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EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region

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SUMMARY

***Fgf8*, which is expressed at the embryonic mid/hindbrain junction, is required for and sufficient to induce the formation of midbrain and cerebellar structures. To address through what genetic pathways FGF8 acts, we examined the epistatic relationships of mid/hindbrain genes that respond to FGF8, using a novel mouse brain explant culture system. We found that *En2* and *Gbx2* are the first genes to be induced by FGF8 in wild-type E9.5 diencephalic and midbrain explants treated with FGF8-soaked beads. By examining gene expression in *En1/2* double mutant mouse embryos, we found that *Fgf8*, *Wnt1* and *Pax5* do not require the *En* genes for initiation of expression, but do for their maintenance, and *Pax6* expression is expanded caudally into the midbrain in the absence of EN function. Since E9.5 *En1/2* double mutants lack the mid/hindbrain region, forebrain mutant explants were treated with FGF8 and, significantly, the EN transcription factors were found to be required for induction of *Pax5*. Thus, FGF8-regulated expression of *Pax5* is dependent on EN proteins, and a factor other than FGF8 could be involved in initiating normal *Pax5* expression in the mesencephalon/metencephalon. The *En* genes also play an important, but not absolute, role in repression of *Pax6* in forebrain explants by FGF8. Previous *Gbx2* gain-of-function studies have shown that misexpression of *Gbx2* in the midbrain can lead to repression of *Otx2*. However, in the absence of *Gbx2*, FGF8 can nevertheless repress *Otx2* expression in midbrain explants. In contrast, *Wnt1* is initially broadly induced in *Gbx2* mutant explants, as in wild-type explants, but not subsequently repressed in cells near FGF8 that normally express *Gbx2*. Thus GBX2 acts upstream of, or parallel to, FGF8 in repressing *Otx2*, and acts downstream of FGF8 in repression of *Wnt1*. This is the first such epistatic study performed in mouse that combines gain-of-function and loss-of-function approaches to reveal aspects of mouse gene regulation in the mesencephalon/metencephalon that have been difficult to address using either approach alone.**

Key words: Engrailed, *Lmx1b*, *Wnt1*, *Gbx2*, *Otx2*, *Pax5*, *Pax6*, Fibroblast growth factor, Organizer, Mouse

INTRODUCTION

Fgf8, which is expressed at the junction between the midbrain (mesencephalon or mes) and anterior hindbrain (metencephalon or met), has been shown in both chick and mouse to have an organizing activity that can induce ectopic expression of many mes/met genes and direct ectopic midbrain and cerebellar (anterior hindbrain) development in the posterior forebrain or midbrain (Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999; Shamim et al., 1999). Mes/met junction, or isthmic, tissue has a similar activity in heterotopic transplantation studies (reviewed by Alvarado-Mallart, 1993; Wassef and Joyner, 1997). Partial loss-of-function studies in mouse and fish support the idea that *Fgf8* is also essential for mes/met development (Brand et al., 1996; Meyers et al., 1998; Reifers et al., 1998). However, how FGF8 signaling is transmitted, and through what genetic pathways it acts, still remain to be determined.

In addition to *Fgf8*, *Wnt1*, *En1/2* and *Pax2/5* are expressed early in the mes/met region, with *Wnt1* expressed in the mes in a band of cells anterior to *Fgf8*, and *En1/2* and *Pax2/5* in mes and met cells surrounding the isthmus (reviewed by Wassef and Joyner, 1997; Joyner et al., 2000). Loss-of-function studies in both mouse and zebrafish have demonstrated that these families of genes are also required for early development of the mes/met region (reviewed by Wassef and Joyner, 1997; Joyner et al., 2000). Furthermore, gain-of-function studies have shown that mis-expression of *En1/2* or *Pax2/5* in chick or fish posterior forebrain results in ectopic expression of mes/met genes including *Fgf8*, and later induction of mes/met development (Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al., 1999; Ristoratore et al., 1999).

Gbx2 and *Otx2* are the first genes known to be expressed in a restricted manner in domains of the mes/met. Their complementary patterns of expression in the anterior or posterior brain with a common border near the mes/met organizer suggested they have antagonistic roles in normal patterning of the midbrain and cerebellum (Wassef and Joyner, 1997; Joyner et al., 2000). Indeed, mouse mutants with no *Otx2* expression in the epiblast fail to maintain rostral neural tissues, including the forebrain and midbrain, whereas ectopic expression of *Otx2* in the anterior hindbrain of mouse and chick embryos results in repression of *Gbx2* in the met, posterior expansion of the midbrain and partial deletion of the cerebellum (Acampora et al., 1998; Rhinn et al., 1998; Broccoli et al., 1999; Katahira et al., 2000). In a complementary manner, in *Gbx2* null mutants, anterior hindbrain tissue is lost and there is a posterior expansion of the *Otx2* expression domain and midbrain tissue (Wassarman et al., 1997; Millet et al., 1999). Furthermore, ectopic expression of *Gbx2* in the posterior midbrain of mouse or chick embryos results in repression of *Otx2* and a rostral shift of the isthmic expression domains of *Fgf8* and *Wnt1*, leading (in mouse embryos) to a transient reduction of the midbrain and expansion of the hindbrain at E9.5 (Millet et al., 1999; Katahira et al., 2000). These genetic studies show that a reciprocal negative interaction between *Gbx2*- and *Otx2*-expressing cells is indeed critical for mes/met patterning through positioning the mes/met border and maintaining a normal organizer.

Epistasis analysis in which gain- and loss-of-function mutants are combined has been extensively performed in many invertebrate species and has proven to be a powerful tool for determining the order of gene action during embryonic development. Owing to technical limitations, epistasis studies have been difficult to perform in vertebrates, although one study that was performed provided evidence that *En1* is a downstream target of WNT1 (Danielian and McMahon, 1996). A simple system that allows for quick epistasis studies in the mouse would be of great value in unraveling the molecular network underlying the formation and function of a normal mid/hindbrain organizer.

We recently described a mouse brain explant culture system that allows for a direct examination of the epistatic relationships between genes that respond to FGF8 (Liu et al., 1999). Using wild-type brain explants, we previously showed that FGF8 can induce *En1, En2* and *Pax5* in E9.5 diencephalic explants, *Gbx2* in both midbrain and diencephalic explants and repress *Otx2* in midbrain explants by 40 hours. In addition, FGF8 induces *Wnt1* in a ring of cells several cell diameters away from the FGF8 bead. In the current study, we found that FGF8 can also induce *Lmx1b* in midbrain explants and repress *Pax6* in posterior forebrain explants. Furthermore, *En2* and *Gbx2* are the first genes to be induced by FGF8 within 8 hours, and alterations in expression of *Pax5, Wnt1, Otx2* and *Pax6* do not occur until 16-40 hours. We extended these studies by determining the epistatic relationships of genes downstream of FGF8 signaling using explants taken from different mutant and transgenic embryos and examined gene expression in early *En1/2* mutant embryos. Diencephalic explant assays using mutant explants showed that the two En genes are required for induction of *Pax5* by FGF8, and in turn PAX5 can upregulate expression of an *En2* mid/hindbrain enhancer that contains PAX2/5-binding sites. Furthermore, while the En genes are not required for regulation of *Gbx2, Wnt1* or *Otx2* by FGF8, they are involved in, but not absolutely required for, repression of *Pax6* in diencephalic explants. In contrast, we found that in *En1/2* double mutant embryos, *Fgf8, Wnt1* and *Pax5* expression is initiated at early somite stages, but lost or greatly reduced by the 11-somite stage and *Pax6* expands into the midbrain. The changes in expression of *Pax5* and *Pax6* in *En1/2* mutants could be due to the early decrease in *Fgf8* expression and a factor other than FGF8 could be responsible for inducing the initial *Pax5* expression. Finally, although *Gbx2* is not required for the induction of *Wnt1, Lmx1b* or *En2* and the repression of *Otx2* by FGF8, it is indeed required for the exclusion of *Wnt1*-expressing cells from around a FGF8 source in midbrain explants. These studies place EN and GBX2 downstream of FGF8 in regulating *Pax5/6* and *Wnt1* expression, respectively, and GBX2 upstream of, and/or parallel to, FGF8 in regulating *Otx2*.

MATERIALS AND METHODS

Breeding and genotyping of the mutant embryos

Both *En1/2* and *Gbx2* mutants were kept on a mixed genetic background between 129 and Swiss Webster. *En1/2* mutants were genotyped by Southern blot hybridization (Millen et al., 1994; Hanks et al., 1995). *Gbx2* mutants were genotyped using a PCR approach (Wassarman et al., 1997). $En1^{+/-}$; $En2^{-/-}$ F_2 males were crossed to either $En1^{+/-}$; $En2^{-/-}$ or $En1^{+/-}$; $En2^{+/-}$ females to obtain *En1/2* double homozygous mutant embryos. $Gbx2^{+/-}$ mice were intercrossed to produce *Gbx2* homozygous mutant embryos.

Generation of transgenic animals

The *En2-CX* and *En2-ΔCX* transgenic lines were generated as described by Song et al. (Song et al., 1996). Transgenic animals were genotyped by a PCR reaction using *lacZ*-specific primers (Liu et al., 1999) and homozygotes were genotyped by comparing the intensities of the Southern blot hybridization signals obtained using a *lacZ*-specific probe and digestion of tail DNA with *Eco*RI, with the ones obtained using an *En2* 3′-probe that detects the endogenous gene (Millen et al., 1994).

Explant cultures, X-gal staining and whole-mount RNA in situ hybridization

Explant cultures were carried out as described previously (Liu et al., 1999) except that the concentration of FGF8b solution was 0.2 mg/ml, unless otherwise indicated. X-gal staining and whole-mount RNA in situ hybridization were performed as described in Liu et al. (Liu et al., 1999). The antisense riboprobes used for RNA in situ hybridization analyses were prepared using previously published mouse sequences, *En1, En2* (Millen et al., 1995), *Fgf8* (Crossley and Martin, 1995), *Gbx2* (Bouillet et al., 1995), *Lmx1b* (Chen et al., 1998), *Pax5* (Asano and Gruss, 1992), *Pax6* (Grindley et al., 1997), *Otx2* (Simeone et al., 1993) and *Wnt1* (Parr et al., 1993).

RESULTS

Gbx2 and *En2* are the first genes to be induced by FGF8

As a first step in dissecting the genetic pathway downstream of FGF8 signaling during mes/met development, the temporal order of gene expression alterations was compared in E9.5 wild type diencephalic explants (*En1, En2, Pax5, Wnt1, Pax6* and *Gbx2* expression), or midbrain explants (*En2, Pax5, Wnt1,*

Lmx1b, *Otx2* and *Gbx2* expression) cultured with FGF8b-soaked beads (Table 1). Among the genes examined, weak expression of *Gbx2* (in midbrain explants) and *En2* was seen in cells around the FGF8-soaked beads after 8 and 16 hours in culture and strong expression by 40 hours. *En1* was discernible at 16 hours and strong at 40 hours. By contrast, by 16 hours, *Wnt1* and *Pax5* expression was not induced, and *Otx2* was not repressed, although alterations in gene expression were seen after 40 hours in culture. *Pax6* expression in diencephalic explants seemed to be partially repressed after 16 hours and was completely repressed by FGF8b but not bovine serum albumin (BSA) at 40 hours (Table 1; Fig. 1A,B). Interestingly, after 16 hours, *Lmx1b* was induced in a broad region surrounding the FGF8 beads in midbrain explants (Fig. 1C), which was followed by transient *Wnt1* expression in scattered cells near the FGF8b-soaked beads in midbrain explants at 24 hours (Fig. 1E). By 40 hours, *Wnt1* expression was restricted to a ring of cells at a distance from the beads (Fig. 1F and Liu et al., 1999), whereas *Lmx1b* expression was more restricted than seen at 16 hours, in cells adjacent to the beads (Fig. 1D). The fact that *En2* and *Gbx2* expression was altered by FGF8 earlier than expression of other genes makes it possible that *En2* and *Gbx2* could be in higher tiers than the other genes in the genetic hierarchy of FGF8 signaling. We therefore used transgenic and mutant mouse brain explants to further examine the roles for En and *Gbx2* in the FGF8 signaling pathway.

Many mes/met genes are initiated but quickly downregulated in *En1/2* double mutants at early somite stages

Previous studies of *En1* (Wurst et al., 1994) and *En2* (Millen et al., 1994) single mutants and an allele in which *En1* was replaced with *En2* (Hanks et al., 1995; Hanks et al., 1998) demonstrated that the two genes have overlapping functions. In order to determine the normal requirement for both En genes during early patterning of the mouse mes/met region, before studying any specific requirements for En genes in mediating FGF8 signaling, gene expression was examined in *En1/2* double mutants. The two En null alleles used in these studies were $En1^{lki}$, referred to as $En1^{-}$, in which *lacZ* replaces part of the first exon of the *En1* gene (Hanks et al., 1995; Matise and Joyner, 1997), and $En2^{ntd}$, referred to as $En2^{-}$, in which a *Neo* gene replaces part of the first exon of the *En2* gene (Millen et al., 1994). For most experiments $En1^{+/-}; En2^{-/-}$ mice were interbred to produce embryos for gene expression studies and for explant assays.

At E9.5, *En1/2* double homozygous mutants were found to have a general deletion of the mes/met region that could be used to distinguish such mutants from their normal-appearing $En1^{+/-}; En2^{-/-}$ littermates by visual inspection of the morphology of the brain. Consistent with the morphology, mes/met genes such as *Pax5*, *Fgf8*, and *Gbx2* were not detected in $En1^{-/-}; En2^{-/-}$ embryos (Fig. 2A,A′,B,B′ and data not shown). *Otx2*, which is normally expressed in the forebrain and midbrain, had a caudal limit of expression in *En1/2* double mutants that was shared with the caudal limit of *Pax6* expression, which normally marks the caudal limit of forebrain (Fig. 2C,C′,D,D′). *lacZ* expression from the *En1* knock-in locus was monitored to identify any cells remaining that express the *En1* mutant allele. *lacZ* expression was seen in a broad transverse band of cells across the mes/met junction in $En1^{+/-}; En2^{-/-}$ E9.5 embryos similar to $En1^{+/-}$ embryos (Fig. 2E and data not shown). In double homozygous *En1/2* mutant embryos, *lacZ* expression was seen strongly only in a small ventral midline patch around the mes/met junction, and weakly in a thin transverse band of cells at what appeared to be the anterior end of the hindbrain (Fig. 2E′). From these studies it appears that most, if not the entire midbrain and rhombomere 1 (r1) are lacking in *En1/2* double homozygous mutants, but that the diencephalon remains, thus providing FGF8-competent tissue for explant cultures.

In order to address whether the deletion of the mes/met region seen at E9.5 was due to lack of initial specification of the mes/met or a failure of the mes/met cells to maintain their identity and/or proliferation, early somite stage *En1/2* mutant embryos were analyzed for gene expression. At the five- to seven-somite stage, the *En1/2* double homozygous mutant embryos appeared similar to their $En1^{+/-}; En2^{-/-}$ littermates. *lacZ* (n=3) expression was found in the mes/met of *En1/2* double homozygous embryos (Fig. 3A′) at a level similar to that in their heterozygous littermates (Fig. 3A), although stronger staining is expected in the homozygous embryos in which two *En1-lacZ* alleles are present. Similarly, *Gbx2* (n=2), *Pax5* (n=2), *Wnt1* (n=2) and *Fgf8* (n=2) were also expressed in the mes/met region of *En1/2* double homozygous mutants, although it seemed that the expression of *Gbx2*, *Pax5* and *Fgf8*

Table 1. Gene expression profiles in brain explants cultured with FGF8b-soaked beads

(A) Diencephalic explants	*En1* i	*En2* i	*Pax5* i	*Pax6* r	*Gbx2* i	*Otx2* r	*Wnt1* i/r
8 hours	0/2	2/3§	nd	nd	nd	nd	nd
16 hours	7/7§	3/3§	0/3	1/2*	2/2§	nd	0/3
40 hours	25/25	17/18	4/4	5/5	8/8	0/21‡	5/7

(B) Midbrain explants	*En2* i	*Pax5* i	*Gbx2* i	*Otx2* r	*Wnt1* i/r	*Lmx1b* i
8 hours	2/2§	0/2	2/3§	nd	nd	nd
16 hours	2/2§	0/2	9/10§	0/3	0/2	2/2§
40 hours	4/4	2/2	10/11	8/8	9/9	2/2

*Incomplete repression was seen.
‡In BSA- and FGF8-treated explants, *Otx2* was variably partially lost.
§At these stages, expression was weak and in a limited domain of cells
i, induced; nd, not determined; r, repressed.

was weaker, with expression of *Pax5* and *Fgf8* found in more restricted domains than in their *En1*$^{+/-}$; *En2*$^{-/-}$ littermates (Fig. 3B,B′,C,C′,D,D′,E,E′). By the 10-12 somite stage, no major morphological deletion of tissue could be detected in *En1/2* double homozygous mutants compared with their *En1*$^{+/-}$; *En2*$^{-/-}$ littermates (compare Fig. 3F with 3F′). Indeed, *lacZ* expression from the *En1* locus in double *En1/2* mutants was in a similar mes/met domain to in *En1*$^{+/-}$; *En2*$^{-/-}$ littermates (Fig. 3F,F′). In contrast to *En1-lacZ* expression in *En1/2* double homozygous mutants, *Pax5* (*n*=2) and *Fgf8* (*n*=2) expression was much weaker and in more restricted domains compared with their littermates (Fig. 3H,H′,J,J′). Furthermore, *Fgf8* expression was not seen in one out of three double mutants examined at this stage (data not shown). *Wnt1* expression in the transverse band seen in the posterior midbrain of *En1*$^{+/-}$; *En2*$^{-/-}$ embryos (Fig. 3I) was not seen in double homozygous 11-somite mutants (Fig. 3I′ *n*=2). Furthermore, in the double homozygous mutants, the lateral expression of *Wnt1* was continuous along the anteroposterior axis, unlike in *En1*$^{+/-}$; *En2*$^{-/-}$ and wild-type embryos, in which *Wnt1* expression along the lateral edge of the neural plate was absent in the anterior hindbrain. *Gbx2* expression in r1 was also much weaker in *En1/2* double homozygous mutant compared with their *En1*$^{+/-}$; *En2*$^{-/-}$ littermates (Fig. 2G,G′).

In chick embryos, the expression domain of the En genes abuts that of the diencephalon gene *Pax6* during early mes/met development. Furthermore, misexpression of *En1* in the diencephalon leads to repression of *Pax6* (Araki and Nakamura, 1999). Based on these observations it was suggested that the En genes are involved in setting up the forebrain/midbrain boundary by repressing the forebrain gene *Pax6*. In mouse, the *En1* mes/met expression domain abuts the *Pax6* forebrain expression domain briefly before the eight-somite stage (A. L. and A. L. J., unpublished observations). To determine whether the En genes are required to maintain the normal *Pax6* expression pattern, *En1/2* early somite double homozygotes were analyzed for *Pax6* expression. In 11-somite *En1/2* mutants, *Pax6* expression was expanded posteriorly compared with that in *En1*$^{+/-}$; *En2*$^{-/-}$ littermates, but the expansion was seen only in the lateral (dorsal) part of the neural plate and formed a decreasing gradient posteriorly, suggesting that EN proteins are involved in, but not the only factors required for, repressing *Pax6* in the mes/met region (Fig. 3K,K′).

EN proteins are required for induction of *Pax5* and involved in the repression of *Pax6* by FGF8

In *En1/2* double homozygous mutant E9.5 embryos, the constriction between the telencephalon and dorsal diencephalon is apparent, and a minor constriction can be seen posterior to *Otx2* and *Pax6* expression domains (arrowheads in Fig. 2C′,D′). Based on our gene expression studies we assume that the region between the two constrictions corresponds to the diencephalon in *En1/2* mutants. Tissue in the anterior two-thirds of this diencephalic region was taken for explant culture to ensure that no hindbrain tissue was included. Such explants were compared with diencephalic explants taken from apparently normal *En1*$^{+/-}$; *En2*$^{-/-}$ littermates. *En1-lacZ* expression was induced in explants taken from double homozygous mutant embryos, or from their wild-type-appearing littermates after 40 hours in culture with FGF8b-soaked beads, showing that EN proteins are not required for induction of transcription from the *En1* locus by FGF8b (Fig. 4A-C). We subsequently examined *Pax5* mRNA expression in the same explants. *Pax5* induction was robust in explants taken from *En1*$^{+/+}$; *En2*$^{+/-}$ and *En1*$^{-/-}$; *En2*$^{+/-}$ embryos (Fig. 4A and data not shown). In contrast, *Pax5* induction was not detected in the double *En1/2* homozygous mutant embryos cultured with beads soaked in either 0.2 mg/ml (Fig. 4C, *n*=2) or 1mg/ml (inset in Fig. 4C, *n*=3) FGF8b protein solution. Interestingly, *Pax5* expression was barely detected in only one out of three *En1*$^{+/-}$; *En2*$^{-/-}$ explants and not detected in the other two (Fig. 4B and data not shown). These studies demonstrate that unlike in *En1/2* mutant embryos at early somite stages where *Pax5* is not dependent on EN function, the En genes are required for induction of *Pax5* by FGF8. Furthermore, EN2, and not EN1, is the limiting factor downstream of FGF8 in the process of activating *Pax5* in forebrain explants.

We next examined whether the EN proteins are required for repression of *Pax6* by FGF8 in diencephalic explants. As described above, when forebrain explants were taken from wild-type embryos and cultured for 40 hours, *Pax6* expression was greatly reduced in cells adjacent to FGF8b-soaked beads (Fig. 4D). In contrast to wild-type explants, *Pax6* was variably

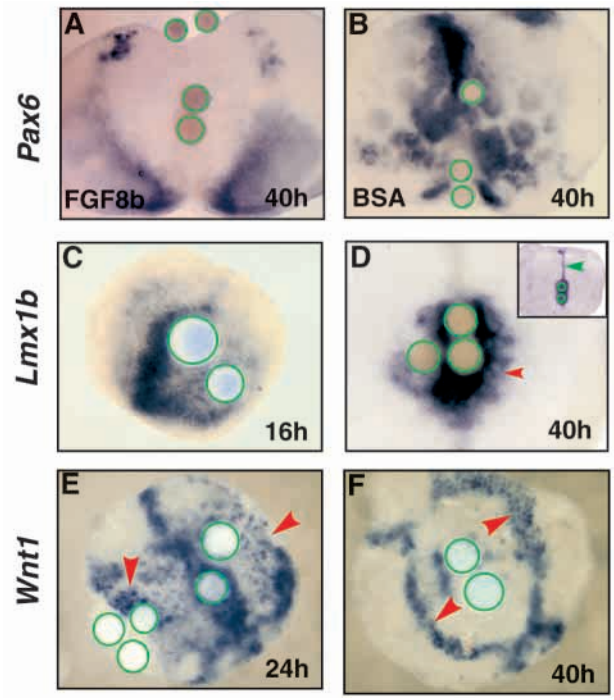

Fig. 1. FGF8b-soaked beads repress *Pax6* in caudal forebrain explants and alter the expression of *Lmx1b* and *Wnt1* in midbrain explants. (A,B) FGF8b-soaked beads (A) but not BSA-soaked beads (B) repress *Pax6* expression in diencephalic explants. (C,D) In midbrain explants, *Lmx1b* expression is induced by FGF8b by 16 hours (C) and its expression is in cells adjacent to the beads by 40 hours (D). The inset in D shows that BSA-soaked beads do not alter the endogenous *Lmx1b* expression (green arrowhead) (E,F) In midbrain explants, *Wnt1* expression is induced in a lot of cells at 24 hour (E, arrowheads), but by 40 hours (F), it is repressed in cells adjacent to the beads, and only expressed in a ring of cells a distance away (arrowheads). Beads are outlined in green.

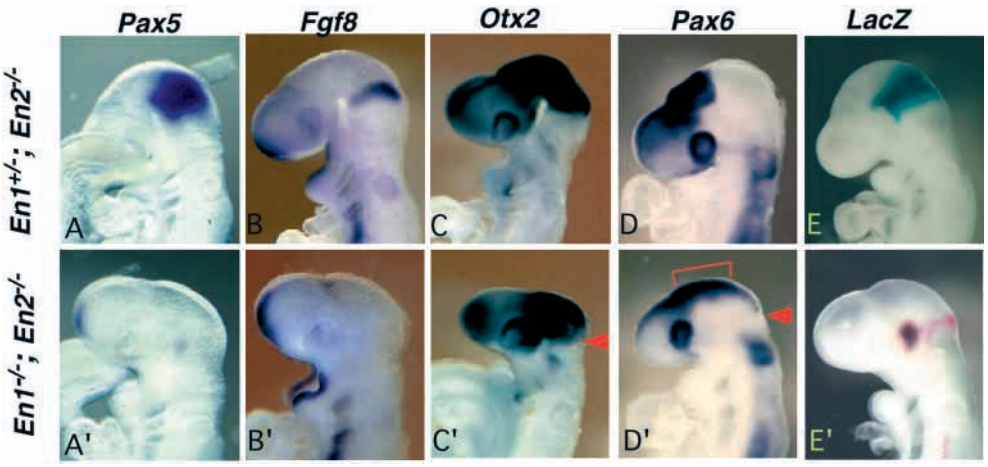

Fig. 2. The midbrain and anterior hindbrain are absent in *En1/2* double homozygous mutant embryos at E9.5.(A,A′) *Pax5*, (B,B′) *Fgf8*, (C,C′) *Otx2*, (D,D′) *Pax6* and (E,E′) *En1-lacZ* expression in E9.5 embryos. The embryos in A-E are $En1^{+/-}; En2^{-/-}$ embryos and the ones in A′-E′ are $En1^{-/-}; En2^{-/-}$ embryos. In $En1^{-/-}; En2^{-/-}$ embryos, a loss of mes/met tissue is morphologically obvious and *Fgf8* and *Pax5* expression in the mes/met region is missing. The caudal boundary of the *Pax6* forebrain expression domain is coincident with the caudal boundary of *Otx2* expression. *En1-lacZ* is only strongly expressed in a ventral patch and weakly expressed in a thin transverse band. The red arrowheads in C′ and D′ point to the constriction at the posterior border of the *Pax6* and *Otx2* domain in the *En1/2* double homozygous embryos. The red bracket in D′ indicates the region that is taken for explant cultures.

and only partially repressed by FGF8 in *En1/2* double homozygous mutant explants (n=4). In one explant, the *Pax6*-negative region was more restricted to cells in the vicinity of the beads than in wild type explants (Fig. 4E). In the other three explants, two of which were cultured with beads soaked in 1 mg/ml FGF8b protein solution, cells adjacent to the beads showed weak *Pax6* expression (Fig. 4F). These results show that the En genes are involved in the repression of *Pax6* by FGF8 in the forebrain, but that other factors must also be involved.

We also examined the induction of *Gbx2* (n=2) and *Wnt1* (n=2) expression in *En1/2* double homozygous mutant explants treated for 40 hours with FGF8b-soaked beads; the two genes showed similar responses to their $En1^{+/-}; En2^{-/-}$ littermates and wild-type embryos (data not shown). This shows that FGF8 can regulate *Gbx2* and *Wnt1* via EN-independent pathways.

PAX2/5-binding sites are required for the upregulation of an *En2* mes/met reporter by FGF8

We have previously shown that two PAX2/5-binding sites within a 1 kb *En2* mes/met enhancer fragment (*En2-CX*) are required for expression of a *lacZ* reporter gene in the region of the mes/met junction in early mouse embryos (Song et al., 1996), indicating that PAX2/5 are involved in regulation of *En2* in the mes/met, in a reciprocal manner to the EN regulation of *Pax5* downstream of FGF8. In order to determine whether the *En2-CX* DNA enhancer fragment is regulated by FGF8 in brain explants, and whether the PAX2/5-binding sites are necessary for such regulation, transgenic embryos were generated containing the 1 kb *En2-CX* enhancer driving *lacZ* (Fig. 5A, here referred to as *En2-lacZ*) and an enhancer, *En2-ΔCX*, lacking the PAX2/5 binding sites (here referred to as $En2^{PBD}$-*lacZ*, where PBD refers to PAX2/5-binding sites deletion, Fig. 5B and Song et al., 1996). As reported previously (Song et al., 1996), E9.5 *En2-lacZ* embryos were found to express *lacZ* in the mes/met region (Fig. 5C), while $En2^{PBD}$-*lacZ* embryos did not (Fig. 5D). Both transgenes express *lacZ* in the spinal cord of embryos due to sequences in the heat shock minimal promoter (Logan et al., 1993). We next determined whether the *En2-lacZ* reporter could respond to FGF8 and more interestingly, whether any induction depended on the PAX2/5-binding sites within the enhancer region. Explants taken from the diencephalic region of *En2-lacZ* and $En2^{PBD}$-*lacZ* transgenics showed scattered low level expression of *lacZ* after 40 hours in culture with control BSA beads (data not shown), indicating that unknown factors in the medium can support a low level of reporter gene expression in a PAX2/5-independent manner. This is not unexpected since binding sequences for many transcription activators are present in the 1 kb regulatory sequence (Song et al., 1996). However, when the explants were cultured for 40 hours with FGF8b-soaked beads, specific expression of the *En2-lacZ* reporter was induced in cells surrounding the beads (Fig. 5E), whereas expression of the $En2^{PBD}$-*lacZ* reporter remained similar to that with BSA control beads (Fig. 5F and data not shown). Furthermore, consistent with the late timing of *Pax5* induction in the brain explants, and unlike endogenous *En2* gene expression, the *En2-lacZ* transgene did not show distinguishable upregulation after 16 hours of explant culture with FGF8b-soaked beads (Fig. 5G and inset). These studies, and our finding that *Pax2* and *Pax8* are not induced by FGF8b (Liu et al., 1999), indicate that the FGF8b-dependent upregulation of the *En2-lacZ* reporter is dependent on PAX5, and the induction is unlike that of the endogenous *En2* gene at 16 hours.

Gbx2 is not required for repression of *Otx2* by FGF8 but is required for late repression of *Wnt1* in cells close to FGF8-soaked beads

We have previously shown that when midbrain explants are cultured with FGF8b-soaked beads, *Gbx2* is induced by 40 hours in *Otx2*-negative cells, and that there is also a ring of *Gbx2*- and *Otx2*-negative cells surrounding the *Gbx2*-expressing cells (Liu et al., 1999). In addition, *Wnt1*-expressing cells are induced adjacent to the *Gbx2*-expressing cells. This spatial relationship of gene expression, and the fact that *Gbx2* is induced before the alterations in *Otx2* and *Wnt1* expression, suggests that *Gbx2* could play a direct role in regulating *Otx2* and *Wnt1* expression by FGF8. Since *Fgf8* mes/met expression is abnormal in *Gbx2* mutant embryos (Wassarman et al., 1997;

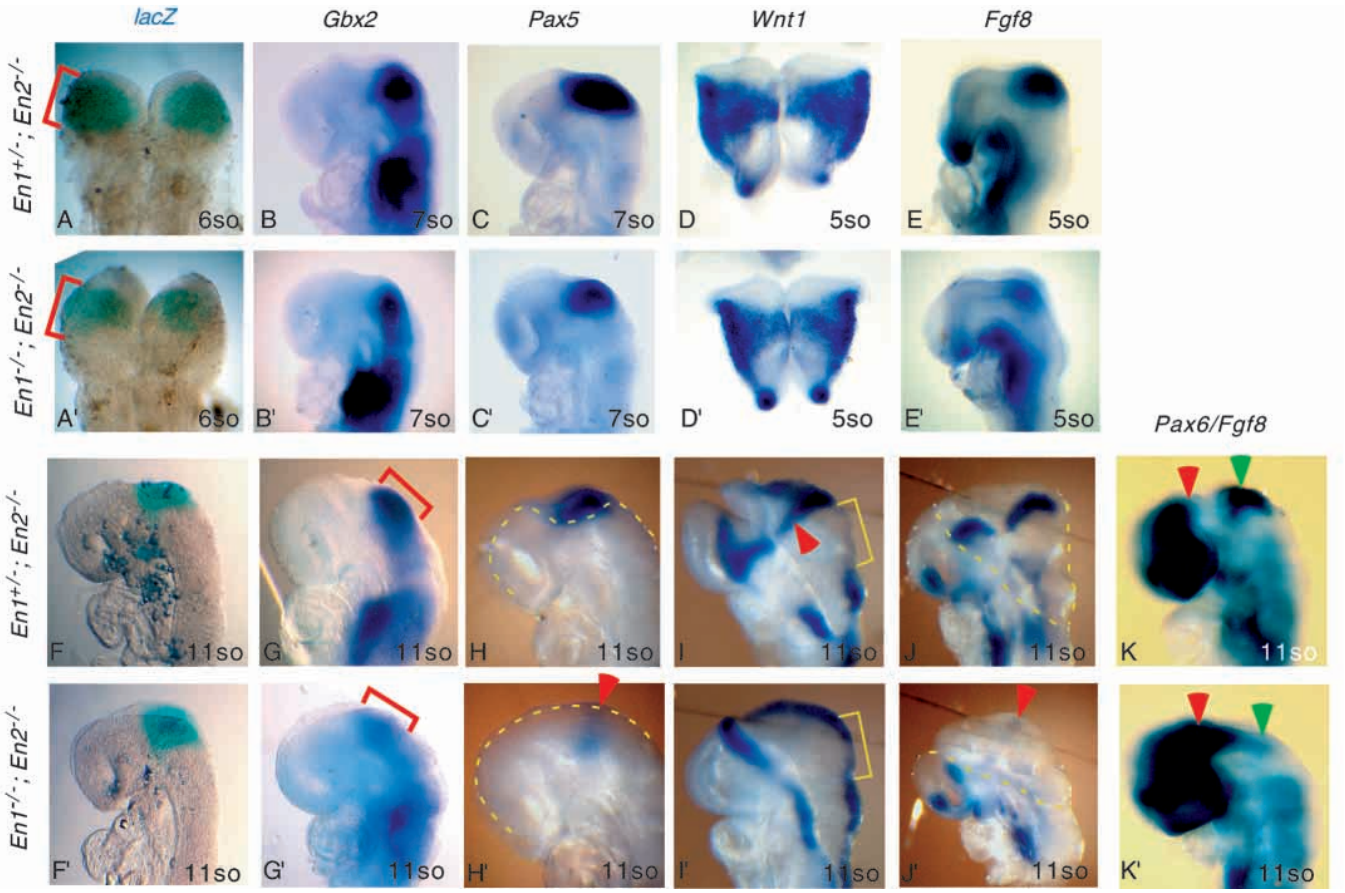

Fig. 3. En genes are not required for initiation, but for maintenance of mes/met gene expression. (A-E) *En1-lacZ*, *Gbx2*, *Pax5*, *Wnt1* and *Fgf8* expression in *En1*$^{+/-}$; *En2*$^{-/-}$ embryos at the five- to seven-somite stage. (A′-E′) *En1-lacZ*, *Gbx2*, *Pax5*, *Wnt1* and *Fgf8* are expressed in the mes/met of *En1/2* double mutants at the five- to seven-somite stage, although it seems that *Gbx2*, *Pax5* and *Fgf8* expression is weaker and more restricted relative to their littermates. Red brackets in A,A′ indicate the *lacZ* expression domain in both *En1/2* homozygous mutant embryos and their littermates. (F-J) *En1-lacZ*, *Gbx2*, *Pax5*, *Wnt1*, *Fgf8* expression in *En1*$^{+/-}$; *En2*$^{-/-}$ embryos at the 10-12 somite stage. (F′-J′) At 10-12 somite stages, *En1*$^{-/-}$; *En2*$^{-/-}$ embryos have similar brain morphology and *En1-lacZ* expression domains to their *En1*$^{+/-}$; *En2*$^{-/-}$ littermates (compare F with F′). (G,G′) *Gbx2* expression in anterior hindbrain is downregulated in the *En1/2* double homozygous mutants (red brackets indicate the anterior hindbrain *Gbx2* expression domain). *Pax5* (H,H′) and *Fgf8* (J,J′) mes/met expression is much weaker and more restricted (red arrowheads) in *En1/2* double homozygous mutants compared with their littermates. The lateral edges of the neural plate are highlighted by dotted lines. (I,I′) In *En1/2* mutant embryos, *Wnt1* is not expressed in the caudal midbrain, but is expressed along the lateral edges of the anterior hindbrain (yellow brackets indicate the anterior hindbrain region). (K,K′) *Pax6* expression expands caudally in *En1/2* double homozygous mutants (K′) compared with littermates (K). Embryos are co-stained for *Fgf8* and *Pax6* RNA in situ. Red arrowheads point to the normal caudal limit of *Pax6* brain expression. Green arrowheads point to the *Fgf8* expression domain. Note that at the 11-somite stage, *Fgf8* expression is barely detectable in *En1/2* double homozygous mutants (see J′) and thus the staining in K′ is primarily due to *Pax6* expression.

Millet et al., 1999), it is not possible to distinguish in the mutants whether the deregulation of *Otx2* and *Wnt1* is due to the abnormal *Fgf8* expression or whether GBX2 is directly required for regulating their expression. We sought to address this question by using midbrain explants taken from *Gbx2* mutants.

Gbx2$^{-/-}$ embryos have a deletion of the anterior hindbrain and an expansion of the midbrain at E9.5, whereas the diencephalon appears normal, based on both morphological landmarks and *Pax6* staining (Wassarman et al., 1997 and data not shown). Explants were taken from the anterior half of the midbrain of *Gbx2* mutants and wild-type E9.5 embryos. After 40 hours in culture with FGF8, *Otx2* expression was completely repressed in cells adjacent to the beads in all wild-type explants (Fig. 6A, *n*=8) or *Gbx2* heterozygotes (not shown, *n*=13). Similarly, *Otx2* was completely repressed in all *Gbx2* homozygous explants by FGF8 (Fig. 6B; *n*=10). This result shows that a *Gbx2*-independent pathway exists for mediating repression of *Otx2* by FGF8, and that the caudal expansion of *Otx2* expression into the *Fgf8* expression domain in *Gbx2* mutant embryos could result at least in part from compromised *Fgf8* expression.

We have shown that in wild-type forebrain and midbrain explants cultured with FGF8b-soaked beads, *Wnt1* expression is consistently found in a ring of cells that are several cell diameters away from the beads after 40 hours (Liu et al., 1999; Fig. 6C). This pattern was also observed in *Gbx2* heterozygous explants treated with FGF8b-soaked beads (not shown, *n*=3). However, in midbrain explants from *Gbx2* homozygous mutants, strong *Wnt1* expression was detected in the cells right adjacent to the beads after 40 hours, showing that *Gbx2* is required for the late repression of *Wnt1* in cells near a source

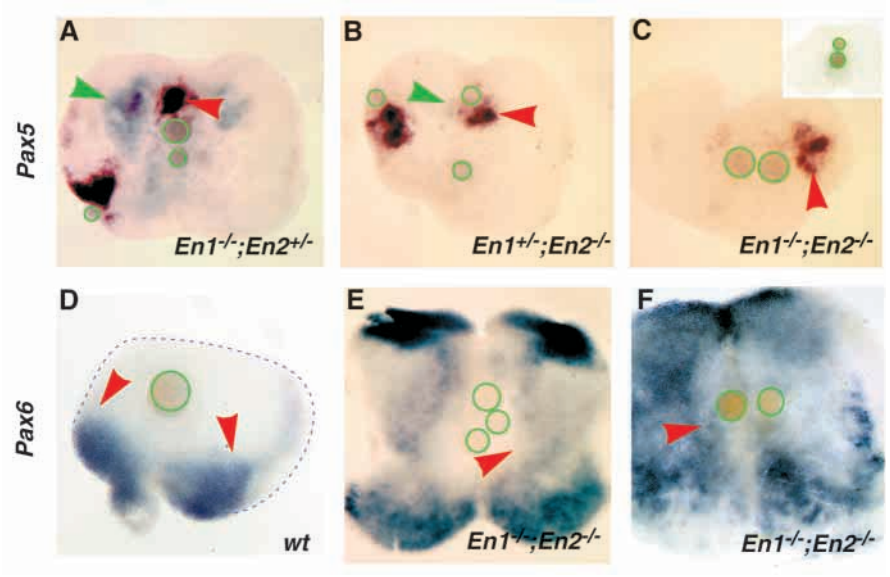

Fig. 4. The En genes are required for *Pax5* induction and involved in *Pax6* repression by FGF8b in E9.5 forebrain explants. (A-C) Forebrain explants cultured for 40 hours with 0.2 mg/ml FGF8b-soaked beads and stained for *En1-lacZ* expression (Red Salmon-gal staining; red arrowheads) and *Pax5* (blue staining; green arrowheads), except that in the inset of C, 1 mg/ml FGF8b-soaked beads were used and the explant was stained for *Pax5* only. Although *En1-lacZ* expression in double *En1/2* homozygous explants shows a similar induction to that in their littermates, *Pax5* induction is not seen in double homozygotes and can be barely seen in one out of three $En1^{+/-}; En2^{-/-}$ explants (B). (D-F) In contrast to the wild type (D), in *En1/2* double homozygous forebrain explants *Pax6* expressing cells are either adjacent to the FGF8b-soaked beads (F) or much closer to them (E) than in wild-type explants. The beads in F were soaked in 1 mg/ml FGF8b. Beads are outlined in green. Dotted line in D outlines the edge of explant.

of FGF8b (Fig. 6D, *n*=4). Finally, expression of *Lmx1b* (*n*=2, Fig. 6F) and *En2* (*n*=2, Fig. 7H) was induced by FGF8 in $Gbx2^{-/-}$ explants at 40 hours, but not by BSA-soaked beads, similar to wild-type explants (Fig. 6E,G and data not shown). Thus, *Lmx1b* and *En2* can be induced by FGF8 independently of *Gbx2*, and *Lmx1b* and *En2* are unlikely involved in the late downregulation of *Wnt1* expression by FGF8.

DISCUSSION

In this study we explored some of the epistatic relationships between mes/met genes using mouse explants from En and *Gbx2* mutants since these are the first genes to be induced by FGF8. Using En mutant explants we show that both En genes are required for FGF8 induction of *Pax5*, but that they are not the only genes required for repression of *Pax6* in diencephalic tissue (Fig. 7A). Since in *En1/2* double homozygous mutant embryos we found that all the mes/met genes examined, including *Pax5*, are initially expressed, a factor(s) other than FGF8 is likely to be responsible for inducing *Pax5* mes/met expression. The downregulation of *Pax5* expression by the 11-somite stage in En mutants, however, could indicate that *Fgf8* and/or the En genes play a later role in maintaining *Pax5* transcription. Using *Gbx2* mutant explants we determined that *Gbx2* plays a specific role in excluding *Wnt1* expression in cells near an FGF8 source. This role of *Gbx2* could account for the normal exclusion of *Wnt1* from cells in the metencephalon and expansion of the *Wnt1* expression domain into the metencephalon in *Gbx2* mutants. Consistent with this, we observed a downregulation of *Gbx2* expression and ectopic *Wnt1* expression in the metencephalon of 11-somite *En1/2* mutant embryos. Surprisingly, although previous experiments showed that *Gbx2* misexpression in the mesencephalon is sufficient to lead to repression of *Otx2* (Millet et al., 1999; Katahira et al., 2000), in our explant system *Gbx2* was not required for a complete repression of *Otx2* by FGF8b. These studies represent one of the first epistasis studies carried out in mice, or other vertebrates, and have uncovered a new level of complexity in the genetic hierarchy of genes downstream of FGF8 that regulate mes/met anteroposterior patterning.

Pax2/5 and *En1/2* are involved in a feedback loop

Previously we showed that two PAX2/5-binding sites are required for the mid/hindbrain expression of an *En2-lacZ* reporter gene, suggesting PAX2/5 might be involved in direct regulation of *En2* (Song et al., 1996). However, further studies showed that transcription of the reporter is much more restricted to the mes/met junction region than the endogenous *En2* gene, indicating that there are other critical DNA regulatory sequences in the *En2* locus (Song and Joyner, 2000). Indeed, deletion of these PAX2/5-binding sites from the endogenous *En2* gene does not abolish *En2* expression, but only decreases its initial expression (Song and Joyner, 2000). We found that the same PAX2/5-binding sites are required for upregulation of the *En2-lacZ* transgene by FGF8 in forebrain explants and the timing of the upregulation is consistent with *Pax5* first being induced and then PAX5 inducing the transgene. In contrast, the endogenous *En2* gene is induced before *Pax5* in forebrain explants by FGF8, and *Pax2* and *Pax8* are not induced at all (Table 1; Liu et al., 1999). Therefore, the normal early induction of *En2* expression by FGF8 must be through a PAX2/5-independent pathway.

More interestingly, *Pax5* is not induced in diencephalic explants by FGF8 in the absence of the En genes, showing that EN proteins could be involved normally in regulating *Pax5*. Taken together with the transgenic studies, this indicates that a positive feedback loop exists between *En2* and *Pax5*. It has been found that misexpression of *En1/2* in chick diencephalic tissue first results in repression of *Pax6* expression and then in induction of *Pax5* expression (Araki and Nakamura, 1999). Consistent with the En misexpression studies, we found in our diencephalic explants, that *Pax6* was partially repressed by 16 hours, whereas *Pax5* was not induced until 40 hours. Furthermore, since *Pax6* was not fully repressed in *En1/2* double mutant diencephalic explants, it is possible that this accounts for the lack of induction of *Pax5* by FGF8 in such mutants. The fact that *Pax5* expression is initiated in *En1/2*

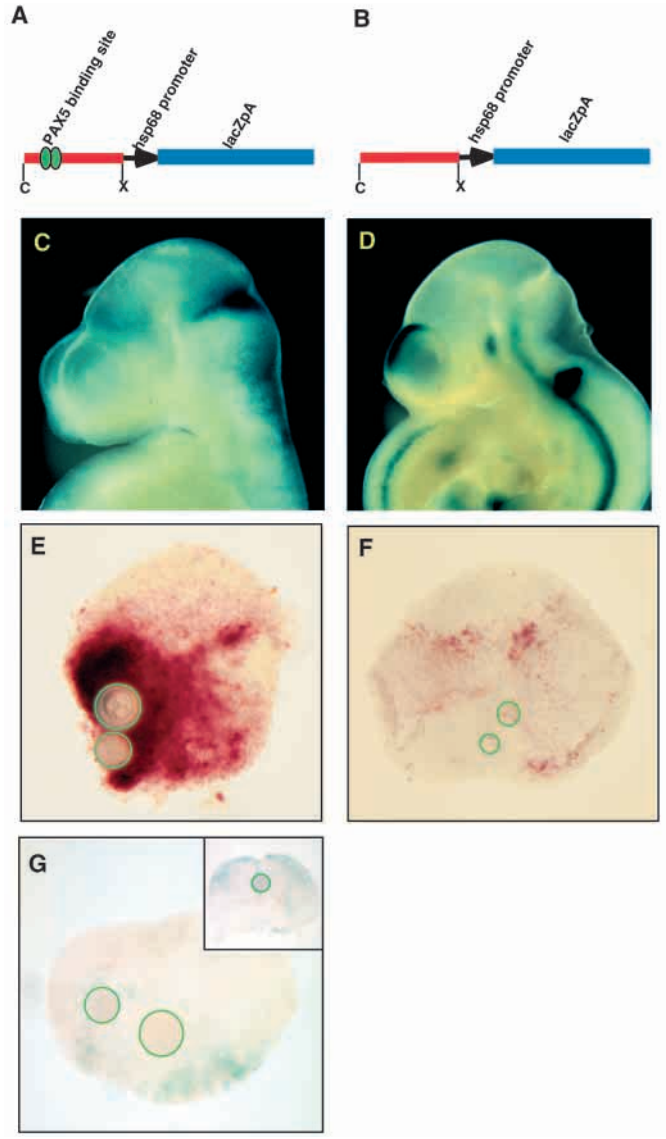

Fig. 5. PAX2/5 DNA-binding sites are essential for upregulation of an *En2-lacZ* reporter in forebrain explants in response to FGF8b. (A,B) Schematic showing the *En2-lacZ* transgene (A) consisting of a 1kb *En2* enhancer (red) with two PAX2/5-binding sites (green ovals) that drives expression of a *lacZ* gene (blue) through a heat shock minimal promoter (black arrow), and the $En2^{PBD}$-*lacZ* transgene (B) in which the PAX2/5-binding sites are deleted. (C) The *En2-lacZ* transgene is expressed in the mouse mes/met region at E9.5 (blue staining). (D) The $En2^{PBD}$-*lacZ* transgene fails to be expressed in the mes/met region. (E,G) FGF8b can upregulate expression of the *En2-lacZ* reporter (red staining) in forebrain explants after 40 hours (E), but not after 16 hours (G). Inset in G shows an explant with BSA-soaked beads showing similar low-level *lacZ* expression to that in G. (F) The $En2^{PBD}$-*lacZ* transgene is not upregulated by FGF8b in forebrain explants. C, *Cla*I; X, *Xba*I.

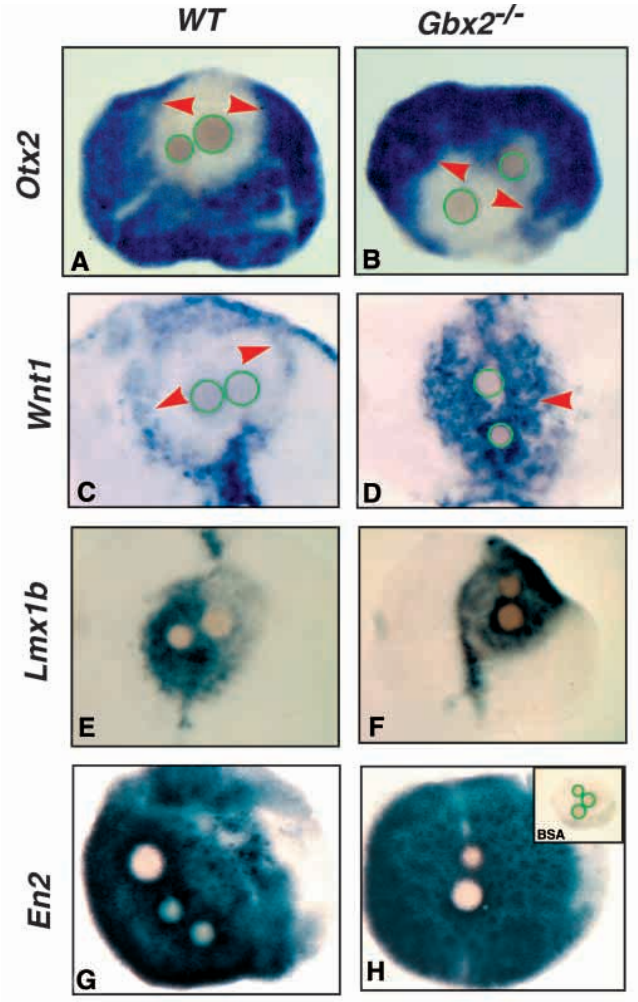

Fig. 6. GBX2 is required for the repression of *Wnt1* but not *Otx2* expression in midbrain explants treated with FGF8b. (A,B) *Otx2* is repressed by FGF8b in both wild-type (A) and $Gbx2^{-/-}$ (B) anterior midbrain explants. (C,D) *Wnt1* is repressed in cells adjacent to the beads by 40 hours in wild-type (C) but not in $Gbx2^{-/-}$ (D) midbrain explants, instead it is expressed in cells near the FGF8b-soaked beads. (E,F) *Lmx1b* is induced in cells adjacent to the FGF8b beads by 40 hours in wild-type midbrain explants (E). This induction is not altered in $Gbx2^{-/-}$ explants (F). (G,H) *En2* is strongly induced in both wild-type (G) and $Gbx2^{-/-}$ (H) anterior midbrain explants by FGF8b-soaked beads but not by BSA-soaked beads (inset in H). Red arrowheads point to regions of strong *Otx2* and *Wnt1* expression. The positions of the beads are highlighted with green rings.

mutant embryos could mean that *Pax5* is regulated not only by FGF8 and EN, but also by other proteins, consistent with multiple transcription regulator-binding sites being present in *Pax5 cis*-regulatory sequences (Pfeffer et al., 2000). Alternatively, *Pax5* can be induced only by FGF8 in *En1/2* mutants in tissue that does not express *Pax6*. Ectopic expression of En genes not only results in repression of *Pax6* and induction of *Pax5* in the diencephalon but also development of midbrain structures in both chick and medaka fish (Araki and Nakamura, 1999; Ristoratore et al., 1999). Furthermore, since expression of an activator form of EN2 in the chick midbrain inhibits midbrain development and results in upregulation of *Pax6*, the primary function of EN2 is likely to be a repressor (Araki and Nakamura, 1999). It has been suggested that a negative feedback loop between En genes and *Pax6* establishes the midbrain/forebrain border (Araki and Nakamura, 1999). We found that in mouse, unlike in chick, the *En1* mes/met expression domain normally only abuts the *Pax6* forebrain expression domain briefly at early somite stages (data

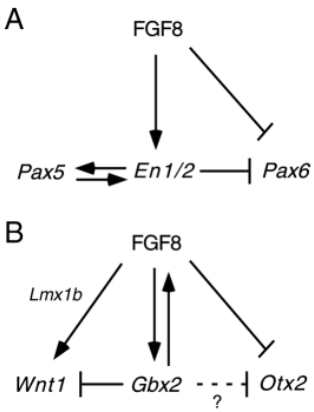

Fig. 7. Model of epistatic relationships between different FGF8 downstream genes during mes/met early patterning
(A) FGF8 upregulates *En1/2*, which in turn upregulate *Pax5* and downregulates *Pax6*. However, PAX5 can directly regulate *En2* transcription. FGF8 might also regulate *Pax6* expression through an EN-independent pathway due to the fact that the repression of *Pax6* by FGF8b-soaked beads varied in *En1/2* double homozygous mutant diencephalic explants. (B) FGF8 upregulates *Gbx2*, and GBX2 in turn represses *Wnt1*. In contrast, FGF8 activates *Wnt1* and represses *Otx2* through a *Gbx2*-independent pathway. *Lmx1b* probably regulates the activation of *Wnt1*.

not shown). In mouse, therefore, *En* genes and *Pax6* can only be involved in establishing the boundary between midbrain and forebrain at early somite stages. Consistent with EN1 repressing *Pax6* early, in 11-somite stage *En1/2* double mutant embryos the *Pax6* forebrain expression domain is expanded caudally. However, since FGF8 is able to partially repress *Pax6* in *En1/2* double mutant explants, and after the eight-somite stage, *En1* and *Pax6* expression do not abut in wild-type embryos, a second pathway must exist, possibly downstream of FGF8, that is *En1/2* independent and mediates repression of *Pax6* after the ten-somite stage.

GBX2 is required for the repression of *Wnt1* by FGF8b, but not for the repression of *Otx2* and activation of *Wnt1*

Previous loss-of-function studies have shown that the expression domains of *Otx2* and *Wnt1* are expanded caudally in *Gbx2* mutants from E8.5 onwards, suggesting that *Gbx2* might be required to repress *Otx2* and/or *Wnt1* (Millet et al., 1999). Furthermore, gain-of-function studies have shown that misexpression of *Fgf8* or *Gbx2* is sufficient to lead to repression of *Otx2* (Liu et al., 1999; Martinez et al., 1999; Millet et al., 1999, Katahira et al., 2000). In contrast, our explant studies showed that the expression domains of *Gbx2* and *Otx2* following FGF8 application are not directly adjacent to each other, but instead that cells expressing neither gene are induced between the two expression domains (Liu et al., 1999). As further direct proof that *Gbx2* is not the only gene involved in repressing *Otx2*, we have shown that FGF8 can repress *Otx2* in midbrain explants taken from *Gbx2* mutant embryos. Our results indicate that either FGF8 induces two pathways that lead to *Otx2* repression, only one of which is dependent of GBX2, or that GBX2 first upregulates *Fgf8* in the *Gbx2* misexpression experiments and this leads to repression of *Otx2* (Fig. 7B). The latter is consistent with the finding that in *Otx1/2* hypomorphic mutants, *Fgf8* expression is first expanded rostrally and then *Otx2* expression is repressed and expression of *Gbx2* and *Wnt1* induced anteriorly (Acampora et al., 1997). Therefore, it is possible that the deregulation of *Otx2* expression in *Gbx2* mutants is an indirect outcome of loss of *Gbx2* function, possibly due to decreased expression of *Fgf8*, as was previously suggested (Wassarman et al., 1997).

In contrast, *Gbx2* plays a more direct role in regulating expression of *Wnt1*. Normal regulation of *Wnt1* expression is extremely important, since it is required for development of the entire mes/met region, as well as for stabilizing the mes/met boundary (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; McMahon et al., 1992; Bally-Cuif et al., 1995). After 40 hours in wild-type midbrain explants, *Wnt1* is expressed in a ring of cells several cell diameters away from FGF8-soaked beads and *Gbx2* is expressed in the *Wnt1*-negative cells close to the bead (Liu et al., 1999). We now show that this expression pattern is the result of two events. Initially, *Wnt1* is induced in a broad region around the FGF8-soaked beads and then *Wnt1* is only maintained in cells at a distance to the beads. The fact that *Wnt1* expression expands caudally in *Gbx2* mutants from E8.5 onwards (Millet et al., 1999), and our finding that in E9.5 *Gbx2* mutant midbrain explants *Wnt1* is induced and maintained in a broad region by FGF8, provide evidence that GBX2 is required for repression of *Wnt1* in cells near an FGF8 source. Consistent with this, the ectopic expression of *Wnt1* in the lateral edges of the mesencephalon in *En1/2* mutants could be due to the decrease of *Gbx2* expression. Our explant studies have revealed an interesting mechanism by which FGF8 regulates *Wnt1* expression, first through activation and then repression, and that this two-step process involves different pathways, since only one is GBX2 dependent.

It was found that *Otx2* is required in a cell-autonomous manner for the expression of *Wnt1* at the mes/met junction, based on studies of mouse chimeras containing $Otx2^{-/-}$ and wild-type cells (Rhinn et al., 1999). From these studies it was not clear whether *Otx2* regulates *Wnt1* directly. Two results with our explant assays suggest that the regulation is likely indirect. First, in midbrain explants *Wnt1* is induced by FGF8 in *Otx2*-negative cells (Liu et al., 1999). Second, we show here that in the absence of *Gbx2*, regulation of *Wnt1* and *Otx2* are dissociated. *Otx2* can be repressed, but *Wnt1* is nevertheless induced and maintained in *Otx2*-negative cells, showing that *Otx2* is not required to directly regulate *Wnt1* expression. Instead, *Otx2* might normally allow *Wnt1* to be expressed in the midbrain by repressing *Gbx2* expression. In early somite embryos, *Wnt1* expression normally progresses from broad mesencephalic expression to expression only at the midbrain/hindbrain junction. Furthermore, *Wnt1* is upregulated in *Otx2*-positive midbrain cells surrounding *Otx2* mutant cells in chimeras. It is therefore possible that *Wnt1* expression can be regulated by a positive signal, and our explant studies indicate that FGF8 is a good candidate. It would therefore be very interesting to know whether the $Otx2^{-/-}$ cells in the midbrain of $Otx2^{-/-}\leftrightarrow$wild-type chimeras do express *Gbx2* and *Fgf8*.

Lmx1b was recently implicated as a positive regulator of *Wnt1* mes/met expression in chick (Adams et al., 2000). Consistent with this, we found that *Lmx1b* was induced by FGF8 several hours before *Wnt1*, and scattered expression of both genes was seen initially. By 40 hours, however, *Wnt1* was

expressed only in *Gbx2*-negative cells at a distance from the beads, whereas *Lmx1b* was in both *Gbx2*-positive and -negative cells (Fig. 1 and data not shown). The latter is consistent with the fact that in chick the *Lmx1b* mes/met domain extends more posteriorly than that of *Wnt1*, overlapping with *Fgf8* and *Gbx2* expression (Adams et al., 2000). Based on our studies, it is possible that in the mes/met border during normal development, FGF8 regulates *Wnt1* expression positively through *Lmx1b* and negatively through *Gbx2*, hence positioning *Wnt1* expression anterior to the mes/met border, adjacent to, but not overlapping with, *Fgf8*.

In summary, by using brain explants taken from the posterior forebrain and anterior midbrain of mutants, we have obtained new insights into the epistatic relationships between different mes/met genes regulated by FGF8 signaling. Such information could not be gained from a direct analysis of the phenotypes of various mutants because of the simultaneous early alterations in expression of multiple genes and, in some cases, loss of tissue. This new information furthers our understanding of how FGF8 functions to pattern the midbrain and cerebellum along the anteroposterior axis, and to maintain a normal boundary between the midbrain and hindbrain. Finally, the studies have revealed that there are multiple pathways and additional factors involved in FGF8 signaling and organizer function that have yet to be identified.

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