Activation of Ras-ERK pathway by Fgf8 and its downregulation by Sprouty2 for the isthmus organizing activity

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Running title: Negative regulation of isthmus organizing signal

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Abstract

In the previous studies, we showed that strong Fgf8 signaling activates the Ras-ERK pathway to induce cerebellum. Here, we show importance of negative regulation of this pathway. 'Prolonged' activation of ERK by misexpression of *Fgf8b* and dominant-negative *Sprouty2* (*dnSprouty2*) did not change the fate of the mesencephalic alar plate. Downregulation of ERK activity using a MEK inhibitor, U0126, or by tetracycline dependent Tet-off system after co-expression of *Fgf8b* and *dnSprouty2*, forced the mesencephalic alar plate to differentiate into cerebellum. We then paid attention to Mkp3. After misexpression of *dnMkp3* and *Fgf8b*, slight downregulation of ERK activity occurred, which may be due to Sprouty2, and the mesencephalon transformed to the isthmus-like structure. The results indicate that ERK must be once upregulated and then be downregulated for cerebellar differentiation, and that differential ERK activity level established by negative regulators receiving Fgf8 signal may determine regional specificity of mesencephalon and metencephalon.

Fgf8 is expressed in the isthmus and acts as an organizer for the mesencephalon and metencephalon[1-6]. It was shown that strong Fgf8 signal activates the Ras-ERK signaling pathway to organize cerebellar differentiation[6, 7]. On the other hand, there are several negative regulators for Ras-ERK signaling pathway, which are expressed overlapping to *Fgf8*[8-21]. *Sprouty2* is one of them[8-10, 13, 15-17, 19, 21-23]. *Sprouty2* is induced by Fgf8, but it downregulates ERK activity[17]. We showed that downregulation of Ras-ERK signaling pathway by *Sprouty2* is indispensable for proper regionalization of the mesencephalon and metencephalon[19, 24]. Upregulation of Ras-ERK signaling pathway by misexpression of dominant negative form of *Sprouty2* (*dnSprouty2*) caused anterior shift of the mes-metencephalon boundary[19]. On the other hand, downregulation of Ras-ERK pathway by misexpression of *Sprouty2* caused posterior shift of the mes-metencephalic boundary to change the fate of the metencephalon to the tectum[19].

We wanted to further elucidate biological significance of negative regulation for Fgf8-Ras-ERK signaling. In normal development, ERK is phosphorylated widely in the mes-metencephalic region around stage 8-9[7, 19, 25]. ERK phosphorylation region become narrower, and ERK phosphorylation level in the metencephalon become very low by stage 12[7, 19]. Then, we preliminarily examined the phosphorylation of ERK in the mesencephalic region that had been transfected with *Fgf8b* to differentiate to the cerebellum. There, ERK was heavily phosphorylated soon after electroporation of *Fgf8b* expression vector (Fig. 1a), but phosphorylation level became very weak by 18 hours after electroporation (Fig. 1f). So we speculated that ERK should be once activated and then be down regulated by negative regulators of Ras-ERK signaling

pathway for cerebellar differentiation.

In order to assess the speculation we carried out experiments paying attention to negative regulators of Ras-ERK signaling, Sprouty2 and Mkp3. We first kept ERK phosphorylation level high by co-electroporation of *Fgf8b* with *dnSprouty2* (dominant negative form of *Sprouty2)* expression vector. Next we down regulated ERK activity after co-transfection of *Fgf8b* and *dnSprouty2*, by inserting a bead soaked in U0126, a MEK inhibitor or by turning off transcription of *dnSprouty2* by tetracycline-controlled method. To our expectation, cerebellum did not differentiate in place of the tectum when ERK activity was kept high by misexpression of *Fgf8b* and dn*Sprouty2* , and that down regulation of ERK activity after its activation by *Fgf8b* and *dnSprouty2* caused differentiation of the cerebellum in the mesencephalic region by changing its fate. We also carried out misexpression of *Fgf8b* and *dnMkp3*, which caused differentiation of isthmic structure in the mesencephalon. By comparing the results, we came to the conclusion that the differential activation level of ERK is established by negative regulators of the Ras-ERK pathway after activation of the pathway by Fgf8, and according to the ERK activation level proper regionalization of the midbrain and hindbrain may occur. Sprouty2 may be indispensable for establishing the region specific ERK activation pattern.

Results

Chronological changes of ERK phosphorylation around the isthmus.

In normal development of chick embryos, ERK phosphorylation is detected in the

metencephalon and the posterior mesencephalon around stages 8 and 9[7, 19, 26, 27], but by stage 12, ERK activity is downregulated in the metencephalon, and phosphorylated ERK is localized only in the posterior mesencephalon[7, 19, 27]. We speculated that this negative regulation of ERK activity is significant for the development of the cerebellum. We then examined temporal changes of ERK phosphorylation in the mesencephalic region that would differentiate into the cerebellum after misexpression of *Fgf8b* by electroporation[4, 6, 7, 23, 27] (Fig. 1). At 3 and 6 hours after electroporation (stage 8 and 9), phosphorylated ERK localized across large areas corresponding to *Fgf8b* misexpression (Fig. 1a, n=4/4; 1b, n=5/5; Sato and Nakamura, 2004). At 12 hours after electroporation, ERK was still phosphorylated in the mesencephalon (Fig. 1d, n=5/5; 1e, n=4/7). At 15 hours after electroporation, phosphorylation level of ERK in the mesencephalon looked a little decreased. At 18 hours after electroporation (stage 13) phosphorylated ERK decreased in the mesencephalic region destined to differentiate into the cerebellum (Fig. 1f, n=3/3). In the metencephalic region, which would differentiate into the cerebellum, ERK phosphorylation was downregulated by 9 hours after electroporation (Fig. 1c, n=13/14). The results support our initial speculation that ERK is activated, then subsequently downregulated when cerebellum is induced by Fgf8b in the mesencephalon.

Continuous activation of ERK in the mesencephalon does not change the fate of the mesencephalon.

In order to test our speculation directly, we tried to keep ERK phosphorylation level high in the mesencephalon, and paid attention to Sprouty2. Misexpression of *dnSprouty2* alone maintained ERK phosphorylation in the metencephalon at 9 hours after electroporation[19] (Fig. 2a). Co-transfection of *Fgf8b* and *dnSprouty2* resulted in augmentation of ERK phosphorylation in the transfected region at 6 hours after electroporation (Fig. 2b, n=6/6). At 9 hours after electroporation, ERK phosphorylation level remained high at the mesencephalic and metencephalic region (Fig. 2c, n=9/11). After 18 hours of electroporation, ERK activity (phosphorylated ERK) remained high in the mesencephalon (Fig. 2d, yellow bracket, n=8/10), but was lost from the metencephalon (Fig. 2d, white bracket, n=6/10).

We next examined the structure induced from the mesencephalon that had been kept ERK activity high by misexpression of *Fgf8b* and *dnSprouty2*. On embryonic day 12 (E12), a swelling in the mesencephalon at the experimental side was smaller than that at the control side, but had the smooth surface, which resembled the surface of the tectum (Fig. 3a-c, n=9/13). Histologically, layers in the mesencephalic swelling at the experimental side are comparable to those at the control side; laminae g and i of SGFS (*stratum griseum et fibrosum superficiale*) are conspicuous (Fig. 3d, f, g), and it was concluded that the alar plate of the mesencephalon differentiated into the tectum when ERK activity was kept continuously high. Examination of the metencephalon under a dissection microscope suggested cerebellar differentiation at both control and experimental side (Fig. 3a-c), and it was confirmed histologically that the external granular layer was continuous from the control to the experimental side (Fig. 3e, h, i).

Otx2 expression as a marker of the mesencephalon and prosencephalon on the experimental side[2, 5, 28-34] was comparable to the control at 48 hours after electroporation (Fig. 3k, l). These results indicate fate change of the mesencephalon and metencephalon did not occur under the condition of prolonged high ERK activity via the co-expression of *Fgf8b* and *dnSprouty2*.

Downregulation of Ras-ERK signaling pathway after after its upregulation changes the fate of the mesencephalon to the cerebellum.

Next we examined if the mesencephalon would change its fate to differentiate into cerebellum if ERK activity, which had been activated by *Fgf8* and *dnSprouty2* co-expression, was downregulated. To do this, we co-electroporated *Fgf8b* and *dnSprouty2* expression vector into the embryos, and then implanted beads soaked in MEK inhibitor, U0126, 17 hours after electroporation (Fig. 4a, e, h). The level of ERK phosphorylation decreased locally around the implanted bead (Fig. 4d, n=2/3) by 48 hours after co-electroporation (31 hours after U0126-bead implantation), *Otx2* expression was repressed in the mesencephalic region (Fig. 4c, n=3/3). Conversely, expression of the metencephalon marker *Gbx2*[2, 28-30, 33] was induced in the mesencephalic region (Fig. 4g, n=6/6). Expression of *Fgf8* shifted anteriorly (Fig. 4j, n=4/4), which is reminiscent of new isthmus formation as observed after *Fgf8b* misexpression altering the fate of the mesencephalon towards the cerebellum[19, 23]. But, we cannot affirm that the cerebellum differentiated in the mesencephalic region, since the treatment of U0126 was associated with high mortality, and embryos did not survive

beyond E8.5.

We then tried to turn off *dnSprouty2* transcription by tetracycline-regulated Tet-off system[35, 36]. pCAGGS-Fgf8b, pTRE-dnSprouty2 and pCAGGS-tTA were co-electroporated at stage 8 or 9, and Dox was administered 5.5 hours later.

By 9 hours after Dox treatment (14.5 hours after electroporation), phosphorylation level of ERK decreased (Fig. 4k-n, n=4/4). On E12.5, the swelling at the midbrain of the experimental side was smaller than that of the control side, and looked like cerebellum (Fig. 4o, p). Histologically, external granular layer was differentiated, and the layer which looks like Purkinje cell layer was also differentiated. Thus, we concluded that the cerebellum differentiated in place of the tectum.

The control embryos, which was electroporated with pCAGGS-Fgf8b, pTRE-dnSprouty2 and pCAGGS-rTA, but were not treated with Dox, showed high level of ERK phosphorylation in the mesencephalon even at 18 hours after electroporation, and the mesencephalon differentiated as the tectum (supplementary Fig. 1).

The finding that prolonging high levels of ERK activity failed to change the fate of the mesencephalon to differentiate to cerebellum, and that reducing ERK activity after its activation succeeded in inducing a fate change proves rightness of our speculation that the Ras-ERK signaling pathway must be once activated and then be negatively regulated for cerebellar differentiation.

Overlapping expression of Gbx2 and Otx2 is allowed in the ERK-activated region

To ascertain the state of the mesencephalon and metencephalon in the presence of

high ERK activity in more detail, we examined the time course of *Gbx2* and *Otx2* expression as a marker of isthmus/metencephalon and mesencephalon/prosencephalon after co-electroporation of *Fgf8b* and *dnSprouty2* expression vector, respectively[2, 5, 28-34]. *Gbx2* expression was induced in the mesencephalic region by 6 hours after electroporation (Fig. 5c, d, n=3/3), but *Otx2* expression persisted strongly (Fig. 5a, b, n=3/3). Consequently, *Otx2* and *Gbx2* expression overlapped in the mesencephalon. Overlapping *Otx2* and *Gbx2* expression was still apparent in the mesencephalon at 9, 12 , 15, 18 and 24 hours after electroporation (9 hours, Fig. 5i-l, *Otx2*; n=4/4, *Gbx2*; n=4/4, and supplementary Fig. 1). The posterior margin of the *Otx2* expression domain was in the normal position by 48 hours after electroporation (compare Fig. 4g and h, n =5/8).

The results indicated that overlapping expression of *Otx2* and *Gbx2* is allowed under an ERK-activated condition. Then we re-examined *Otx2* and *Gbx2* expression in embryos of normal and after *Fgf8b* misexpression, which forces mesencephalic alar plate to differentiate to cerebellum. *Fgf8b* misexpression also induced overlapping expression of *Otx2* and *Gbx2* in the mesencephalon initially[19] (Fig. 5m, n), followed by repression of *Otx2* expression in the mesencephalic region, and *Gbx2* expression covered the mesencephalon by 12 hours after electroporation[19] (Fig. 5o, p). In normal development, *Otx2* and *Gbx2* expression overlaps around stage 8 and 9[2] (Fig. 5e, f), then *Otx2* and *Gbx2* expression abuts at the isthmus by stage 10[2, 29, 30, 32, 33].

Importance of Sprouty2 in transduction of isthmus organizing signal

Mkp3 is also induced by Fgf signaling pathway, and de-phosphorylates ERK, thus functions as a negative regulator of Fgf8 signaling pathway in the isthmic region[11, 22]. We then pursued distinct role of Mkp3 from Sprouty2. It was reported that *Mkp3* and *Sprouty2* were induced by Fgf8 but via different intracellular signaling pathway[11]. Expression of *Mkp3* after *Fgf8b* misexpression was first examined. *Mkp3* expression was induced ectopically in the diencephalon and myelencephalon after *Fgf8b* misexpression (Fig. 6a-b, e-g, and k-m), which is contrasted by *Sprouty2* expression after *Fgf8b* misexpression[19]. *Sprouty2* was induced in the place where *Fgf8b* was expressed. Furthermore, after Fgf8b and dnSprouty2 misexpression *Mkp3* was also induced in the mesencephalon (Fig. 6c-d, h-j, n-p). This may indicate that *Mkp3* is induced in the place where ERK is activated strongly, because ERK activity is augmented by antagonistic activity of dnSprouty2 against Sprouty2.

Then we co-electroporated *Fgf8b* and *dnMkp3* expression vectors to see if Mkp3 functions as Sprouty2 does or differently. At 3 and 6 hours after co-electroporation, phosphorylation of ERK occurred widely as co-expression of *Fgf8b* and *dnSprouty2* (Supplementary Fig. 2a-f, 3 hours, n=1/1; 6 hours, n=4/4). By 9 hours after co-electroporation, dephosphorylation of ERK appeared in the metencephalic region, but its dephosphorylation level was weaker than that after transfection with *Fgf8b* (Supplementary Fig. 2g-i, n=5/7). By 18 hours after co-electroporation of *Fgf8b* and *dnMkp3*, dephosphorylation of ERK occurred in the isthmus and posterior mesencephalon, but dephosphorylation level was weaker than that after *Fgf8b* transfection (Supplementary Fig. 2p-r, n=3/3). Since *Sprouty2* is induced by Fgf8,

dephosphorylation in the mesencephalic region may be due to Sprouty2.

Forty eight hours after co-electroporation of *Fgf8b* and *dnMkp3* expression vector, new *Fgf8* expression line was induced in the diencephalic region parallel to the intrinsic isthmic Fgf8 expression (Supplementary Fig.3a, n=4/4). Along the induced *Fgf8*, axons were ascending from the ventral to the dorsal (Supplemetary Fig. 3b-c). Fiber trajectory indicates that ascending fibers are trochlear nerve fibers (Supplementary Fig. 3d-f, at 72h after electroporation), which indicates that the mesencephalic region differentiated as the isthmus.

Discussion

Negative regulation of Ras-ERK signaling for cerebellar differentiation is necessary

Our previous studies showed that misexpression of *Fgf8b* resulted in the fate change of the mesencephalic alar plate from the tectum to the cerebellum, and that disruption of Ras-ERK signaling by dominant negative form of Ras changed the fate of the metencephalon to differentiate into the tectum[6, 7]. These studies indicated that the region where Ras-ERK signaling is activated by strong Fgf8 differentiates into cerebellum. But careful observation revealed that in the proper metencephalic region ERK is activated around stage 8-9 but that its activity is down regulated by stage 12[7, 19]. It was also shown that in the mesencephalic region that would differentiate to cerebellum after *Fgf8b* misexpression ERK was activated till 12 hours after electroporation but that ERK phosphorylation level was decreased from 15 hours after electroporation. From these

observations we speculated that ERK should be first activated then be downregulated for cerebellar differentiation.

In order to prove our speculation, we misexpressed Fgf8b and dnSprouty2 to keep ERK phosphorylation level high in the mesencephalon, where ERK phosphorylation level was kept still high at 18 hours after electroporation. As we expected, both gene expression pattern and histology showed that mesencephalon did not change its fate when ERK activity was kept high. Although the swelling in the mesencephalon at E12 was smaller than the control tectum, the swelling had layers specific to the tectum.

Then we down regulated ERK activity by inserting a U0126-bead, a MEK inhibitor, or by adopting tetracycline-regulated Tet-off system for *dnSprouty2* expression. ERK phosphorylation level was indeed decreased after a U0126-bead implantation. In embryos that were inserted with a U0126-bead after co-transfection of *Fgf8b* and *dnSprouty2*, gene expression pattern of the mesencephalon changed to that of metencephalon, that is, *Otx2* expression was repressed, *Gbx2* expression was induced in the mesencephalic region, and *Fgf8* expression shifted anteriorly indicating new isthmus formation[19]. High mortality of embryos after the treatment of U0126 bead made it difficult to identify the histological property. We could successfully shut down *dnSprouty2* expression by adopting tetracycline-controlled Tet-off system. ERK was once activated in our system (6 hours after electroporation), and phosphorylation level decreased by 15 hours after electroporation (Dox treatment at 6 hours after electroporation), and the mesencephalon differentiated into the cerebellum, not to the tectum. The results indicate that negative regulation of Ras-ERK signaling for

cerebellar differentiation is necessary.

Role of sprouty 2 in the isthmus organizing signal

By 6 hours after co-electroporation of *Fgf8b* and *dnSprouty2* expression vector, *Gbx2* expression was induced in the mesencephalic region but *Otx2* expression persisted strongly so that *Gbx2* and *Otx2* were expressed overlapping in the mesencephalic region at 6-24 hours after electroporation. This result was a surprise since it had been accepted that *Otx2* and *Gbx2* expression abuts at the isthmus[2, 29, 30, 32, 33]. Careful examination revealed that *Fgf8b* misexpression, which forces mesencephalic alar plate to differentiate to cerebellum, also induced overlapping expression of *Otx2* and *Gbx2* in the mesencephalon initially, followed by repression of *Otx2* expression in the mesencephalic region. Consequently, *Gbx2* expression covered the mesencephalon by 12 hours after electroporation of *Fgf8b* expression vector, which may be concomitant of downregulation of ERK activity. In normal development, *Otx2* and *Gbx2* expression overlaps around stage 8 and 9[2] (Fig. 7), then *Otx2* and *Gbx2* expression abuts at the isthmus by stage 10[2, 29, 30, 32, 33] (Fig. 7). The results indicate that Fgf8-Ras-ERK signaling may induce an activated and unstable state, where overlapping expression of *Otx2* and *Gbx2* is allowed, and consequently the tissue may have potential to differentiate into tectum, isthmur or cerebellum. Downregulation of ERK after its activation by negative regulators may repress *Otx2* expression at the site where *Gbx2* is expressed overlapping. Under the condition of overlapping expression of *Otx2* and *Gbx2*, *Otx2* may be dominant, and the final fate may correspond to *Otx2*.

Discussion above indicates importance of negative regulation of Ras-ERK signaling for the proper regionalization. We paid attention to *Sprouty2* and *Mkp3,* which are expressed overlapping to *Fgf8*, and induced rapidly by Fgf8, but function as a negative regulators for Ras-ERK pathway[8-11, 13, 15-19, 21-23]. We previously showed that Sprouty2 plays a role in proper placing mes/metencephalon boundary by regulating Ras-ERK signaling (Suzuki-Hirano, 2005). When Ras-ERK signaling was repressed by Sprouty2, mes/metencephalon boundary shifted posteriorly, resulting in fate change of the metencephalic alar plate to the tectum. On the other hand, when excess Ras-ERK signaling flowed by misexpression of *dnSprouty2*, mes/metencephalic boundary shifted anteriorly.

We further extended analysis of negative regulation of Fgf8-ERK signaling by Sprouty2 and Mkp3, and propose that activation of ERK and subsequent downregulation of it in response to the isthmic organizing signal is indispensable for the proper regionalization.

Sprouty2 and *Mkp3* are induced by Fgf8, but they may be induced via different transduction pathway. It was reported that *Mkp3* was induced via PI3K-AKT signaling pathway[11], while that *Sprouty2* was induced via Ras-ERK signaling pathway. Our previous study of Fgf8b misexpression showed that *Sprouty2* was induced overlapping to *Fgf8*[19] (Fig. 7). In the present study, *Mkp3* was ectopically induced in the diencephalon and myelencephalon, but induction of Mkp3 in the mesencephalon was weak (Fig. 7). These results indicate that Sprouty2 is responsible for down regulation of ERK activity in the mesencephalon after *Fgf8b* misexpression. The notion is further

supported by the results after co-transfection of *Fgf8b* with *dnMkp3* or with *dnSprouty2*. ERK activity was kept high up to 18 hours after electroporation throughout the misexpression site of Fgf8b and dnSprouty2. After *Fgf8b* and *dnMkp3* misexpressed, slight down regulation of ERK activity occurred in the mesencephalic region. Considering induction of *Sprouty2* and *Mkp3* by Fgf8b (Fig. 7), we suggest that Sprouty2 plays crucial role in down regulation of ERK after *Fgf8b* misexpression, and *Fgf8b* and *Mkp3* misexpressed. We also suggest that Sprouty2 is responsible to set differential ERK activity in the mesencephalon to metencephalon in normal develoment, and that Mkp3 regulates ERK where its phosphorylation level is high.

Activation of Ras-ERK signaling pathway is an important step of the isthmic organizer. Differential down regulation of this pathway may determine the regional specificity. Continuous activation of ERK by Fgf8b and dnSprouty2 did not change the fate of the mesencephalon, and the brain vesicles developed according to the prepattern (Fig. 7). Down regulation of ERK activity by MEK inhibitor U0126 or turn off dnSprouty2 expression adopting Tet-off system after its activation by *Fgf8b* and *dnSprouty2* misexpression resulted in ectopic cerebellar differentiation in place of the tectum. When *Fgf8b* and *dnMkp3* were misexpressed, slight down regulation of ERK activity in the mesencephalon occurred, and the isthmic structure differentiated in the midbrain (Fig. 7). In the presence of *dnMkp3*, ERK activation level may have been kept slightly higher than that in the former case (application of U0126 or turning off dnSprouty2 after *Fgf8b* and *dnSprouty2* misexpression). Considering all together, it is proposed that the differential activation level of ERK is established by Sprouty2 after

activation of Ras-ERK signaling pathway by Fgf8, and according to the ERK activation level the isthmus and cerebellum may differentiate.

Methods

In ovo electroporation.

In ovo electroporation was carried out at stage 8-9 (5-7 somite stage, Hamburger and Hamilton, 1951) as described previously[37-39]. GFP expression vector (pCA-GAP-GFP) was co-electroporated to ascertain the efficiency of transfection. GFP was detected by whole-mount immunostaining with anti-GFP antibody or *in ovo* imaging. *Fgf8b* was inserted in pMiwIII vector or pCAGGS vector. *Fgf8b* expression vector and dominant negative form of *Sprouty2* (*dnSprouty2*) expression vector inserted in pMiwIII vector were described previously[6, 19]. pMiwIII vector has Rous sarcoma virus enhancer and chick β-actin promoter, and assures ubiquitous expression of the transgene[40-42]. *dnMkp3* expression vector which was inserted in pCS2 vector was provided by Kawakami[14].

Histology.

Embryos were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline), and embedded in Technovit (Kulter). Serial 5 μm sections were stained with Hematoxylin and Eosin.

Beed implantation.

A bead soaked in U0126 (Wako), a MEK1/2 inhibitor, was implanted into the

mesencephalic region 17 hours after electroporation. U0126 was diluted in DMSO to a final concentration of 10 mM, where Affigel Blue beads (Bio-Rad) or AG1-X2 ion-excharge resin beads (Bio-Rad) were placed as described previously[7].

Tet-off

HA-tagged dnSprouty2[19] was inserted in TRE-driven BI-EGFP vector (BI-EGFP-dnSpry2), which has pminCMV promotor[36] (Constructed by Nakagawa, and we got licence from Clonetec). pBI-EGFP-dnSpry2 vector and pCAG-tTA (Tet-controled transcriptional activator) (Tet-OFF) vector and pCAGGS-Fgf8b vector were co-electroporated at HH 8 embryos. The embryos were administered with 0.2 mg/ml Doxycyclin (Dox) in PBS at 5.5 hours after co-electroporation, and then Dox was administered at every 24 hours. We confirmed that *dnSpry2* was expressed by 6 hours after co-electroporation, and was not expressed by 12 hours after DOX administration by immunostaining for HA-tag (data not shown).

***In situ* hybridization.**

In situ hybridization was carried out according to the method as described previously[19], which is a modification of Wilkinson (1992)[43]. Probes for *Otx2, Gbx2, Fgf8* and *Mkp3* are described in Katahira et al. (2000)[30] or in Kawakami et al. (2003)*[14]*.

Immunohistchemistry.

For Immunohistochemistry, anti-GFP rabbit polyclonal antibody (Molecular Probe),

anti-diphosphorylated ERK antibody, (Sigma) and anti-neurofilament antibody (3A10, DSHB) were used as primary antibodies. As secondary antibodies, Alexa488-conjugated anti-rabbit IgG (Molecular Probe), biotinylated anti-mouse IgG antibody (VECTOR) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson) were used. For detection of biotinylated antibody, the ABC-*Elite* system (VECTOR) was adopted.

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Figure legends

Figure 1. Time course analysis of ERK activity after *Fgf8b* misexpression.

ERK activity was assessed by whole-mount immunostaining with anti-dpERK (diphosphorylated ERK) antibody. Misexpression was monitored by GFP fluorescence co-transfected (a'-f'). The right side of the embryo is the experimental side (upper side), and the left side is the control side. At 3 or 6 hours after electroporation of *Fgf8b*, phosphorylated ERK localization corresponded broadly to *Fgf8* misexpression (a-b'). By 9 hours after electroporation and afterwards (c-f), ERK phosphorylation level at the metencephalon was reduced (white bracket on c, d). By 15 hours after electroporation (e, e'), ERK phosphorylateion looked a little reduced, and by 18 hours after electroporation (f, f'), reduction of ERK phosphorylation became clear in the mesencephalic region, which was destined to become cerebellum (white bracket on f).

mes, mesencephalon; is, isthmus; met, metencephalon; tel, telencephalon; di, diencephalon. Scale bars are 200 μm.

Figure 2. Time course analysis of ERK activity after *dnSprouty2* or *Fgf8b* and *dnSprouty2* misexpression.

(a) 9 hours after electroporation of *dnSprouty2* expression vector.

(b-d) 6 hours (b), 9 hours (c) and 18 hours after co-electroporation of *Fgf8b* and *dnSprouty2* expression vector.

ERK activity was assessed by whole-mount immunostaining with anti-dpERK antibody. Misexpression was monitored by GFP fluorescence co-transfected. The right side of the

embryo is the experimental side (upper side), and the left side is the control side. At 9 hours after electroporation of *dnSprouty2 in ovo*, ERK phosphorylation levels remained higher compared to the control side (yellow bracket on a). At 6 hours after electroporation of *Fgf8b* and *dnSpryty2* (b*)*, ERK phosphorylation augmented corresponding to the transfection site. ERK phosphorylation in the metencephalon remained at 9 hours after electroporation (yellow bracket on c). ERK phosphorylation in the mesencephalon remained at 18 hours after electroporation (d-d", yellow bracket).

mes, mesencephalon; is, isthmus; met, metencephalon; tel, telencephalon; di, diencephalon. Scale bars are 200 μm.

Figure 3. Continuous activation of ERK does not change the fate of the mesencephalon.

(a-i) An E12 embryo co-transfected with *Fgf8b* and *dnSpry2*. Dorsal view (a), lateral views of the control (b) and experimental side (c). H & E staining on the horizontal sections indicated on a (d-i). Higher magnification images of the areas indicated on d and e (f, g and h, i, respectively). Although the mesencephalic swelling at the experimental side is small, comparison of structures indicates that the swelling is the tectum.

(j) GFP fluorescence that was co-transfected with *Fgf8b* and *dnSpry2 in ovo* at 18 hours after electroporation. (k, l) Whole-mount *in situ* hybridization on the embryo shown on j for *Otx2* at 48 hours after electroporation. The mes/metencephalon boundary is placed at the normal position.

tel, telencephalon; tect, optic tectum; cer, cerebellum; fp, floor plate; mes,

mesencephalon; is, isthmus; met, metencephalon. Scale bars are 1 mm (a) and 200 μm (d, e, j, l).

Figure 4. Indication of fate change of the mesencephalon to cerebellum by downregulation of Ras-ERK signaling after its activation

(a-j) Down regulation of ERK activity by U0126 (MEK1/2 inhibitor). A U0126-soaked bead (yellow circle on a, e, h, asterisk on d) was inserted in the mecencephalic region at 17 hours after co-electroporation of *Fgf8b* and *dnSpry2*.

(a, e, h) GFP fluorescence image at 20 hours after electroporation.

(d) Immunohistochemistry with anti-dpERK. ERK phosphorylation level is decreased around the U0126 bead (asterisk). Broken line indicates the area where ERK activity is repressed by U0126.

(b, c, f, g, i, j) Whole-mount *in situ* hybridization for *Otx2* (b, c) , *Gbx2* (f, g) , and *Fgf8* (i, j) at 48 hours after electroporation (17 hours after bead implantation). Expression of *Otx2* was repressed (c) and expression of *Gbx2* was induced (g) in the mesencephalic region. Ectopic expression line of *Fgf8* was formed at the mesencephalic region, as observed after *Fgf8b* misexpression (j).

(k-n) Turn-off of *dnSprouty2* transcription by tetracycline regulation Tet-off system.

Dox was first administered at 5.5 hours after electroporation of *Fgf8b* and *dnSprouty2* expression vector, and administered every 24 hours.

(k, l) 15 hours and (m, n) 18 hours after electroporation of Fgf8b and dnSprouty2 expression vector. (k, m, n) Immunohistochemistry with anti-dpERK, and fluorescent

image of GFP whose expression vector was coelectroporated.

(o) Dorsal and (p) lateralview of the brain of an E12.5 embryo, and HE stained section at the line indicated on o (q: lower magnification, and R: higher magnification).

ERK activity in the mesencephalon was down regulated by 15 hours after electroporation of Fgf8b and Sprouty2 expression vector (9.5 hours after Dox administration), and ERK phosphorylation level at the experimental side is comparable to the control side at 18 hours after electroporation. On E12.5, the swelling in the midbrain was smaller and looked like cerebellum. Histologically. The swelling did not have tectal laminae (q), and higher magnification show external granular layer (egl, on r) and Purkinje cell layer (arrow on r), which are characteristics to the cerebellum.

tel, telencephalon; di, diencephalon; mes, mesencephalon; is, isthmus; met, metencephalontect; ov, otic vesicle; fp, floor plate; cer, cerebellum; ect-cer, ectopic cerebellum differentiated in the midbrain region; tect, tectum; cont, control side; exp, experimental side; dotted line on o represents the midline of the brain, which is bent at the diencephalon-midbrain junction. Scale bars are 1 mm (o, q); 200 μm (others).

Figure 5. Changes in expression of *Otx2* and *Gbx2* in the mes/metencephalic region.

Whole-mount *in situ* hybridization for *Otx2* (a, b, f, i, j, m, o), *Gbx2* (c, d, h, k, l, n, p), and *Fgf8* (e, g).

(e-h) HH stage 9+ normal embryos (e, f, and g, h are the same embryos). *Otx2* and *Gbx2* expression is overlapping around the isthmus in normal embryos around stage 9 (e to h).

(a-d, i-l) Embryos at 6 hours (a-d) and 12 hours (i-l) after *Fgf8b* and *dnSprouty2* co-electroporation. *Otx2* and *Gbx2* expression is overlapping in the mesencephalon since *Otx2* expression remained (a, b, i, j) and *Gbx2* expression was induced in the mesencephalon (c, d, k, l).

(m-p) Embryos at 6 hours (m, n) and 12 hours (o, p) after *Fgf8b* electroporation. The right side of the embryo is the experimental side. *Otx2* and *Gbx2* expression is overlapping around the isthmus at 6 hours after electroporation (m, n), since *Otx2* expression remained (m) and *Gbx2* expression was induced in the mesencephalon (n). *Otx2* and *Gbx2* expression abuts at the isthmu at 12 hours after electroporation (arrows on o, p).

Arrowheads indicate the position of the isthmus. mes, mesencephalon; is, isthmus; met, metencephalon; Scale bars are 200 μm.

Figure 6. Induction of *Mkp3* expression by misexpression of Fgf8b or co-expression of *Fgf8b* and *Sprouty2*.

Whole-mount *in situ* hybridization for *Mkp3* (a, c, e, f, h, i, k, l, n, o), and immunohistochemistry with anti-GFP antibody (b, d, g, j, m, p). Dorsal view (a-e, h, k, n) or lateral view (f, g, i, j, l, m, o, p).

a-d: Embryos at 15 hours after *Fgf8b* (a, b) or *Fgf8b* and *dnSprouty2* misexoression (c, d). a, b or c, d are the same embryos.

e-j: Embryos at 18 hours after *Fgf8b* (e-g) or *Fgf8b* and *dnSprouty2* misexoression (h-j). e-g or h-j are the same embryos.

k-p: Embryos at 24 hours after *Fgf8b* (k-m) or *Fgf8b* and *dnSprouty2* misexoression (n-p). k-m or n-p are the same embryos.

At 15 and 18 hours after electroporation of *Fgf8b* expression vector, *Mkp3* expression was induced in the diencephalon, where ERK activity had been kept high (a, e, f). Induction of Mkp3 expression after Fgf8b and dnSprouty2 expression was also stronger in the diencephalon (c, h, i, n, o), which may explain that down regulation of ERK activity did not occur.

Figure 7. Negative regulation for the isthmic organizing signal.

In normal development, *Otx2* (blue) and *Gbx2* expression (green) is overlapping around stage 8 and 9, when ERK is rather widely activated around the isthmus (brown), but by HH stage 12, ERK activity is down regulated in the metencephalon, and *Otx2* and *Gbx2* expression is not overlapping, when the fate of the metencephalon is fixed to cerebellum (fix).

After *Fgf8b* misexpression (+Fgf8b), ERK is activated corresponding to the *Fgf8b* misexpression. ERK is activated in the mesencephalic region, where *Sprouty2* expression is induced (pale blue). Mkp3 expression (purple) is induced in the diencephalon and myelencephalon and weakly induced in the mesencephalic region after *Fgf8b* misexpression. When only *Fgf8b* is misexpressed (Fgf8b only), ERK activity in the mesencephalic region is downregulated, and the alar plate of the

mesencephalon differentiates into the cerebellum. When *Fgf8b* and *dnMkp3* are co-expressed (Fgf8b + dnMkp3) ERK activity is slightly downregulated in the mesencephalon, and the mesencephalon differentiates as the isthmus. When *Fgf8b* and *dnSprouty2* are co-expressed (Fgf8b + dnSprouty2), ERK activity in the mesencephalon is kept high, and the alar plate of the mesencephalon differentiates into the tectum.

The results of the present study indicates that Sprouty2 plays a crucial role in determining ERK activation pattern for the proper regionalization of the mesencephalon and metencephalon.

mes, mesencephalon; is, isthmus; met, metencephalon.

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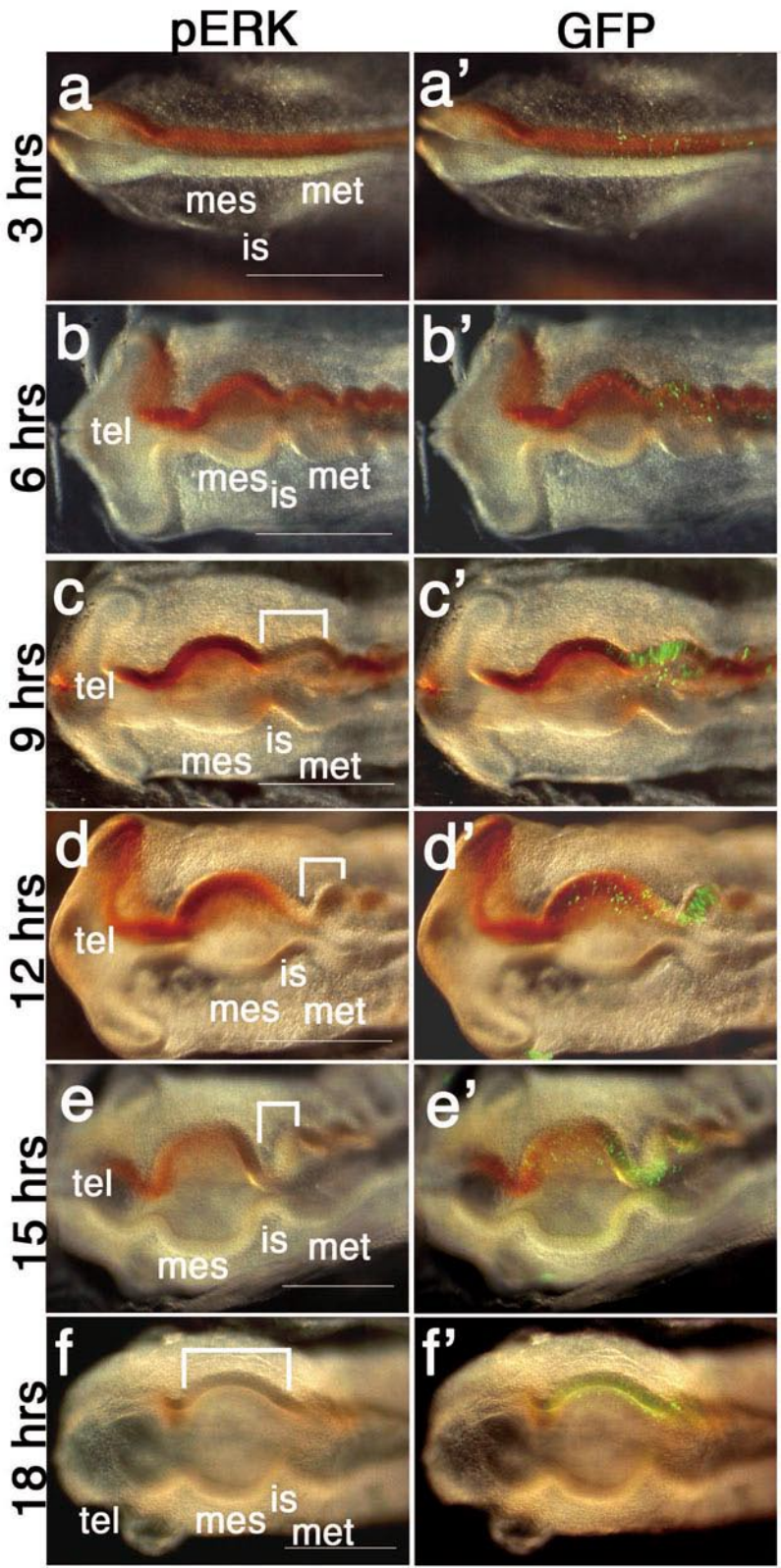
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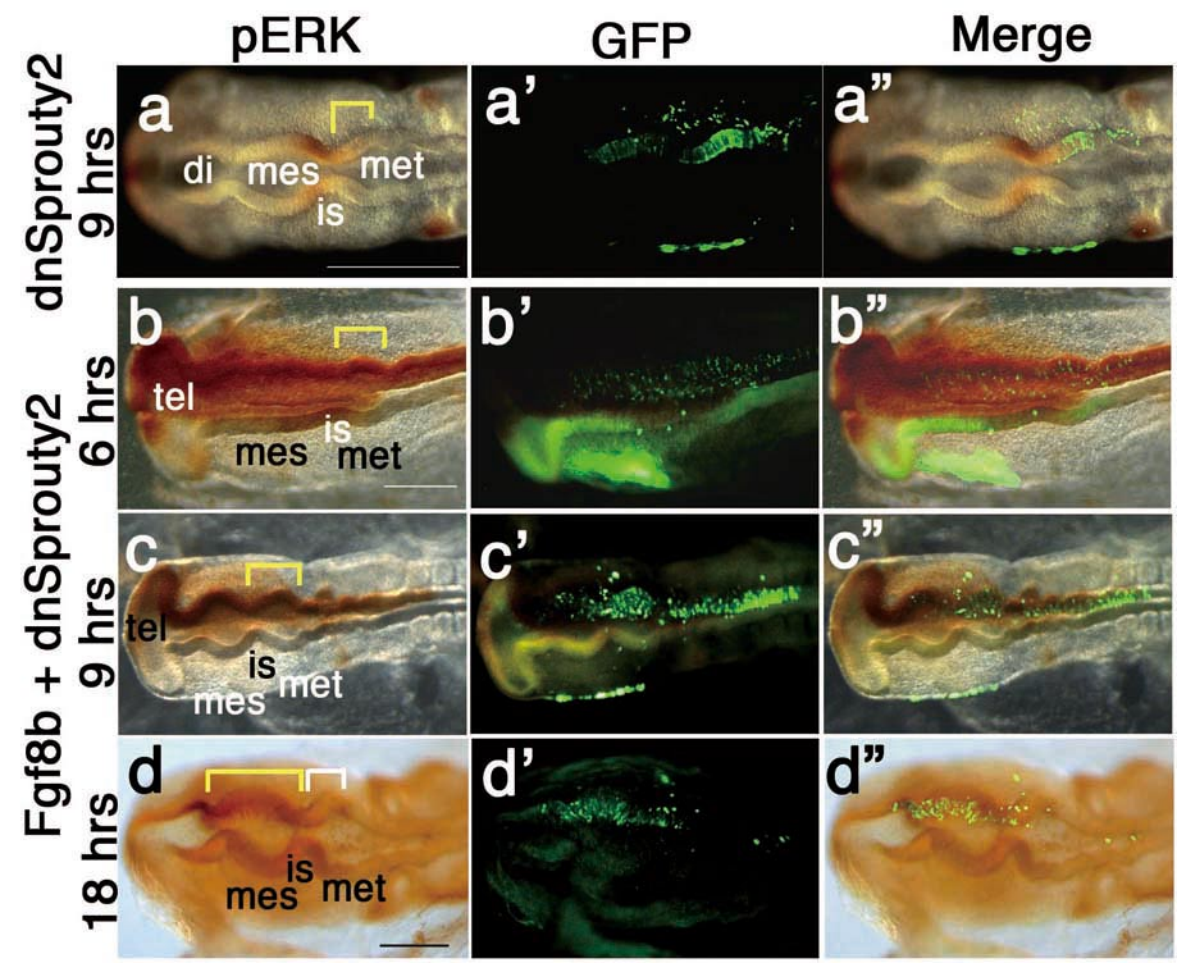
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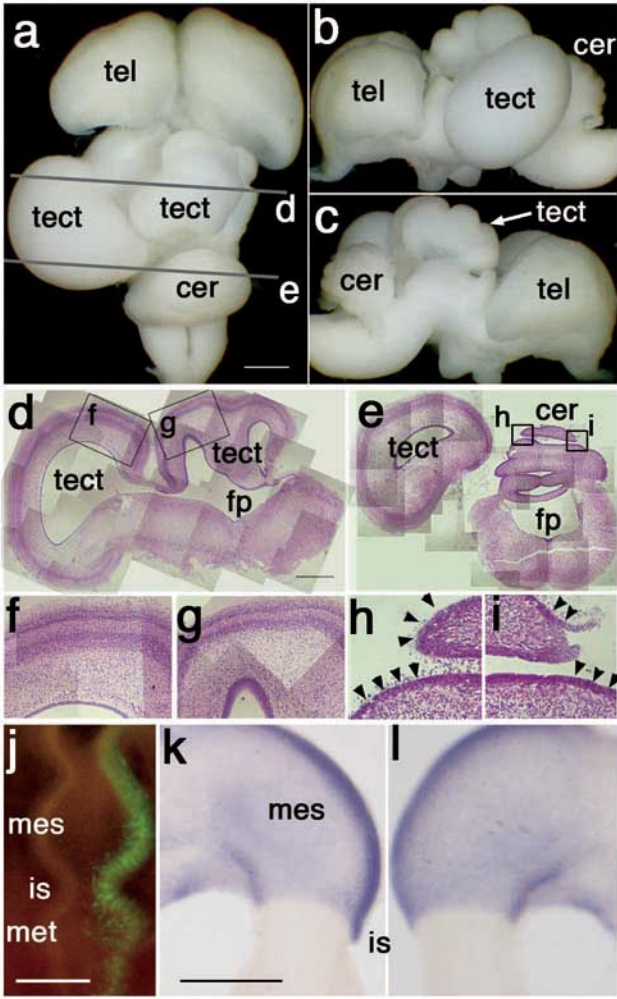
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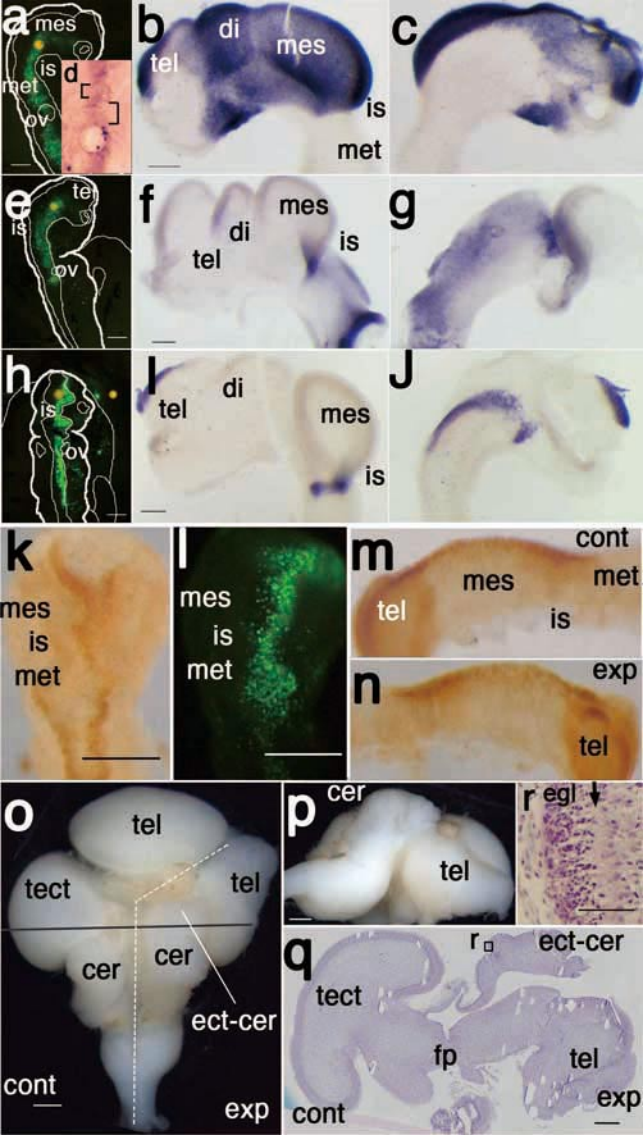
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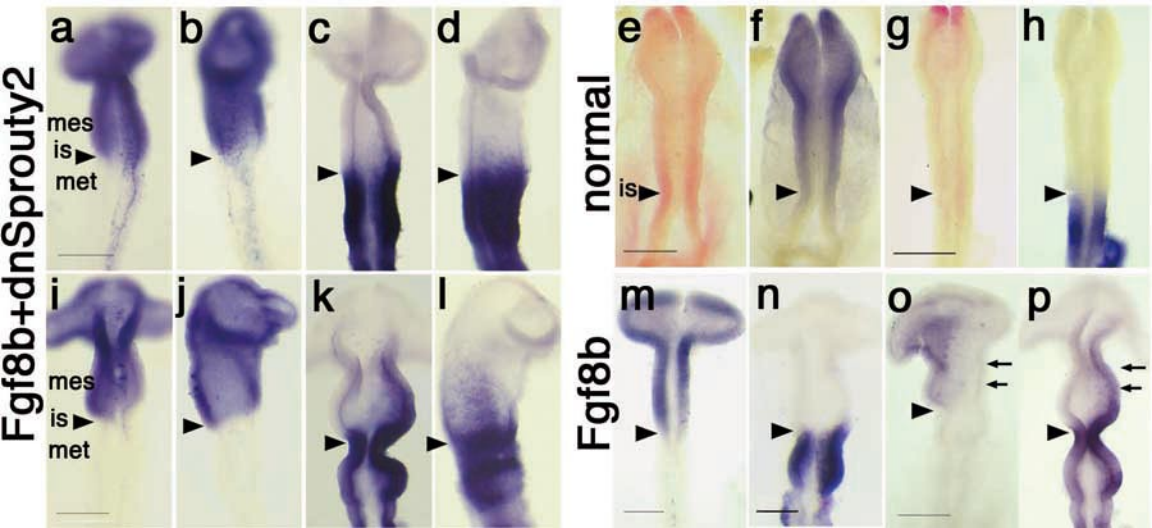
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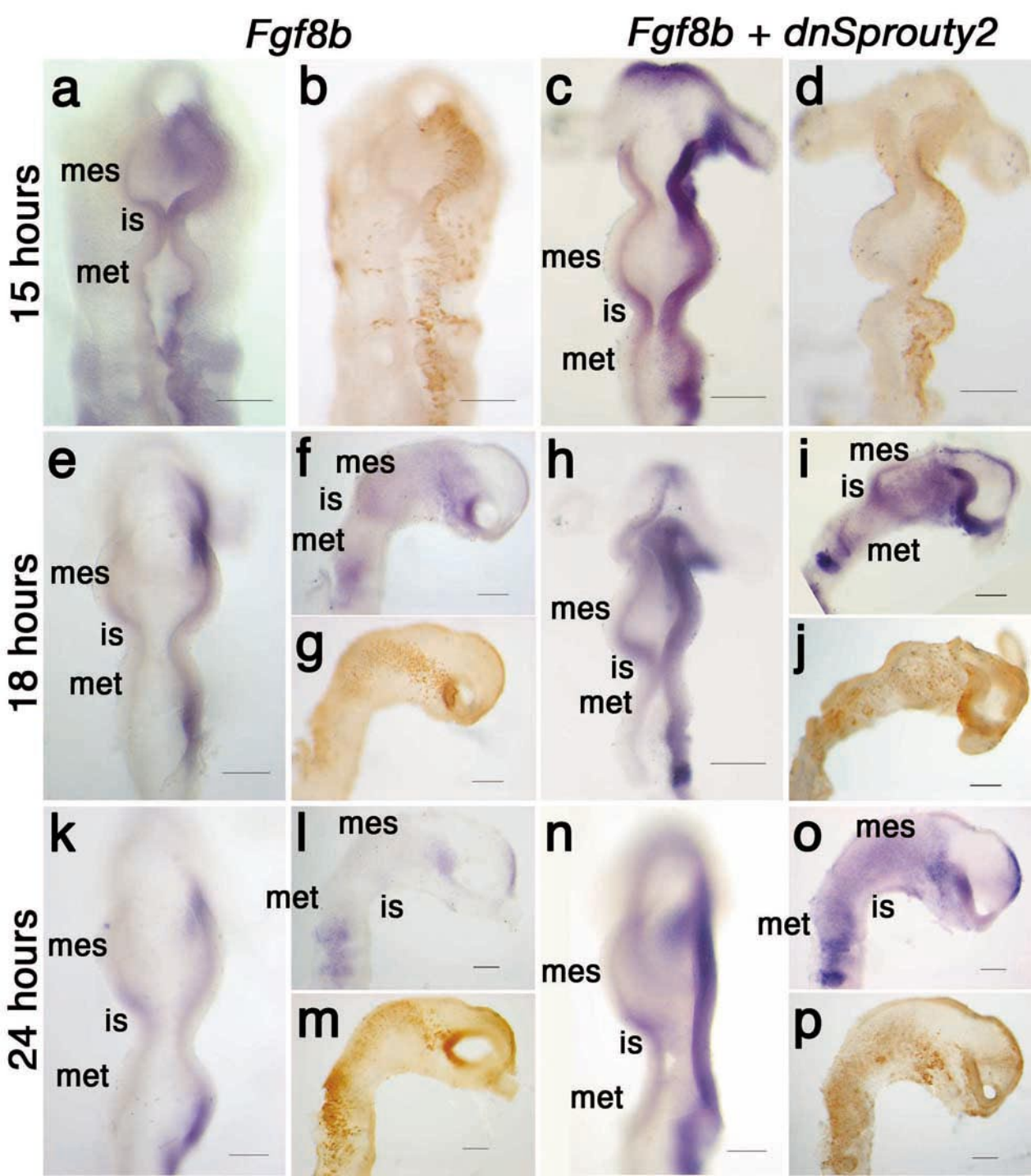
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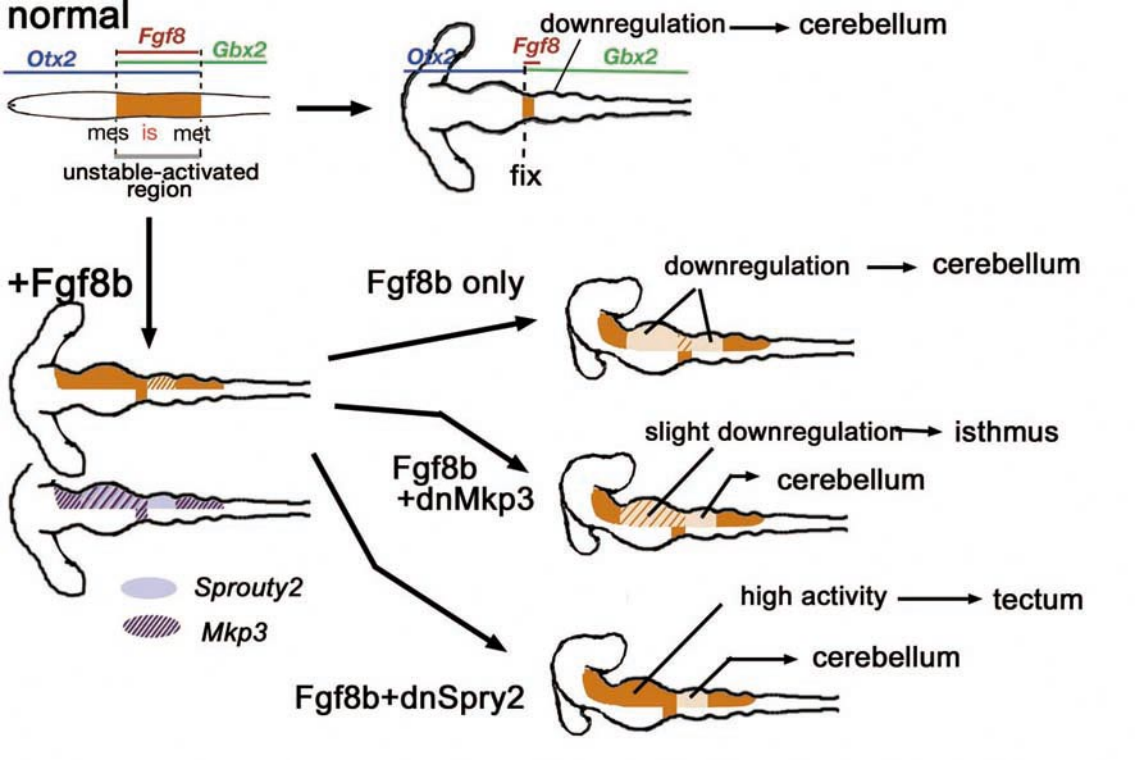
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