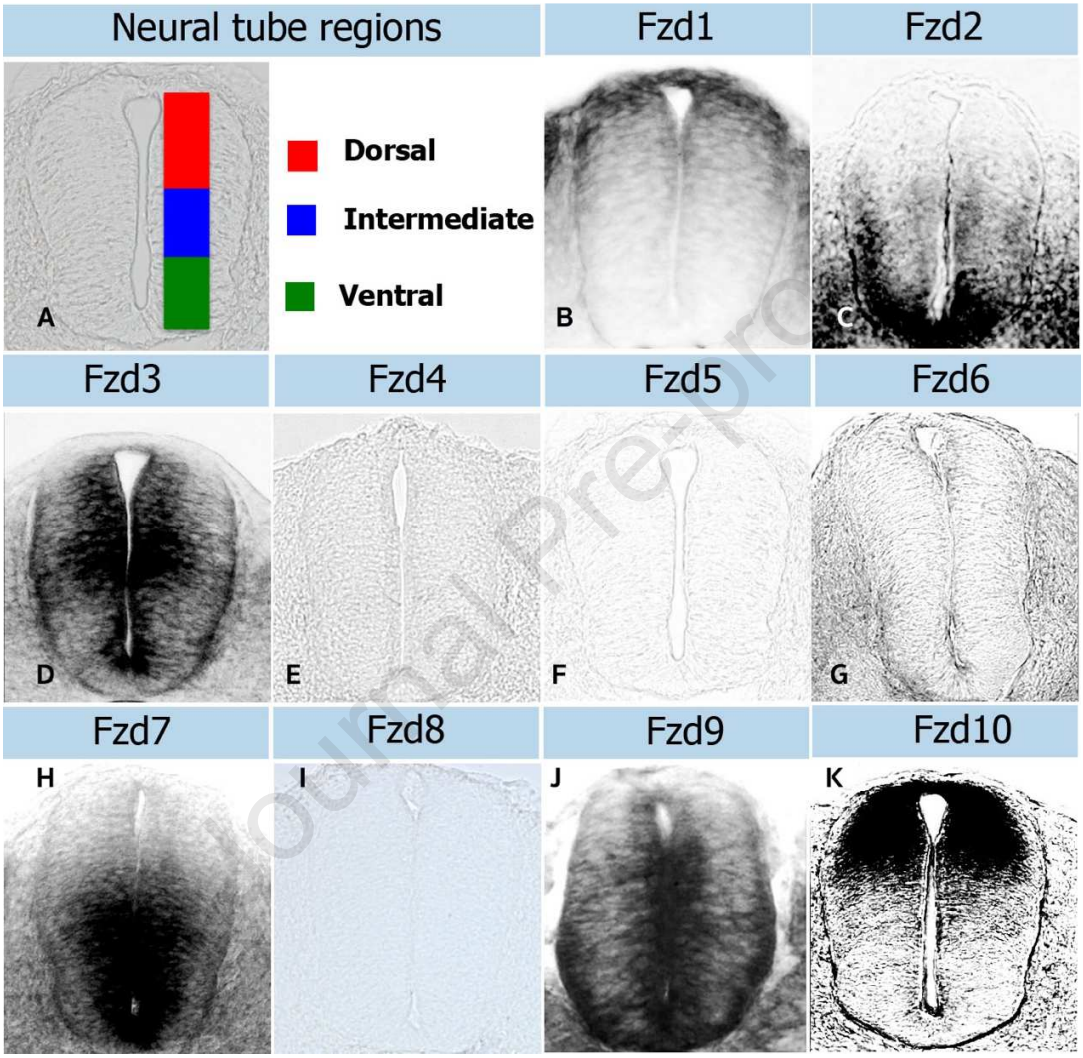
Neural tube regions
Dorsal
Intermediate
Ventral
A
Fzd1
B
Fzd2
C
Fzd3
D
Fzd4
E
Fzd5
F
Fzd6
G
Fzd7
H
Fzd8
I
Fzd9
J
Fzd10
K

Figure 1

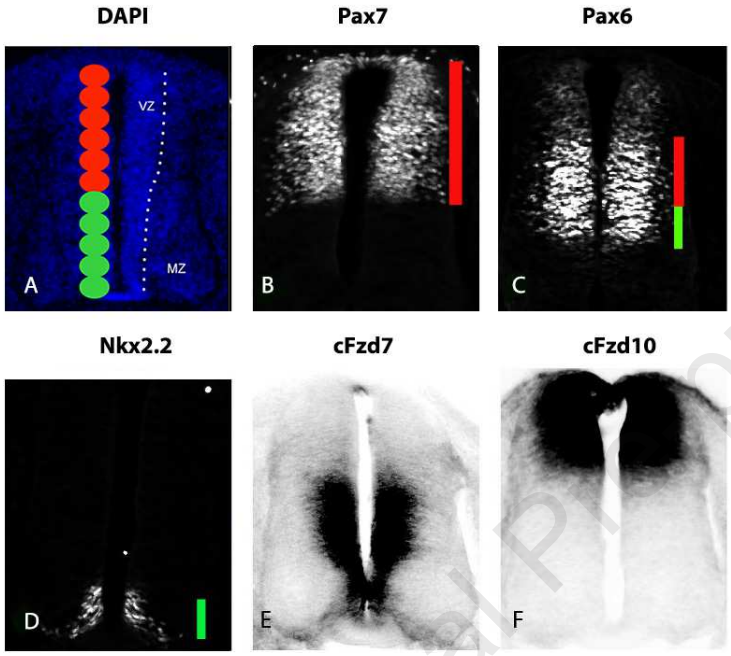

Figure 2

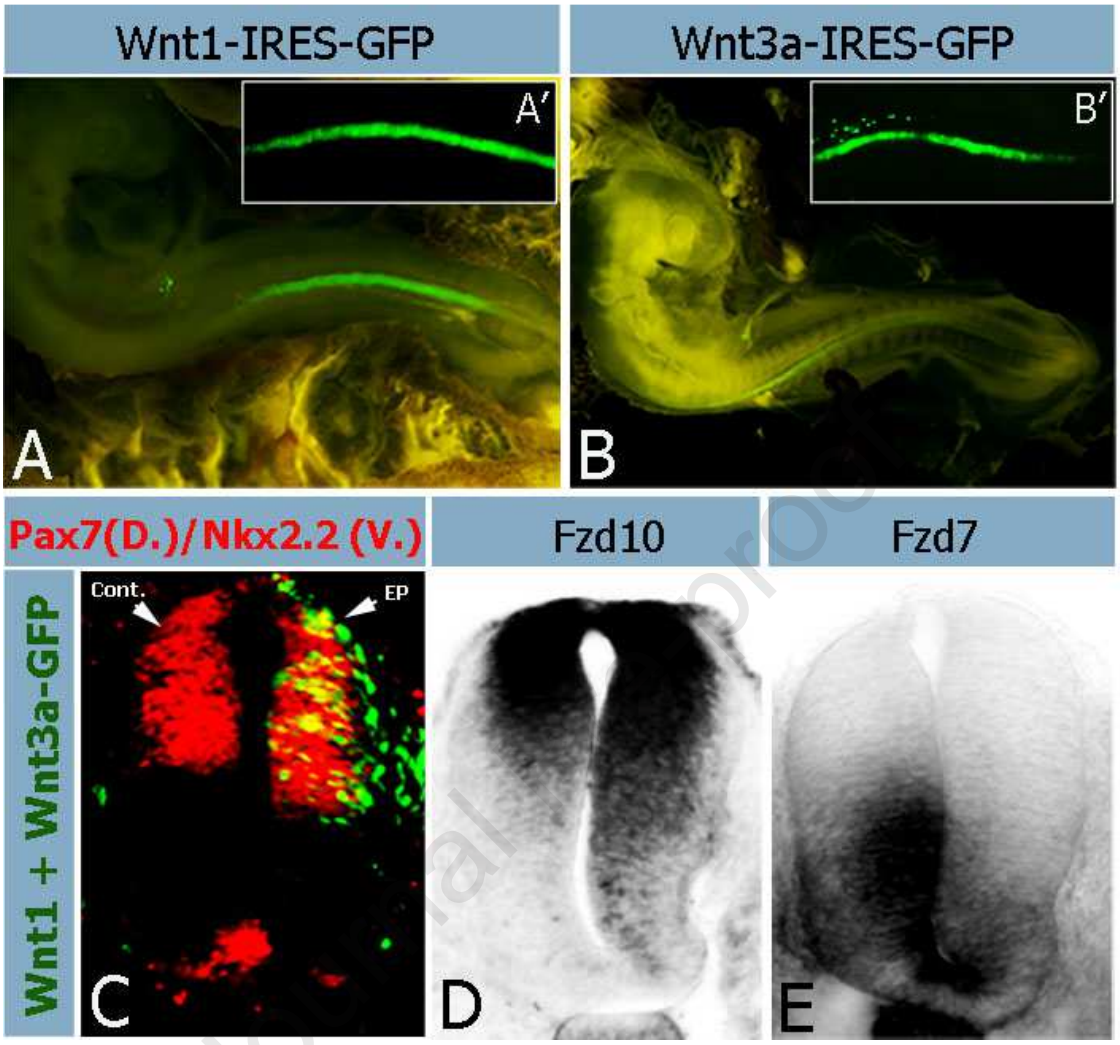
Wnt1-IRES-GFP
Wnt3a-IRES-GFP
A′
B′
A
B
Pax7(D.)/Nkx2.2 (V.)
Fzd10
Fzd7
Wnt1 + Wnt3a-GFP
Cont.
EP
C
D
E

Figure 3

The authors declare that there is no conflict of interests regarding the publication of this paper.