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# Graded Levels of FGF Protein Span the Midbrain and Can Instruct Graded Induction and Repression of Neural Mapping Labels

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## SUMMARY

**Graded guidance labels are widely used in neural map formation, but it is not well understood which potential strategy leads to their graded expression. In midbrain tectal map development, FGFs can induce an entire midbrain, but their protein distribution is unclear, nor is it known whether they may act instructively to produce graded gene expression. Using a receptor-alkaline phosphatase fusion probe, we find a long-range posterior > anterior FGF protein gradient spanning the midbrain. Heparan sulfate proteoglycan (HSPG) is required for this gradient. To test whether graded FGF concentrations can instruct graded gene expression, a quantitative tectal explant assay was developed. *Engrailed-2* and *ephrin-A*s, normally in posterior > anterior tectal gradients, showed graded upregulation. Moreover, *EphA*s, normally in anterior > posterior countergradients, showed coordinately graded downregulation. These results provide a mechanism to establish graded mapping labels and more generally provide a developmental strategy to coordinately induce a structure and pattern its cell properties in gradients.**

## INTRODUCTION

Topographic maps are found throughout the nervous system. As proposed by Sperry (Sperry, 1963), initial formation of these maps can be explained by graded recognition labels that are specified genetically. This requires, first, setting up positional labels in gradients and, second, reading the labels out to form a map. Studies over the last 15 years have extensively characterized the labeling molecules and downstream guidance mechanisms (McLaughlin and O'Leary, 2005; Luo and Flanagan, 2007). However, the upstream mechanisms that set up these labels in gradients are not well understood. More generally, it is not well understood how cells may be instructed by upstream patterning cues to produce a final output of graded intrinsic cell properties such as gene expression.

The midbrain tectum offers a favorable system to understand patterning of graded cell properties because it has been an extensively studied model for both developmental patterning (Raible and Brand, 2004; Sato et al., 2004; Partanen, 2007) and topographic mapping (Knoll and Drescher, 2002; McLaughlin and O'Leary, 2005; Luo and Flanagan, 2007). During map formation, ephrin-A and EphA proteins are cell surface labels expressed respectively in posterior > anterior and anterior > posterior tectal gradients and guide retinal axons to form a topographic map. The homeodomain protein Engrailed-2 (En2) shows a posterior > anterior tectal distribution and can induce *ephrin-As* when expressed in ectopic patches (Friedman and O'Leary, 1996; Logan et al., 1996; Shigetani et al., 1997), indicating a role upstream of *ephrin-A*s. In the initiation of midbrain development, a key role is played by *Fgf8* subfamily members. *Fgf8* mRNA forms a sharp band at the midbrain-hindbrain boundary (MHB) (Figure 1A), while *Fgf17* and *Fgf18* mRNAs are in slightly broader domains at the MHB (Sato et al., 2004). Strikingly, an FGF8-soaked bead implanted into the diencephalon can induce an entire ectopic midbrain (Crossley et al., 1996), demonstrating that FGF can act as an organizer signal for midbrain formation. In vivo studies have led to a model where different FGF signaling levels would induce discrete midbrain-hindbrain structures such as the tectum and cerebellum (Crossley et al., 1996; Lee et al., 1997; Liu et al., 1999, 2003; Martinez et al., 1999; Xu et al., 2000; Sato et al., 2001; Trokovic et al., 2003; Olsen et al., 2006; Basson et al., 2008). While FGF protein distribution is significant for such models, it has remained unclear; nor is it known what mechanism, following tectal induction, may produce gene expression in gradients (Figure 1A).

Particularly analogous to midbrain patterning is the classical model system of proximodistal patterning of discrete limb structures: both are polarized structures with a signaling center at one end; *Fgf8* RNA is expressed in a sharp band at one end of the structure; FGF-soaked beads can induce the structure; *Fgf* knockout results in cell death, size reduction, and malformed patterns (Sato et al., 2004; Tabin and Wolpert, 2007). The mechanism for limb proximodistal patterning is still under active investigation (Tabin and Wolpert, 2007). One model is the classic temporally based progress zone mechanism (Summerbell et al., 1973), where undifferentiated cells measure the time they spend in a zone near the distal tip, and their fates are specified in a proximal-to-distal order. In this model, FGFs are thought to be permissive for patterning by keeping distal cells alive and able to change fates and would only need to be localized at the distal tip. Other models have also been proposed,

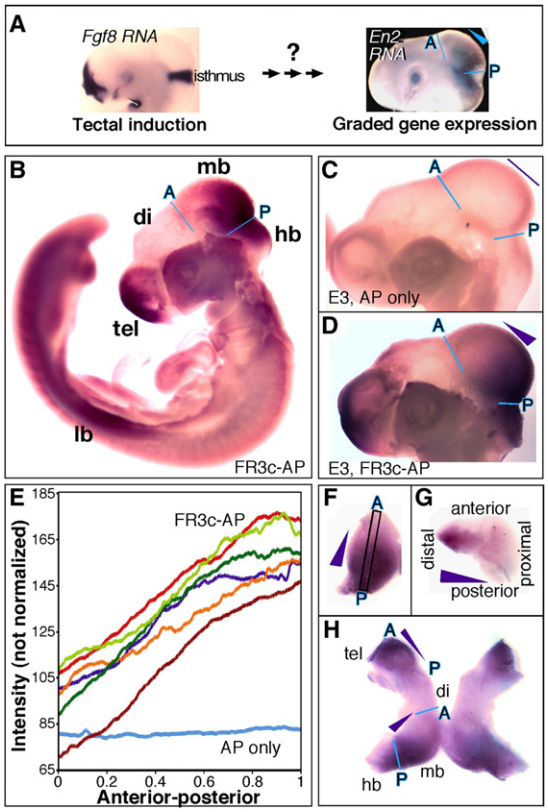


**Figure 1. FR3c-AP Binding to the Chick Embryo**

(A) Illustration of the question. FGF8 can induce an entire midbrain, and *Fgf8* RNA is expressed at the isthmus at the MHB (left, E3). Within the tectum, genes such as *En2* (right, E3), *ephrin-A*s, and *EphA*s appear in gradients. However, it is unclear what mechanisms may lead from tectal induction to gene expression in gradients. (B–D) FR3c-AP binding on E3 embryo whole mounts. (B and D) Binding can be seen in a posterior > anterior gradient in the midbrain, and in the telencephalon, hindbrain, and limb buds. (C) Unfused AP negative control. (E) Intensity plots across E3 tecta with FR3c-AP (n = 6) or AP control (n = 1), showing a reproducible posterior > anterior tectal gradient. Each curve represents one embryo. (F–H) Flat-mounted tectum (F), limb bud (G), or brain (H) from FR3c-AP in situ on E3 embryos, showing posterior > anterior midbrain gradient (F and H), anterior > posterior telencephalic distribution (H), and distal > proximal limb bud distribution (G). The 50 pixel wide rectangle placed on the tectum (F) was used to quantify intensity. Blue lines mark the anterior (A) and posterior (P) ends of the tectum. Purple schematics mark the presence or absence of a gradient. di, diencephalon; hb, hindbrain; lb, limb bud; mb, midbrain; tel, telencephalon.

notably a more recent prespecification model, where cell types are specified in early progenitors, which would then expand to produce discrete limb structures (Dudley et al., 2002; Sun et al., 2002). In this model, FGFs instruct cell fates (Mariani et al., 2008), which could potentially be mediated by a graded distribution of FGF proteins. Ongoing research has provided both evidence and challenges for each of these models (Tabin and Wolpert, 2007).

By analogy with the limb, following midbrain induction, there could be multiple mechanisms to generate graded gene expression. One set of models could involve FGF proteins acting permissively, by triggering patterning processes such as a downstream instructive gradient or a progress zone mechanism, where FGFs only need to be localized around the MHB. Alternatively, since FGFs are secreted proteins, they might be in a spatial gradient and directly instruct graded gene expression. Previous work has shown central developmental roles for graded molecules such as Hedgehogs, BMPs, and Bicoid, which can instruct an output of cell fates that are discrete (Kerszberg and Wolpert, 2007). However, it is unclear whether graded instructive cues provide a suitable strategy to generate an output that is graded, especially since engineering principles show that robustness can be difficult to achieve in conversion of graded input to graded output (Shannon, 1948; Oppenheim et al., 1997).

Previously, in vivo manipulations of FGF signaling have resulted in deletion, duplication, or expansion of midbrain structures (Sato et al., 2004), and it remains unknown whether FGFs might instruct graded gene expression. These in vivo phenotypes, and other previous findings such as differential midbrain distribution of MAP kinase activation (Sato and Nakamura, 2004), dominant-negative FGFR phenotypes beyond the MHB (Scholpp et al., 2003), or reduction in tectal expression domains of *Spry2*, *En*, and *ephrin-A2* in response to FGF signaling reduction (Carl and Wittbrodt, 1999; Basson et al., 2008), would be consistent with a spatial FGF gradient or with other mechanisms, such as growth coupled with temporal integration of signals. Finally, a previous study using an antibody raised against FGF8b reported a band at the MHB (Inatani et al., 2003), but it has remained unclear whether FGF protein might be in a gradient or whether the distribution extends throughout the tectum.

Here, we find that an FGFR-alkaline phosphatase fusion protein probe can be used to detect FGF proteins in situ and show a long-range gradient spanning millimeters across the embryonic midbrain. For a molecule to act as a graded instructive cue, two conditions must be met: (1) it should exist in a gradient, and (2) graded input levels should produce an appropriate cellular response. Thus, we developed an assay to systematically vary input concentrations and examine response of chick tectal explants to FGFs 8a, 8b, 17, and 18. The results show graded induction of *En2* and *ephrin-A* and coordinately graded repression of *EphA*, with the opposing responses matching the orientation of the tectal gradients in vivo. These results provide a mechanism to establish graded mapping labels in the tectum and potentially other neural maps throughout the brain. More generally, they can provide a principle for a patterning cue to coordinately induce a structure and produce within it graded cell properties.

## RESULTS

### In Situ Detection of FGFs with an FGFR-AP Fusion Probe

Genes downstream of FGFs are known to exist in midbrain gradients, but no upstream molecules are known to be in gradients (Figure 1A), so we were interested to know the FGF protein distribution itself. Detecting the distributions of secreted proteins, including gradients, has commonly been difficult by antibody staining, so we tested here whether a receptor-alkaline phosphatase (AP) fusion protein probe could be used to detect FGF

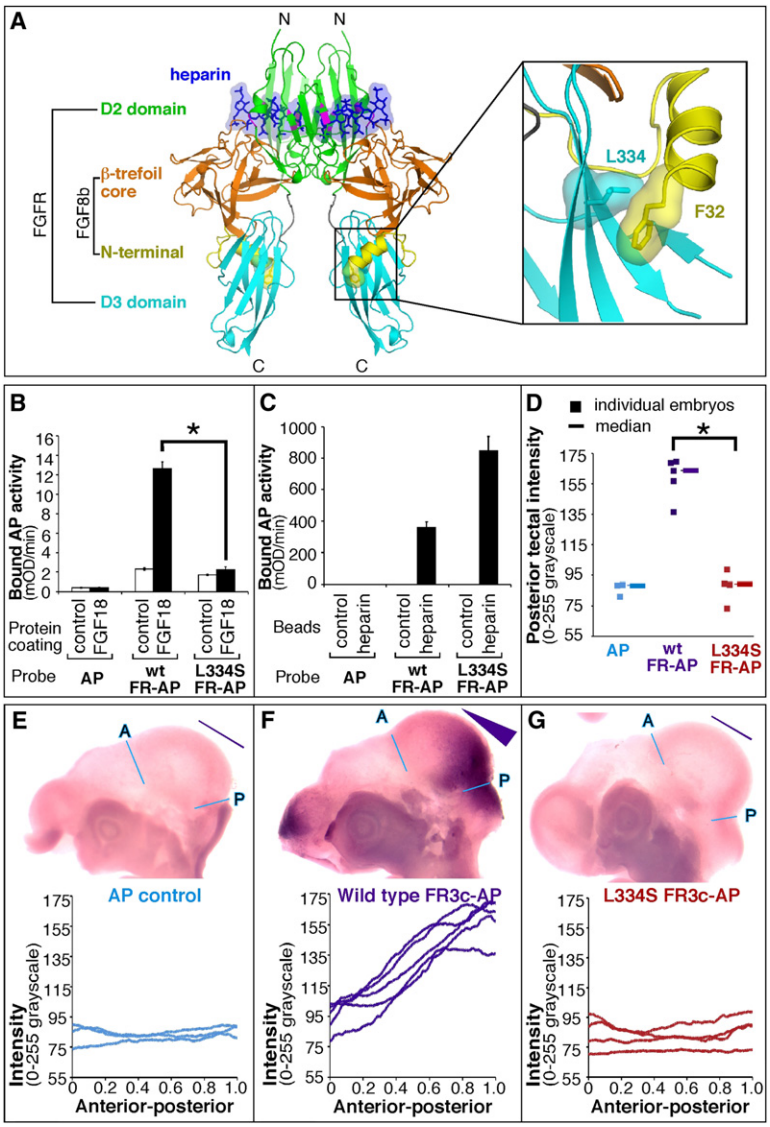


**Figure 2. Dependence of Tectal Gradient on FGF Binding Capacity of FR3c-AP**

(A) Structural representation of predicted hydrophobic interaction between L334 of FGFR3c and F32 of FGF8b, illustrating that this site is located at the interface for FGF8 subfamily members but separated from the heparin interface. Heparin binding residues on the receptor are in magenta. (B and C) Mutant FR3c-AP binding to FGF was greatly reduced, compared with wild-type (*$p < 0.001$); control: IgG. Binding to heparin-agarose beads was greater than wild-type ($p < 0.02$); control: sepharose beads. After binding, beads were washed with buffer containing 150 mM NaCl. Histograms are mean ± SEM. (D) Mutant FR3c-AP showed lower-intensity values for binding at the posterior tectal end than wild-type (*$p < 0.02$) and similar to AP control. Each square represents one tectum. The medians are shown (horizontal lines) because binding intensities are ordinal but not necessarily linear representations of concentrations. (E–G) Upper panels: affinity probe in situ on E3 embryos. Blue lines mark anterior (A) or posterior (P) ends of the tectum. Lower panels: tectal intensity plots: each curve represents one tectum. The tectal gradient was reduced to background level by the L334S mutation. wt, wild-type; FR-AP, FR3c-AP.

proteins in situ. Because this methodology involves native protein interactions, it has generally been used on lightly fixed or unfixed tissues to detect integral membrane proteins (Flanagan et al., 2000). It was therefore unclear whether it could detect the distribution of secreted FGFs. Indeed, FGFR-AP was previously reported not to detect FGFs in fixed tissues (Allen and Rapraeger, 2003).

A construct was made with the FGFR3c ectodomain linked to AP (FR3c-AP) (see Figure S1A available online). FGFR3c binds several FGFs, including members of the FGF8 subfamily (FGFs 8, 17, and 18) involved in midbrain-hindbrain patterning (Olsen et al., 2006; Zhang et al., 2006). In initial experiments, FR3c-AP was shown to bind purified FGF8 subfamily proteins ($p < 0.04$; Figure S1B). In addition, increased surface binding was seen with unfixed *Fgf8b*-transfected cells compared to vector-transfected controls ($p < 10^{-5}$; Figures S1C and S1D). Thus, FR3c-AP can be used to detect FGF8 subfamily members, including on cell surfaces in situ.

## FGF Protein Gradient in the Tectum

We next tested the FR3c-AP probe on unfixed embryos. On embryonic day 3 (E3) chick whole mounts, FR3c-AP bound prominently to the midbrain. Binding spanned the entire midbrain and appeared to be in a posterior > anterior gradient (Figures 1B–1D). A gradient was seen by E3 and continuing through E5; from E6 onward, tectal binding was present but no longer in an obvious gradient (data not shown). In other parts of the embryo, although not assessed in depth, FR3c-AP binding appeared to be in an anterior > posterior distribution in the forebrain, distal > proximal in the limb bud, and was also present in the hindbrain (Figures 1B, 1G, and 1H), all of which are locations where *Fgf* mRNA is expressed and plays important roles during development (Grove and Fukuchi-Shimogori, 2003; Sato et al., 2004; Tabin and Wolpert, 2007). Quantitation of the tectal pattern confirmed a long-range gradient (Figures 1E and 1F).

The gradient perceived by FR3c-AP is likely attributable to FGFs, since FR3c-AP binds FGFs 8, 17, and 18 and since the corresponding mRNAs are expressed around the MHB (Sato et al., 2004), where the tectal binding gradient was most intense (Figures 1A and 1B). Further confirmation came from several approaches. A specific point mutation, L334S, was made in FR3c-AP. This mutation inhibits FGFR binding to the FGF8 subfamily and is distant from the heparin interface of FGFR (Figure 2A) (Schlessinger et al., 2000; Olsen et al., 2006; Pitteloud et al., 2007). As expected, the mutation reduced recombinant FGF binding to background levels ($p < 0.001$; Figure 2B) and did not reduce heparin binding (Figure 2C). When the mutant FR3c-AP was tested on embryos, binding was reduced to background levels ($p < 0.02$ for wild-type versus mutant intensity in

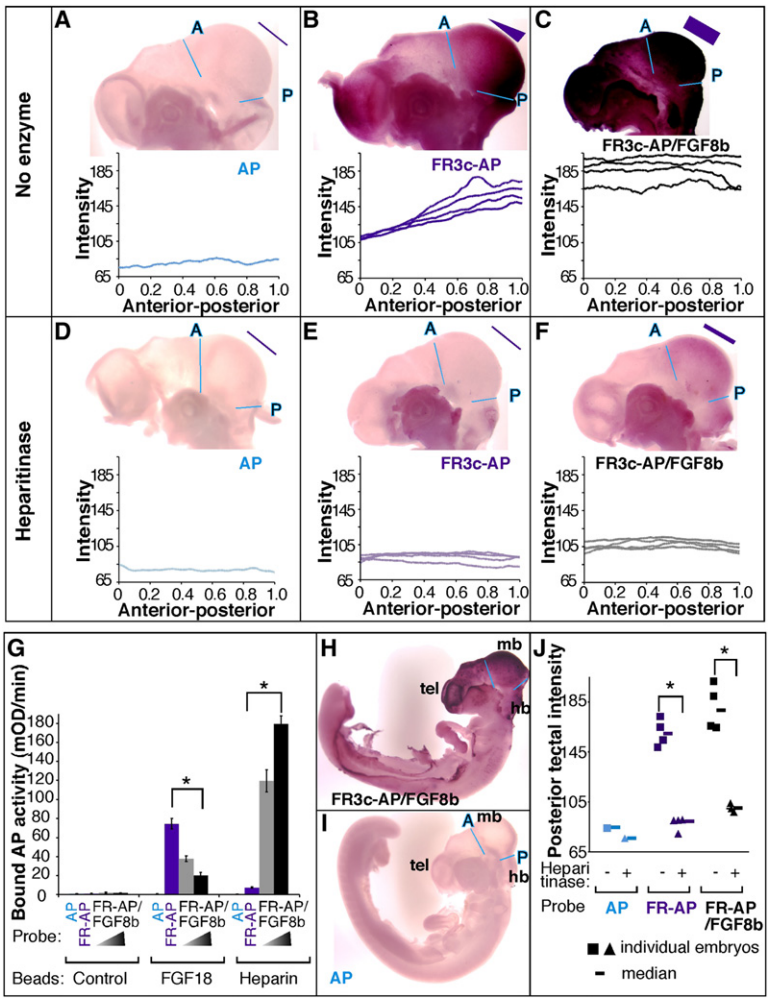


**Figure 3. Cognate HSPG Involvement and Distribution**

(A–F) Upper panels: binding of E3 chick brains with AP control, FR3c-AP, or in a modified LACE assay with FR3c-AP/FGF8b complex to detect cognate HSPGs. Lower panels: tectal intensity plots; each curve represents one tectum. Heparitinase pretreatment reduced FR3c-AP tectal binding gradient to background level and also greatly reduced FR3c-AP/FGF8b binding. (G) Preincubation with FGF8b increased FR3c-AP binding to heparin-agarose beads. Conversely, it reduced binding to FGF-agarose beads. Grey and black bars: 3.4 μg/ml and 13.6 μg/ml FGF8b, respectively. Control: BSA-agarose beads. After heparin binding, beads were washed with buffer containing 500 mM NaCl. Histograms show mean ± SEM, *$p < 0.005$. (H and I) E3 chick embryos treated with FR3c-AP/FGF8b probe or AP control. The colorimetric reaction was developed for a shorter time than in panel (C). Binding was seen in the telencephalon (tel), midbrain (mb), and hindbrain (hb). The midbrain binding did not appear graded. (J) FR3c-AP or FR3c-AP/FGF8b binding intensity at posterior ends of tecta, showing reduction in binding by pretreatment with heparitinase (*$p < 0.04$). Each square or triangle represents one tectum: horizontal lines show medians. Blue lines mark the anterior (A) or posterior (P) ends of the tectum. FR-AP, FR3c-AP.

posterior tectum; Figures 2D–2G), confirming FGF involvement. Another line of evidence came from an antibody-blocking approach: when a mixture of antibodies against FGF8b, 17, and 18 was applied to embryos prior to FR3c-AP, binding was greatly reduced (Figures S2A and S2B), further confirming FGF involvement. Additionally, tectal FGF distribution was examined in an approach independent of FR3c-AP. Proteins bound to the anterior or posterior tectal cell surface were extracted with salt, then tested by western blot with an antibody against FGF17, confirming a posterior > anterior difference (Figures S2C–S2E). Finally, given the FGF involvement in FR3c-AP binding, any additional tectal partner should bind to a complex of FR3c-AP with FGF, and experiments using an FR3c-AP/FGF probe did not detect a gradient, as described below.

## Cognate HSPG Involvement and Distribution

Detecting a gradient with FR3c-AP in unfixed embryos indicated that tectal FGFs are immobilized by interaction with cell surfaces or extracellular matrix. A good candidate to mediate gradient localization would be HSPG, which binds FGFs (Ornitz, 2000). Previously, gene disruption of HSPG synthesis was found to eliminate a band of FGF8b antibody staining at the MHB (Inatani et al., 2003), although not examining graded tectal FGF localization or formally addressing whether HSPG disruption could affect FGFs by an indirect developmental mechanism. Here, we used a complementary biochemical approach with the FR3c-AP probe. FR3c-AP binding was reduced to background levels by pretreatment either with heparitinase ($p < 0.04$; Figures 3B, 3E, and 3J) or with heparin as a competitor (Figures S2F and S2G). These results show an involvement of HSPG and support a model where HSPG localizes the FGF distribution in the midbrain-hindbrain region.

Next, the distribution of cognate HSPGs in the embryo was examined using a modified ligand and carbohydrate engagement (LACE) assay (Allen and Rapraeger, 2003). This technique exploits the greater avidity for HSPG of an FGFR/FGF complex than FGFR alone, which was confirmed by in vitro binding ($p < 0.005$ for increased binding to heparin, and for decreased binding to FGF; Figure 3G). When the FR3c-AP/FGF8b complex was applied to E3 whole mounts, strong binding was seen throughout the tectum and was not detectably graded (Figures 3H and 3I). Similar binding patterns were observed when FR3c-AP was preincubated with FGF8a, 17, or 18 (data not shown). The binding was greatly reduced by pretreatment of embryos with heparitinase ($p < 0.04$ for posterior intensity; Figures 3C, 3F, and 3J), demonstrating its HSPG dependence, as seen with other applications of LACE (Allen and Rapraeger, 2003). Taken together, the results indicate that both FGF and HSPG are required for FR3c-AP binding to the tectum and indicate a uniform distribution of cognate tectal HSPG, which could serve to bind and localize the posterior > anterior distribution of FGF. Interestingly, preferential binding by the FR3c-AP/FGF

complex was found in the midbrain, hindbrain, forebrain, and limb bud (Figures 3C, 3H, and S2H), regions where FGF8 subfamily members function (Grove and Fukuchi-Shimogori, 2003; Sato et al., 2004; Tabin and Wolpert, 2007).

## Graded Induction of *En2* by Increasing Concentration of FGFs

After finding a tectal gradient of FGF proteins, we wanted to test if different FGF concentrations could regulate expression of tectal genes in a graded manner. Since this is a quantitative question, we set up an assay where FGF concentrations could be varied systematically and the resulting gene expression measured quantitatively. Specifically, anterior tectal explants were grown on a membrane with a specified concentration of purified FGFs in the medium (Figure 4A), and 2 days later the explants were tested for downstream gene expression by quantitative RT-PCR.

Initial experiments tested the effect of FGF18 on *En2*. FGF18 caused a large induction of up to 26-fold in *En2* mRNA (Figure 4C). The *En2* expression appeared to increase in a graded manner with increasing FGF concentrations, and this was confirmed with two statistical tests. The first test assessed whether *En2* showed an increase with increasing FGF concentrations, and a significant positive correlation was found (correlation coefficient $\rho = 0.92$, $p < 0.0001$). Second, to assess if the response was graded, the data were tested against a two-region threshold model, and no position could be found where the results showed a discrete response above and below a threshold concentration ($p < 0.007$). Heparin addition produced a more than 10-fold increase in sensitivity (compare Figure S3A with 4E), lowering the FGF concentration requirement and showing that heparin can facilitate FGF signaling as in other systems. In control experiments, we developed a dissociated tectal cell culture system and still found graded *En2* induction by FGFs (Figure S3B), confirming that the graded response is not simply explained by limited penetration into explants. These results show that increasing FGF concentrations produced a graded increase of *En2*. Moreover, the direction of the response matched the direction of their in vivo gradients (Figures 4B, 4C, S4A, and S4D).

Previous work has found that four FGF8 subfamily proteins (FGFs 8a, 8b, 17, and 18) can differentially induce discrete midbrain versus hindbrain fates (Crossley et al., 1996; Lee et al., 1997; Liu et al., 1999, 2003; Sato et al., 2001). Here, we tested the effects of these different FGFs on *En2*. Rather than producing qualitatively different responses in this assay, all four FGFs appeared to produce a graded output of *En2* induction (Figures 4C and 4E). This appears consistent with previous studies that have led to a model attributing induction of discrete structures to different strengths of FGF signaling (see Discussion).

The FR3c-AP binding gradient is found throughout the tectum. Therefore, to test if a response to FGFs could be found at different positions along the anterior-posterior axis, the tectum was divided into three portions. Like the anterior explants (Figure 4C), middle and posterior thirds also showed induction of *En2* in response to increasing FGF ($\rho = 0.73$ for middle, $\rho = 0.67$ for posterior, $p < 0.02$ for both; Figure S3C). Thus, *En2* can be induced in response to increasing FGF levels throughout the region with an in ovo tectal FGF gradient.

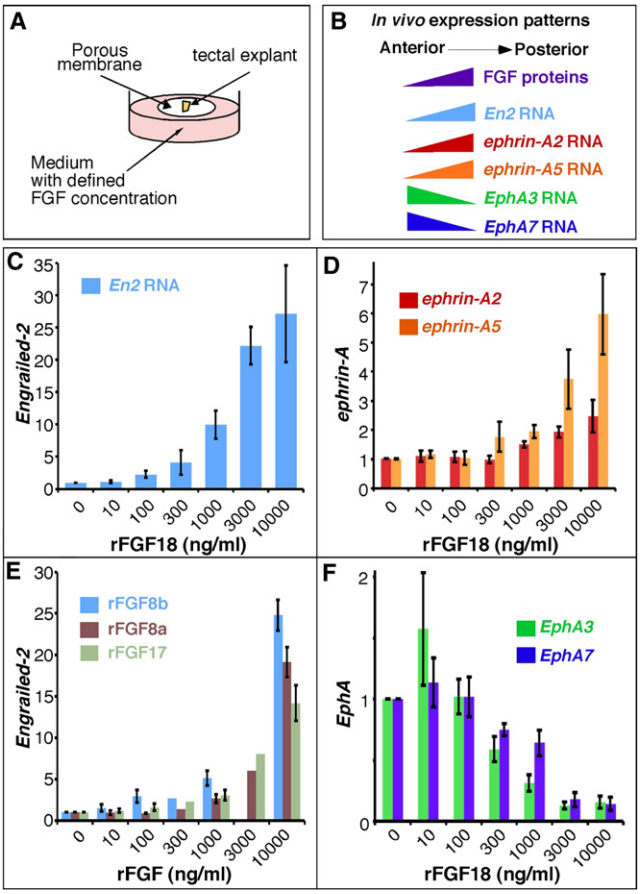


**Figure 4. Graded *En2* and *ephrin* Induction and Graded *Eph* Repression by FGFs**

(A) Illustration of the assay. Tectal explants from E3 chick embryos were cultured on a membrane floating in medium with a defined FGF concentration for 2 days. Gene expression was then determined by quantitative RT-PCR. (B) Schematic illustration of the tectal distribution of FGF proteins, *En2*, *ephrin-A*, and *EphA* RNA. (C–F) A graded increase in FGF18 concentrations produced a graded increase of *En2* (C), *ephrin-A* (D), and a graded decrease of *EphA* (F) mRNA in anterior tectal explants. Similar *En2* induction was also observed in response to FGFs 17, 8a, and 8b (E). The data point is absent at 3 μg/ml of rFGF8b. Histograms show mean ± SEM.

## FGFs Can Produce Graded Induction or Repression of Mapping Labels

The ultimate molecular output for topographic map formation is the graded expression of mapping labels. We therefore tested the effect of FGFs on expression of the best-characterized of these labels, the Ephs and ephrins. Increasing concentrations of FGF18 produced induction of *ephrin-A5* mRNA in a graded manner (Figure 4D; $\rho = 0.87$, $p < 0.0001$; $p < 0.01$ for two-region threshold model). *Ephrin-A2* also showed an increase in expression ($\rho = 0.68$, $p < 0.0001$); the two-region threshold test did not reach statistical significance for *ephrin-A2* ($p = 0.17$), although visual inspection of the data suggested a graded trend (Figure 4D). Interestingly, the fold response to FGF was greater for *ephrin-A5* than *ephrin-A2* ($p = 0.03$ at 10 μg/ml rFGF18; Figure 4D), correlating with the steeper tectal gradient for *ephrin-A5* than *ephrin-A2* in vivo (Monschau et al., 1997; Frisen

et al., 1998; Feldheim et al., 2000). This suggests the distinctive in vivo gradient shapes may be explained by differential gene responses to FGF.

Ephrin gradients are typically associated with a countergradient of Eph receptors (Knoll and Drescher, 2002; McLaughlin and O'Leary, 2005; Luo and Flanagan, 2007). We therefore tested the effect of FGFs on *EphA* expression. Increasing concentrations of FGF18 resulted in a graded decrease of *EphA3* ($\rho = -0.79$, $p < 0.0001$; $p < 0.05$ for two-region threshold model; Figure 4F). *EphA7* also showed a decrease ($\rho = -0.70$, $p < 0.0001$); the two-region threshold test did not reach statistical significance ($p = 0.14$), although visual inspection of the data suggested a graded trend (Figure 4F). From a technical perspective, the opposite outputs of *EphA* and *ephrin-A* provide further confirmation that our assay is not merely detecting global effects on gene expression or cell physiology but rather that different targets are regulated with specificity. In terms of biological implications, these results reveal a regulatory role of FGFs on graded *EphA* expression. Moreover, as with *En2*, the direction of change in *ephrin-A* and *EphA* expression in the assay matched the orientation of all the in vivo FGF and mapping label gradients (Figures 4B, 4D, 4F, and S4).

## DISCUSSION

Topographic maps are found throughout the brain, and a fundamental strategy to generate such maps is to use guidance labels in genetically specified gradients. While the labels themselves have been studied extensively, the upstream mechanism that leads to their expression in gradients has remained a gap in our understanding of the principles that lead from genes to maps. Here, we find that an FGF protein gradient spans the embryonic tectum and that graded levels of FGFs produce in tectal explants graded induction of *En2* and *ephrin-A*, as well as graded repression of *EphA*. The directions of these responses fit the polarity of all the corresponding gradients in vivo. Thus, FGF proteins have suitable properties to act as graded instructive cues to produce graded mapping labels in the tectum, and similar mechanisms are likely to be used in other neural maps. More generally, these results, together with previous studies, provide a model for a patterning cue to both induce a structure and instruct within it graded cell properties.

### A Gradient of FGF Proteins in the Midbrain

The spatial distribution of cell-cell signaling molecules is critical for developmental patterning. However, a great number of them are secreted, and antibody detection of their distribution has generally been difficult. We find here that the receptor-AP fusion technique (Flanagan et al., 2000), widely used to detect integral membrane proteins, can detect a gradient of secreted FGFs. The fusion probe approach may be generally useful to detect the distribution of secreted molecules, including other gradients that are difficult to detect with antibodies.

Due to the key role of FGF in midbrain induction and patterning, its protein distribution is important for understanding midbrain development. Here, using the FR3c-AP fusion probe approach, we have found a long-range FGF protein gradient spanning the tectum. Interestingly, a previous study detected a concentrated band of staining at the MHB with an antibody raised against FGF8b (Inatani et al., 2003). One possible explanation for the difference may be that the antibody staining had lower sensitivity, therefore detecting high levels of FGFs only, and correlating with the highest FR3c-AP probe binding here. Alternatively, the antibody may have specifically detected FGF8b or some subset of FGF8 subfamily members, while FR3c-AP may detect a broader composite of FGFs, including 8a, 8b, 17, and 18, since the fusion protein mimics native ligand-receptor interactions and therefore provides biological information about the overall distribution of cognate ligands (Flanagan et al., 2000). Potentially consistent with this explanation, FGF8b has higher affinity for HSPG than FGFs 17b and 18 (M.M., unpublished data), which might restrict FGF8b close to the MHB, while allowing other FGFs to spread further across the tectum. Such a mechanism might help shape the overall gradient.

The tectal gradient of FR3c-AP binding was seen by E3 and continued until E5. The uniform tectal distribution seen from E6 may be consistent with previous evidence that FGFs are involved in allowing retinal axons to enter the tectum from the optic tract, which was reported to show higher bFGF labeling than the tectum (McFarlane et al., 1995). Thus, FGFs may have initial roles in tectal induction and patterning and an additional later role in axon guidance.

### Mechanisms to Generate a Polarized Tectum

The ability of FGFs to induce an entire midbrain (Figure 5A) implies at least a permissive role in the expression of downstream tectal genes. Here, using a quantitative assay, we show that FGFs can also instruct graded gene expression. Moreover, they can produce both gene induction and repression. This is unlikely to be explained by *ephrin-A*s or *EphA*s regulating one another, since tectal *EphA* was not noticeably affected in *ephrin-A* knockout mice (Feldheim et al., 2000), nor was *ephrin-A* expression in *EphA* knockouts (Feldheim et al., 2004; Rashid et al., 2005). Ephs and ephrins show complementary expression patterns in multiple neural maps and many other tissues (Knoll and Drescher, 2002; Poliakov et al., 2004; McLaughlin and O'Leary, 2005; Picker and Brand, 2005; Flanagan, 2006). Thus, the finding here of FGF counterregulation of *ephrin-As* and *EphAs* may help provide insight into the mechanisms for their complementary expression in multiple contexts.

Spatial patterning of polarized tissues such as the tectum is a central feature of development, but it has been unclear which mechanism leads from initial tectal induction to production of graded cell properties. Drawing lessons from the analogous limb system, several mechanisms could potentially operate in midbrain patterning. Previous studies have provided important evidence on FGF actions in the midbrain and could be consistent with an FGF gradient mechanism, while also consistent with other mechanisms (see Introduction). Here, our finding that FGF proteins exist in a tectal gradient and can produce appropriately graded gene expression supports a model with a tectal FGF protein gradient acting instructively to produce an output of graded cell properties (Figure 5B). Such a model allows coordination between tissue induction and patterning of graded cell properties and could be used in multiple developmental contexts.

Our finding of a graded output of gene expression provides a complement to previous studies that led to a model where

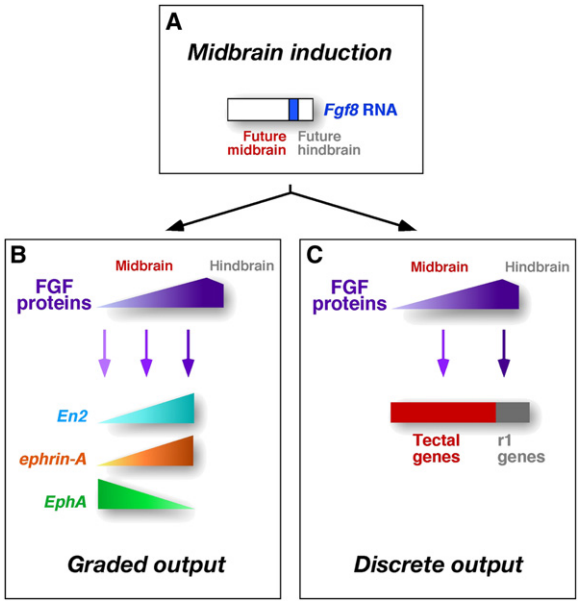


**Figure 5. Models of FGF Action in the Midbrain-Hindbrain Region**

(A) FGF8 can induce formation of an entire midbrain, and its RNA is expressed near the MHB. (B and C) As shown in this study, a posterior > anterior gradient of FGF proteins spans the midbrain. (B) Graded levels of FGFs produce in tectal explants a graded output of gene expression, including *En2*, *ephrin-A*, and *EphA*. (C) Graded levels of FGFs may also produce discrete midbrain versus hindbrain fates by inducing tectal versus rhombomere (r1) genes. This is likely to involve interactions of the FGF signal with other genes (see text). Processes illustrated in panels (A)–(C) may occur simultaneously or sequentially.

different strengths of FGF signaling can produce discrete midbrain versus hindbrain fates (Sato et al., 2001, 2004; Liu et al., 2003; Olsen et al., 2006). Together with the results described here, this leads to an overall model where FGF protein can produce both a discrete and a graded output (Figures 5B and 5C): high FGF signaling above a threshold would induce hindbrain structures; lower FGF signaling would induce midbrain; and within this range of FGF concentrations, graded FGF levels would produce graded gene expression. The graded versus discrete outputs could be generated via diverging downstream signaling pathways or by different *cis* elements for transcriptional regulation. Also, other genes are likely to act in concert with *Fgf*s. For example *Otx2*, which is expressed in the midbrain but not the hindbrain, can inhibit mesencephalon from differentiating into cerebellum at high FGF8b levels (Sato et al., 2001), and gene interactions of this type could allow FGF to have different actions on the two sides of the MHB.

### Graded Cues as a Developmental Patterning Strategy

Gradients of molecular cues are a fundamental strategy for developmental patterning. For an output of discrete cell fates, initial identification of molecules with morphogen actions has given insight into pattern formation and provided the basis for many subsequent studies of gradient formation and action. FGFs constitute a large family of patterning molecules, and here we find that they can fulfill the two basic requirements of a graded instructive cue for production of graded cell properties. First, they form a spatial gradient, with a remarkably long range spanning millimeters across the tectum, and second, a graded FGF input can produce a graded output of mapping labels. Thus, a graded input of cues can not only produce discrete cell fates, as shown previously, but also instruct a final output of graded cell properties. These findings can provide a developmental principle to pattern graded labels in the tectum and potentially other neural maps throughout the brain. More generally, taken together with previous findings, the results can provide a mechanism for a patterning cue to induce a structure and coordinately produce both discrete and graded cell properties.

## EXPERIMENTAL PROCEDURES

For whole-mount affinity probe in situ, after color development, tecta were flat mounted, imaged, and analyzed with NIH ImageJ. For quantitation, a 50 pixel wide rectangle was placed from the anterior to posterior end of one tectal lobe (Figure 1F). Fifty-nine pixels from each end were removed to reduce optical edge effects. Intensities were measured on a grayscale of 0 (white) to 255 (black). A-P tectal positions were normalized to a scale of [0, 1]. Quantitation was on original digital images; images in the figures were adjusted for color, brightness, and contrast, with the same adjustments for all images in a comparison group. Since intensities are ordinal but not necessarily linear representations of molecular concentration, statistical analyses used the nonparametric Mann-Whitney rank-sum test.

For tectal explant culture, the anterior, middle, or posterior 1/3 of E3 chick tecta were cultured on Nuclepore Track-Etch Membrane floating on medium for 2 days before quantitative RT-PCR. Two statistical tests were used to analyze gene expression. Spearman's correlation coefficient was used to test a correlation between gene expression and FGF concentration. To assess if the response was graded, data were tested for the null hypothesis that there is a point between two neighboring FGF concentrations that would fit a two-region threshold model, using the Kruskal-Wallis test to examine uniformity of response within regions below and above each possible threshold.

For more detailed experimental procedures, see Supplemental Data.

## SUPPLEMENTAL DATA

Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/neuron/supplemental/S0896-6273(09)00398-5.

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## REFERENCES

Allen, B.L., and Rapraeger, A.C. (2003). Spatial and temporal expression of heparan sulfate in mouse development regulates FGF and FGF receptor assembly. J. Cell Biol. *163*, 637–648.

Basson, M.A., Echevarria, D., Petersen Ahn, C., Sudarov, A., Joyner, A.L., Mason, I.J., Martinez, S., and Martin, G.R. (2008). Specific regions within the embryonic midbrain and cerebellum require different levels of FGF signaling during development. Development *135*, 889–898.

Carl, M., and Wittbrodt, J. (1999). Graded interference with FGF signalling reveals its dorsoventral asymmetry at the mid-hindbrain boundary. Development *126*, 5659–5667.

Crossley, P.H., Martinez, S., and Martin, G.R. (1996). Midbrain development induced by FGF8 in the chick embryo. Nature *380*, 66–68.

Dudley, A.T., Ros, M.A., and Tabin, C.J. (2002). A re-examination of proximodistal patterning during vertebrate limb development. Nature *418*, 539–544.

Feldheim, D.A., Kim, Y.I., Bergemann, A.D., Frisen, J., Barbacid, M., and Flanagan, J.G. (2000). Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron *25*, 563–574.

Feldheim, D.A., Nakamoto, M., Osterfield, M., Gale, N.W., DeChiara, T.M., Rohatgi, R., Yancopoulos, G.D., and Flanagan, J.G. (2004). Loss-of-function analysis of EphA receptors in retinotectal mapping. J. Neurosci. *24*, 2542–2550.

Flanagan, J.G. (2006). Neural map specification by gradients. Curr. Opin. Neurobiol. *16*, 59–66.

Flanagan, J.G., Cheng, H.J., Feldheim, D.A., Hattori, M., Lu, Q., and Vanderhaeghen, P. (2000). Alkaline phosphatase fusions of ligands or receptors as in situ probes for staining of cells, tissues, and embryos. Methods Enzymol. *327*, 19–35.

Friedman, G.C., and O'Leary, D.D. (1996). Retroviral misexpression of engrailed genes in the chick optic tectum perturbs the topographic targeting of retinal axons. J. Neurosci. *16*, 5498–5509.

Frisen, J., Yates, P.A., McLaughlin, T., Friedman, G.C., O'Leary, D.D., and Barbacid, M. (1998). Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. Neuron *20*, 235–243.

Grove, E.A., and Fukuchi-Shimogori, T. (2003). Generating the cerebral cortical area map. Annu. Rev. Neurosci. *26*, 355–380.

Inatani, M., Irie, F., Plump, A.S., Tessier-Lavigne, M., and Yamaguchi, Y. (2003). Mammalian brain morphogenesis and midline axon guidance require heparan sulfate. Science *302*, 1044–1046.

Kerszberg, M., and Wolpert, L. (2007). Specifying positional information in the embryo: looking beyond morphogens. Cell *130*, 205–209.

Knoll, B., and Drescher, U. (2002). Ephrin-As as receptors in topographic projections. Trends Neurosci. *25*, 145–149.

Lee, S.M., Danielian, P.S., Fritzsch, B., and McMahon, A.P. (1997). Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. Development *124*, 959–969.

Liu, A., Losos, K., and Joyner, A.L. (1999). FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development *126*, 4827–4838.

Liu, A., Li, J.Y., Bromleigh, C., Lao, Z., Niswander, L.A., and Joyner, A.L. (2003). FGF17b and FGF18 have different midbrain regulatory properties from FGF8b or activated FGF receptors. Development *130*, 6175–6185.

Logan, C., Wizenmann, A., Drescher, U., Monschau, B., Bonhoeffer, F., and Lumsden, A. (1996). Rostral optic tectum acquires caudal characteristics following ectopic engrailed expression. Curr. Biol. *6*, 1006–1014.

Luo, L., and Flanagan, J.G. (2007). Development of continuous and discrete neural maps. Neuron *56*, 284–300.

Mariani, F.V., Ahn, C.P., and Martin, G.R. (2008). Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. Nature *453*, 401–405.

Martinez, S., Crossley, P.H., Cobos, I., Rubenstein, J.L., and Martin, G.R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development *126*, 1189–1200.

McFarlane, S., McNeill, L., and Holt, C.E. (1995). FGF signaling and target recognition in the developing Xenopus visual system. Neuron *15*, 1017–1028.

McLaughlin, T., and O'Leary, D.D. (2005). Molecular gradients and development of retinotopic maps. Annu. Rev. Neurosci. *28*, 327–355.

Monschau, B., Kremoser, C., Ohta, K., Tanaka, H., Kaneko, T., Yamada, T., Handwerker, C., Hornberger, M.R., Loschinger, J., Pasquale, E.B., et al. (1997). Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons. EMBO J. *16*, 1258–1267.

Olsen, S.K., Li, J.Y., Bromleigh, C., Eliseenkova, A.V., Ibrahimi, O.A., Lao, Z., Zhang, F., Linhardt, R.J., Joyner, A.L., and Mohammadi, M. (2006). Structural basis by which alternative splicing modulates the organizer activity of FGF8 in the brain. Genes Dev. *20*, 185–198.

Oppenheim, A.V., Willsky, A.S., and Nawab, S.H. (1997). Signals & Systems, Second Edition (Upper Saddle River, NJ: Prentice Hall).

Ornitz, D.M. (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays *22*, 108–112.

Partanen, J. (2007). FGF signalling pathways in development of the midbrain and anterior hindbrain. J. Neurochem. *101*, 1185–1193.

Picker, A., and Brand, M. (2005). Fgf signals from a novel signaling center determine axial patterning of the prospective neural retina. Development *132*, 4951–4962.

Pitteloud, N., Quinton, R., Pearce, S., Raivio, T., Acierno, J., Dwyer, A., Plummer, L., Hughes, V., Seminara, S., Cheng, Y.Z., et al. (2007). Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. J. Clin. Invest. *117*, 457–463.

Poliakov, A., Cotrina, M., and Wilkinson, D.G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. Dev. Cell *7*, 465–480.

Raible, F., and Brand, M. (2004). Divide et Impera–the midbrain-hindbrain boundary and its organizer. Trends Neurosci. *27*, 727–734.

Rashid, T., Upton, A.L., Blentic, A., Ciossek, T., Knoll, B., Thompson, I.D., and Drescher, U. (2005). Opposing gradients of ephrin-As and EphA7 in the superior colliculus are essential for topographic mapping in the mammalian visual system. Neuron *47*, 57–69.

Sato, T., and Nakamura, H. (2004). The Fgf8 signal causes cerebellar differentiation by activating the Ras-ERK signaling pathway. Development *131*, 4275–4285.

Sato, T., Araki, I., and Nakamura, H. (2001). Inductive signal and tissue responsiveness defining the tectum and the cerebellum. Development *128*, 2461–2469.

Sato, T., Joyner, A.L., and Nakamura, H. (2004). How does Fgf signaling from the isthmic organizer induce midbrain and cerebellum development? Dev. Growth Differ. *46*, 487–494.

Schlessinger, J., Plotnikov, A.N., Ibrahimi, O.A., Eliseenkova, A.V., Yeh, B.K., Yayon, A., Linhardt, R.J., and Mohammadi, M. (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. Mol. Cell *6*, 743–750.

Scholpp, S., Lohs, C., and Brand, M. (2003). Engrailed and Fgf8 act synergistically to maintain the boundary between diencephalon and mesencephalon. Development *130*, 4881–4893.

Shannon, C.E. (1948). A mathematical theory of communication. The Bell System Technical Journal *27*, 379–423, 623–656.

Shigetani, Y., Funahashi, J.I., and Nakamura, H. (1997). En-2 regulates the expression of the ligands for Eph type tyrosine kinases in chick embryonic tectum. Neurosci. Res. *27*, 211–217.

Sperry, R.W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA *50*, 703–710.

Summerbell, D., Lewis, J.H., and Wolpert, L. (1973). Positional information in chick limb morphogenesis. Nature *244*, 492–496.

Sun, X., Mariani, F.V., and Martin, G.R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. Nature *418*, 501–508.

Tabin, C., and Wolpert, L. (2007). Rethinking the proximodistal axis of the vertebrate limb in the molecular era. Genes Dev. *21*, 1433–1442.

Trokovic, R., Trokovic, N., Hernesniemi, S., Pirvola, U., Vogt Weisenhorn, D.M., Rossant, J., McMahon, A.P., Wurst, W., and Partanen, J. (2003). FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals. EMBO J. *22*, 1811–1823.

Xu, J., Liu, Z., and Ornitz, D.M. (2000). Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures. Development *127*, 1833–1843.

Zhang, X., Ibrahimi, O.A., Olsen, S.K., Umemori, H., Mohammadi, M., and Ornitz, D.M. (2006). Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J. Biol. Chem. *281*, 15694–15700.