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Meis2 competes with the Groucho co-repressor Tle4 for binding to Otx2 and specifies tectal fate without induction of a secondary midbrain-hindbrain boundary organizer

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The transcription factor *Otx2* is expressed throughout the anterior neuroectoderm and is required for the formation of all forebrain- and midbrain-derived structures. The molecular determinants that cooperate with *Otx2* to subdivide its expression domain into distinct functional units are, however, poorly understood at present. We show here that the TALE-homeodomain protein Meis2 is expressed in the chick tectal anlage and is both necessary and sufficient for tectal development. Unlike known tectum-inducing genes, the ability of Meis2 to initiate tectal development does not involve the formation of a secondary midbrain-hindbrain boundary organizer, but instead requires direct interaction with Otx2. Using an Otx2-dependent reporter assay we demonstrate that Meis2 competes with the Groucho co-repressor Tle4 (Grg4) for binding to Otx2 and thereby restores Otx2 transcriptional activator function. Together, our data suggest a model in which the balance between a co-repressor and a co-activator, which compete for binding to Otx2 in the mesencephalic vesicle, provides spatial and temporal control over tectal development. Controlled formation of Meis2-containing higher order protein complexes might thus serve as a general mechanism to achieve subdivision of the anterior neuroectoderm into distinct functional units during embryogenesis.

KEY WORDS: Groucho, Meis2, Midbrain, Otx2, Tle4 (Grg4), Chick

INTRODUCTION

Otx2 is a member of a highly conserved family of homeodomain-containing transcription factors that control early development of anterior brain structures in different animal phyla. Mice mutant for *Otx2* lack fore- and midbrain and *D. melanogaster* embryos mutant for the related gene *orthodenticle* (*ocelliless* – FlyBase) do not form the anterior-most part of the head (Acampora et al., 1995; Finkelstein and Perrimon, 1990). *Otx2* controls multiple steps during vertebrate brain development, beginning with the specification of the anterior visceral endoderm, which acts as a head organizer by inducing *Otx2* expression in the anterior part of the adjacent neuroectoderm (Simeone, 2002). Subsequently, this *Otx2* domain becomes subdivided into molecularly distinct units, which eventually give rise to the different brain structures of the fore- and midbrain. Thus, whereas *Otx2* is required for the specification of general characteristics of the anterior vertebrate brain, additional factors need to cooperate with *Otx2* in the specification of individual brain structures.

Meis1-3 belong to the TALE (three amino acid loop extension) family of homeodomain-containing transcription factors and function as regulators of cell proliferation and differentiation of several organs and tissues during development. *Meis1* and *Meis2*, for instance, are involved in proximal-distal limb patterning, skeletal muscle differentiation, hindbrain, lens and retina development (Berkes et al., 2004; Bessa et al., 2008; Capdevila et al., 1999; Dibner et al., 2001; Heine et al., 2008; Mercader et al., 1999; Vlachakis et al., 2001; Zhang et al., 2002). To date, only mice mutant for *Meis1* have been generated, and these display defects in angiogenesis and eye development (Azcoitia et al., 2005; Hisa et al., 2004). TALE homeodomain proteins form dimeric or trimeric complexes with other transcription factors. Complex formation not only influences the DNA-binding affinity and specificity of the interacting proteins, but has also been shown to control nuclear import of the partner protein (Mercader et al., 1999; Moens and Selleri, 2006; Vlachakis et al., 2001). Meis-interacting proteins have been isolated from non-neuronal tissue and from the hindbrain and include the Meis-related Pbx family, members of the Hox clusters, several homeodomain-containing proteins, and the myogenic bHLH proteins MyoD and Myf5 (Chang et al., 1997; Knoepfler et al., 1999; Shen et al., 1997; Swift et al., 1998; Vlachakis et al., 2001). Meis-interacting proteins in the anterior neural tube, by contrast, have thus far remained elusive. Here we identify Otx2 as a direct interaction partner of Meis2 in the tectal anlage and show that Meis2 is both necessary and sufficient for tectal fate specification.

MATERIALS AND METHODS

Expression constructs and in ovo electroporation

pMIWIII-*Meis2HA*, pMIWIII-*Meis2EnR* and the RNAi targeting vector pSTRIKE-*Meis2_siRNA* were described previously (Heine et al., 2008). Each construct (1-2 μg/μl) was electroporated together with 0.5 μg/μl of a plasmid expressing enhanced green fluorescent protein (pMIWIII-*GFP*) into the right-hand wall of the neural tube of Hamburger-Hamilton stage (HH) (Hamburger and Hamilton, 1992) 9-11 chick embryos (White Leghorn) as described (Heine et al., 2008). To control for possible non-specific effects, pMIWIII-*GFP* or a fusion protein of the unrelated homeodomain (HD)-containing protein SOHo1 to the *EnR* domain were electroporated (Schulte and Cepko, 2000). Retroviral transfection employing RCASBP(B)-*Meis2HA* was used for widespread, long-term expression (Heine et al., 2008).

RNA in situ hybridization and immunohistochemical analysis

The cDNAs used to generate in situ probes were gifts from C. Cepko and C. Tabin (Harvard Medical School, Boston, MA, USA), D. O'Leary (Salk Institute, La Jolla, CA, USA), C. Logan (University of Calgary, Alberta, Canada), A. Pierani (Institut J. Monod, Paris, France) or were cloned from

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chick HH10-12 whole-head total RNA by RT-PCR with gene-specific primers (sequences available upon request). In situ hybridization was performed as described (Heine et al., 2008). Immunohistochemical detection of the misexpressed transgene after in situ hybridization was carried out with a rabbit antibody against GFP (1:5000; Molecular Probes, Eugene, OR, USA), followed by detection with a horseradish-peroxidase-coupled secondary antibody (1:1000; Roche Diagnostics, Mannheim, Germany) and visualization with the DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA).

Primary antibodies used were rabbit polyclonal anti-GFP (1:5000; Molecular Probes), rat polyclonal anti-HA (1:1000; Roche), rabbit polyclonal anti-Meis2 (1:5000; gift of A. Buchberg, Kimmel Cancer Center, University of Philadelphia Medical School), goat polyclonal anti-Otx2 (1:40; R&D Systems, Minneapolis, MN, USA), mouse monoclonal anti-Pax6 purified IgGs [1:5000; Developmental Studies Hybridoma Bank (DSHB), IA], mouse monoclonal anti-Pax7 (1:5; DSHB, IA), and mouse monoclonal anti-neurofilament RM270.7 (1:8000; gift of V. Lee, University of Pennsylvania Medical School, Philadelphia).

Isolation and analysis of Meis2-interacting proteins

Preparation of tectal lysates

Approximately 30-40 HH15-18 chick tecta per experiment were resuspended in 10 mM Hepes pH 8, 10 mM KCl, 0.1 mM EDTA, 2 mM DTT and Complete protease inhibitor tablets (Roche) and lysed by adding Igepal (Sigma Aldrich, Steinheim, Germany) to a final concentration of 1%. Cell nuclei were collected by brief centrifugation. The supernatant contained the cytosolic fraction ('cyto'). The nuclei were reconstituted in 10 mM Hepes pH 8, 10 mM KCl, 0.1 mM EDTA, 2 mM DTT, 400 mM NaCl, 1% Igepal and Complete protease inhibitors and incubated for 15 minutes at 4°C under constant rotation. Cellular debris was removed by brief centrifugation ('nucl'). Cytosolic and nuclear fractions were used separately or combined (designated input, 'in'). Lysates were pre-cleared by incubation with empty glutathione sepharose 4B beads (GE Healthcare-Amersham, Piscataway, NJ, USA) or empty Protein G-agarose beads (Roche) for 30-60 minutes under constant rotation at 4°C.

In vitro translation of ^{35}S-radiolabeled proteins

^{35}S-radiolabeled proteins were in vitro translated using the TNT T7/T3 Reticulocyte Lysate System (Promega, Mannheim, Germany) according to the manufacturer's instructions. For each pull-down experiment, 15 μl of radiolabeled protein were diluted in buffer BP (10 mM Hepes pH 8, 10 mM KCl, 0.1 mM EDTA, 2 mM DTT, 150 mM NaCl, 0.4% Igepal and Complete protease inhibitors).

GST pull-down experiments

GST fusion proteins (in pGEX4T1) were purified following standard procedures. Immobilized GST fusion proteins were incubated with pre-cleared tectal lysates or with ^{35}S-radiolabeled proteins for 2 hours under constant rotation at 4°C. Following extensive washes in buffer BP, the protein complexes were analyzed by SDS-PAGE followed by autoradiography or western blot following standard procedures.

Co-immunoprecipitation assay

Pre-cleared tectal lysates were incubated with anti-Meis2 antibody overnight at 4°C under constant rotation. Protein G-agarose beads (Sigma Aldrich) were added for 4 hours at 4°C with rotation. After extensive washes, the immunoprecipitates were separated by SDS-PAGE and analyzed by western blot using a goat polyclonal Otx2 antibody (1:2000; R&D Systems). The input ('in') loading control corresponded to less than 10% of the total protein used in the precipitation.

Cell transfection and transient reporter assays

The Gal4-responsive construct plGC-luc and the expression vectors pMC-Gal4Otx2, pMC-Gal4Otx2Δ1-100 and pKW-Tle4 were described previously (Eberhard et al., 2000; Heimbucher et al., 2007). Mouse NIH 3T3 fibroblasts were transfected in 24-well plates using Lipofectamine (Invitrogen, Karlsruhe, Germany) with 300 ng plGC-luc and 50 ng of the *Renilla* luciferase expression plasmid phRG-TK (Promega) together with empty vector and the expression vectors indicated. Luciferase activities were measured 48 hours after transfection with the Dual Glo Luciferase Assay System (Promega). For normalization, firefly luciferase values were standardized to *Renilla* luciferase activity. Experiments were performed in at least triplicate.

RESULTS

The spatial-temporal expression of *Meis2* suggests a role in midbrain development

Meis2 transcripts were first detected in the mesencephalic vesicle at HH11-12 (13- to 16-somite stage; data not shown). From its onset, *Meis2* expression was strong in the dorsal mesencephalon, but was absent from the diencephalon, midbrain-hindbrain boundary (MHB) region and rhombomere 1 (Fig. 1A,B). Additional domains of expression at these developmental stages included the neural retina, lens and rhombomeres 2/3 (Fig. 1A,B) (Heine et al., 2008). Within the mesencephalic vesicle, *Meis2* was strongly expressed in the alar plates (Fig. 1C,D, arrows), which give rise to the optic tecta, and more weakly in the mesencephalic basal plates, which give rise to the tegmenta. Expression was excluded from the roof plate and floor plate (Fig. 1C,D). By HH19, *Meis2* transcripts were absent from the mesencephalic basal plates, with the exception of two lateral groups of cells close to the ventral midline, which were presumably newly generated neurons of the nucleus oculomotoris (Fig. 1D, arrowheads). In the late embryonic tectum, *Meis2* transcripts marked distinct tectal layers (Fig. 1E). Comparable expression was observed in mouse embryos (Fig. 1F). *Meis1* transcripts, by contrast, were present at very low levels in the mesencephalic vesicle (data not shown) (Shim et al., 2007).

Meis2 is both necessary and sufficient for tectal development

The *Meis2* splicing isoform 2a is prominently expressed in the anterior neural tube of HH11-15 chick embryos (Heine et al., 2008). Similar to the related Meis1a, we identified a transcriptional activation domain in the C-terminus of Meis2a (see Fig. S1 in the supplementary material) (Huang et al., 2005). To investigate a possible role for *Meis2* in tectal fate specification, we introduced full-length *Meis2a* fused to an HA tag (*Meis2HA*) into the diencephalic alar plate at HH10-11 and monitored changes in diencephalic brain morphology 6 and 12 days later. Prominent bulges indicative of a diencephalic-to-mesencephalic fate change were readily observed in all electroporated embryos (Fig. 2A,A′,G,G′) (n=5/5 for E7; n=5/5 for E13). These ectopic bulges were populated by cells that were immunoreactive for the tectal markers Pax7 and Meis2 (Fig. 2B-B″,H). Pax7- and Meis2-expressing cells in the diencephalon were always restricted to the electroporated, right-hand side of the brain and were never found on the contralateral, non-electroporated side of the same embryo (Fig. 2E). Notably, Pax7- and Meis2-expressing cells were arranged in distinct laminae, which although not perfectly recapitulating the lamination of a normal optic tectum, nevertheless strongly resembled the cellular organization characteristic of the mesencephalic alar plate at the respective developmental stages (compare Fig. 2B″,C with 2H,I). Ectopic midbrain-like structures in *Meis2HA*-transfected brains also failed to express the diencephalic marker Pax6, a further indication of a true cell fate shift of diencephalic to mesencephalic tissue (Fig. 2D,D′). In addition, the structures induced by *Meis2* transfection in the diencephalon possessed a superficial, neurofilament-immunoreactive axonal layer similar to the stratum opticum of the endogenous optic tecta (Fig. 2J,K). For a uniform misexpression of *Meis2* throughout the mesencephalic vesicle (including the roof and basal plates), we turned to retroviral transfection with the replication-competent

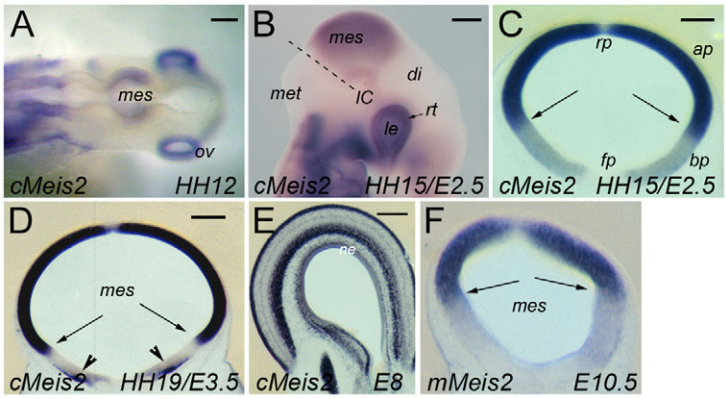

Fig. 1. Developmental expression of *Meis2*. (**A**) Whole chick embryo at HH12. (**B**) Whole chick embryo at HH15. (**C**) Coronal vibratome section of the midbrain vesicle at HH15. (**D**) Coronal vibratome section of a chick midbrain at HH19. (**E**) Distribution of *Meis2* transcripts in a cross-section through an E8 chick mesencephalon. Expression is strong in the neuroepithelium (ne) and in discrete tectal layers (**F**) *Meis2* expression in the mouse midbrain at E10.5. The arrows in C, D and F point to the border of mesencephalic alar and basal plate; the arrowhead in D points to two isolated domains of *Meis2* expression in the mesencephalic basal plate, presumably neurons of the nucleus oculomotoris; the dashed line in B marks the isthmic constriction. ap, alar plate; bp, basal plate; di, diencephalon; fp, floor plate; IC, isthmic constriction; le, lens; mes, mesencephalon; met, metencephalon; ov, optic vesicle; rp, roof plate; rt, neural retina: Scale bars: 200 μm in A-D; 500 μm in E.

retrovirus RCASBP(B)-*Meis2HA* (Heine et al., 2008). This strategy resulted in a marked increase in tectal volume at the expense of tegmental structures and in the complete absence of the mesencephalic roof plate and dorsal midline-derived structures at mid- to late embryonic stages (Fig. 2L-N). Transfection of *Meis2HA* into the metencephalic vesicle, by contrast, never initiated the formation of tectum-like structures, nor induced corresponding molecular changes (see Fig. S2 in the supplementary material; data not shown).

Because of the abundance of Meis2-immunoreactive cells in the ectopic tectal structures, we examined whether they corresponded to cells that had retained expression of the *Meis2HA* transgene, or whether Meis2 expression in these cells reflected upregulation of the endogenous Meis2 protein. Labeling with an HA-specific antibody revealed only a few, randomly distributed *Meis2HA*-transfected cells at E7 and none at E13 (Fig. 2F; data not shown). When Meis2- and HA-immunoreactive cells were monitored at different times following *Meis2HA* transfection, we found that expression of the electroporated Meis2HA protein decreased after 24 hours post-transfection, whereas the endogenous Meis2 protein continued to be expressed (see Fig. S3 in the supplementary material). Thus, Meis2 stabilizes the diencephalic-to-mesencephalic cell fate change through positive regulation of its own expression.

To test whether Meis2 is not only capable of inducing a diencephalic-to-mesencephalic fate shift, but is also required for normal tectal development, we expressed a fusion of *Meis2a* to the repressor domain of *D. melanogaster* Engrailed (*Meis2EnR*), which we had previously shown to function as a Meis function-blocking construct, or vectors expressing *Meis2*-specific small interfering RNAs (siRNAs) in the mesencephalic vesicle (Heine et al., 2008). *Meis2EnR* transfection into the midbrain vesicle resulted in a marked distortion of the optic tectum as compared with non-transfected or mock-transfected brains (n=5/7) (Fig. 3A,C). Coronal sections through *Meis2EnR*-transfected tecta stained with DAPI revealed that the lumen of the optic tectum, which is normally still prominent at this stage, was often filled with ectopic cell masses and the characteristic tectal lamination was markedly disturbed (Fig. 3A′,A″,C′,C″). Areas with aberrant tectal morphology also failed to express the tectal markers Pax7 and Meis2 (Fig. 3B,D). When mesencephalic *Meis2* expression was repressed by RNAi-mediated knockdown, the optic tecta displayed abnormalities and frequently failed to separate (Fig. 3F-H). At later embryonic stages, tectal lamination was disrupted in such specimens (compare Fig. 3A″ with 3H). In addition to these structural defects, the lateral longitudinal fascicle (LLF), a prominent axonal trajectory in the midbrain, was disrupted following transfection with *Meis2* function-blocking constructs (Fig. 3J-N′). To control for possible non-specific effects, we targeted *Meis2EnR* or the *Meis2*-specific knockdown constructs to regions of the neural tube that do not express endogenous *Meis2* (Fig. 3E-E″,I). *Meis2EnR* transfection did not notably compromise diencephalic development, as evident in the persistent expression of the diencephalic marker Pax6 in transfected cells, nor did it affect axonal trajectories in the hindbrain (Fig. 3E-E″; data not shown). Similarly, diencephalic morphology was unaltered following *Meis2* knockdown (Fig. 3I). Taken together, these results demonstrate that *Meis2* is necessary for normal tectal development and can induce the formation of tectal structures at the expense of diencephalic, tegmental or roof plate-derived structures when experimentally expressed in the anterior neural tube.

Meis2 transfection induces tectum-specific gene expression changes

To assess early events following *Meis2* transfection, the expression of several genes that are specific for the mesencephalic, metencephalic and diencephalic anlagen was monitored. The cell surface protein ephrin B1 (Efnb1) is expressed in the mesencephalic alar plate at early somite stages, making it a suitable marker for the tectal anlage (Braisted et al., 1997). When *Meis2HA* together with *GFP* was introduced into the neural tube at the level of the future diencephalon, *Efnb1* transcripts were induced in patches of transfected cells (n=10/12, 24 hours post-transfection; n=8/9, 48 hours) (Fig. 4A-D; data not shown). Transfection of *GFP* alone did not induce *Efnb1* (n=0/14; data not shown). *Pax6* expression, which we had found to be repressed by *Meis2HA* at 6 days post-transfection, was already lost 20 hours after *Meis2HA* misexpression (Fig. 4E-F″) (n=5/5).

Consistent with the loss of tectal features (Fig. 3), transfection of Meis2 function-blocking constructs, but not transfection of a control-EnR fusion protein, in the midbrain vesicle effectively repressed *Efnb1* expression (Fig. 4G-I; data not shown) (n=22/28; n=0/13 for SOHoEnR as control) (Schulte and Cepko, 2000). Similarly, *Dbx1* transcripts, which are normally present in the dorsal mesencephalon, were undetectable in *Meis2EnR*-transfected regions of the neural tube (n=8/9) (Fig. 4J-K″). By contrast, expression of the homeodomain transcription factor *Otx2*, which specifies the anterior neural plate and its derivatives, and expression of the paired-box transcription factors *Pax3* and *Pax7*, which mark the alar plate along much of the length of the neural tube, were unaffected by *Meis2EnR* 24 hours following transfection (Fig. 4L-M′; see Fig. S4 in the supplementary material). In addition, although transfection of *Meis2*-interfering constructs had induced aberrant foliation of the transfected tissue at mid-embryonic stages, this was not

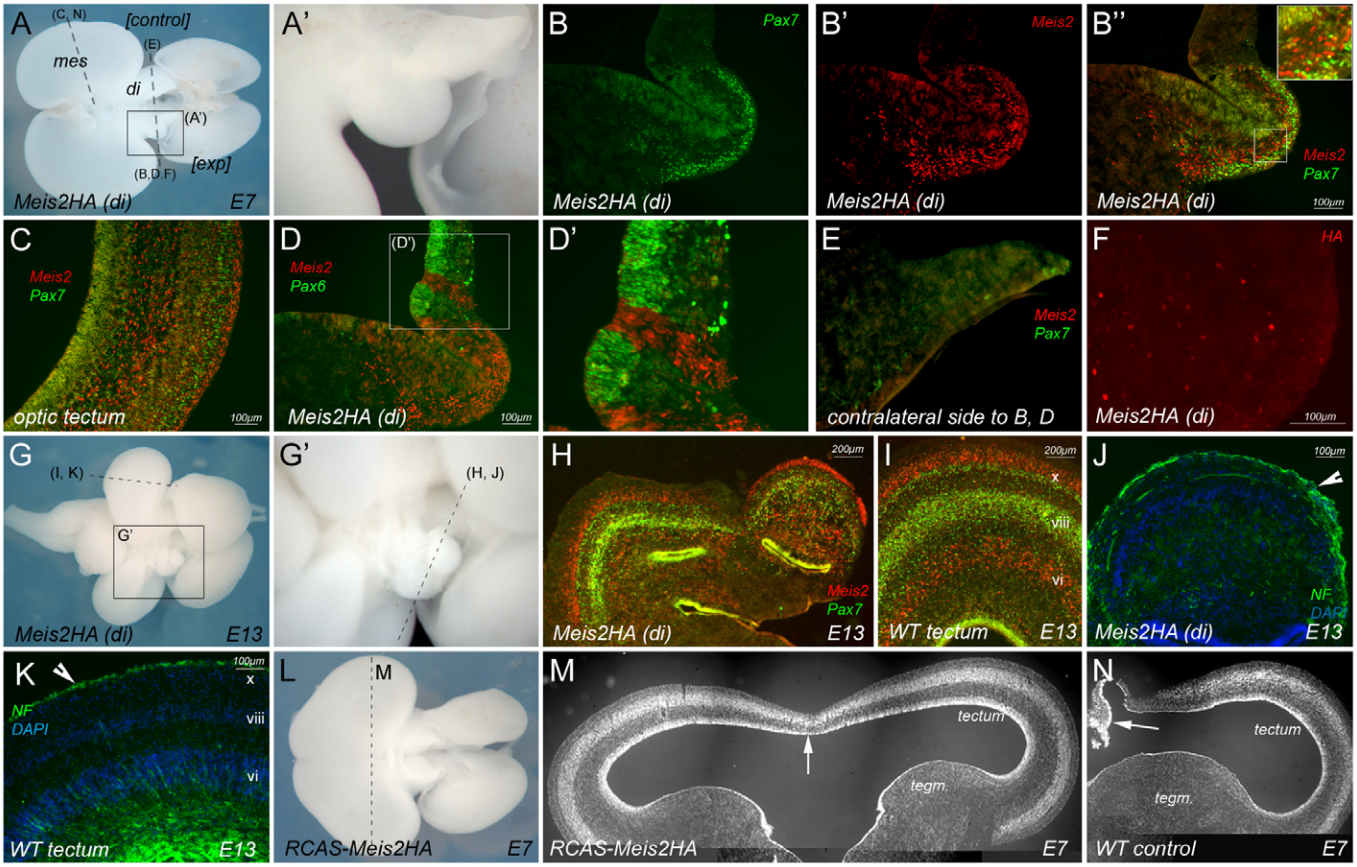

Fig. 2. *Meis2* transfection into the diencephalic vesicle induces tectal structures. (**A**,**A′**) Ectopic tectal structures in the chick diencephalon 6 days following *Meis2HA* transfection (A). The dashed lines indicate the plane of section shown in B,D-F and C,N. (A′) Higher magnification of the boxed area in A. (**B-C**) Distribution of Pax7- and Meis2-immunoreactive cells in the electroporated side of the diencephalon (B-B″) or the optic tectum (C). (**D**,**D′**) Loss of Pax6 immunoreactivity in *Meis2HA*-transfected cells in the diencephalon. (**E**) Meis2 and Pax7 are not expressed in the non-electroporated, contralateral side of the diencephalon shown in A. (**F**) Few cells still express the HA epitope 6 days after *Meis2HA* transfection. (**G**,**G′**) Ectopic tectum-like structures in the diencephalon 12 days after *Meis2HA* transfection. (**H**,**I**) Distribution of Pax7- and Meis2-immunoreactive cells in the ectopic (H) and normal (I) optic tectum. (**J**,**K**) Neuronal processes stained for neurofilament and compared with cell nuclei stained with DAPI in the ectopic (J) and normal (K) optic tectum. The arrowheads mark a superficial axonal layer in both structures, presumably a stratum opticum. The roman numerals in I and K indicate tectal layers (LaVail and Cowan, 1971). (**L**) Morphology of a brain 6 days after retroviral misexpression of *Meis2HA* throughout the neural tube. (**M**,**N**) Cross-section through both hemispheres of the embryo shown in L and stained with DAPI to visualize cell nuclei (M), as compared with wild-type (WT) control (N). The midbrain alar plates are dorsally fused; the tecta appear enlarged and the tegmenta reduced in volume compared with the wild type. Note the fused optic tecta and loss of dorsal midline-derived structures (arrows).

accompanied by ectopic induction of hindbrain-associated genes, such as *Ath1* (*Tnfsf4* – Mouse Genome Informatics), *Irx2* or *Zic1* (Fig. 4N-Q′; data not shown) (Ben Arie et al., 1997; Lin and Cepko, 1998; Matsumoto et al., 2004). We also analyzed whether loss of *Meis2* function induces expression of the diencephalic marker Pax6 in the midbrain. The rationale behind this experiment was the observation that diencephalon and mesencephalon share expression of *Otx2* but differ in the expression of *Meis2*. This raises the possibility that loss of *Meis2* in the midbrain might entail diencephalic differentiation as a consequence of the persistent *Otx2* expression. However, we did not observe ectopic *Pax6* expression in the midbrain under these conditions (data not shown).

Otx2 is essential for the development of all anterior brain structures, including the midbrain, and can induce midbrain development when ectopically expressed in rhombomere 1 (Katahira et al., 2000). Transfection of *Otx2* into rhombomere 1 rapidly induced *Meis2* expression, placing *Meis2* downstream of *Otx2* in tectal development (see Fig. S5 in the supplementary material). In addition, co-transfection of *Meis2* function-blocking constructs together with *Otx2* prevented the metencephalic-to-mesencephalic fate change, which is otherwise triggered by *Otx2* misexpression (see Fig. S6 in the supplementary material). We conclude from these results (1) that *Meis2* acts upstream of the tectal markers *Dbx1* and *Efnb1* but downstream of *Otx2*, and (2) that loss of *Meis2* function disrupts normal tectal development, but is not sufficient to induce a mesencephalic-to-metencephalic or a mesencephalic-to-diencephalic transdifferentiation.

Meis2 instructs tectal development without participating in MHB organizer function

Midbrain development is under the control of the MHB organizer, a signaling center located just caudal to the *Otx2/Gbx2* junction, which forms during late gastrulation. An interdependent loop of nuclear factors and secreted proteins, including *Fgf8/17/18*, *Wnt1*, *Pax2/5* and *En1/2*, shape and maintain the MHB organizer (Wurst and Bally-Cuif, 2001). Ectopic expression of any of these genes in the diencephalic vesicle of chick or zebrafish embryos induces expression of other MHB-associated factors and initiates

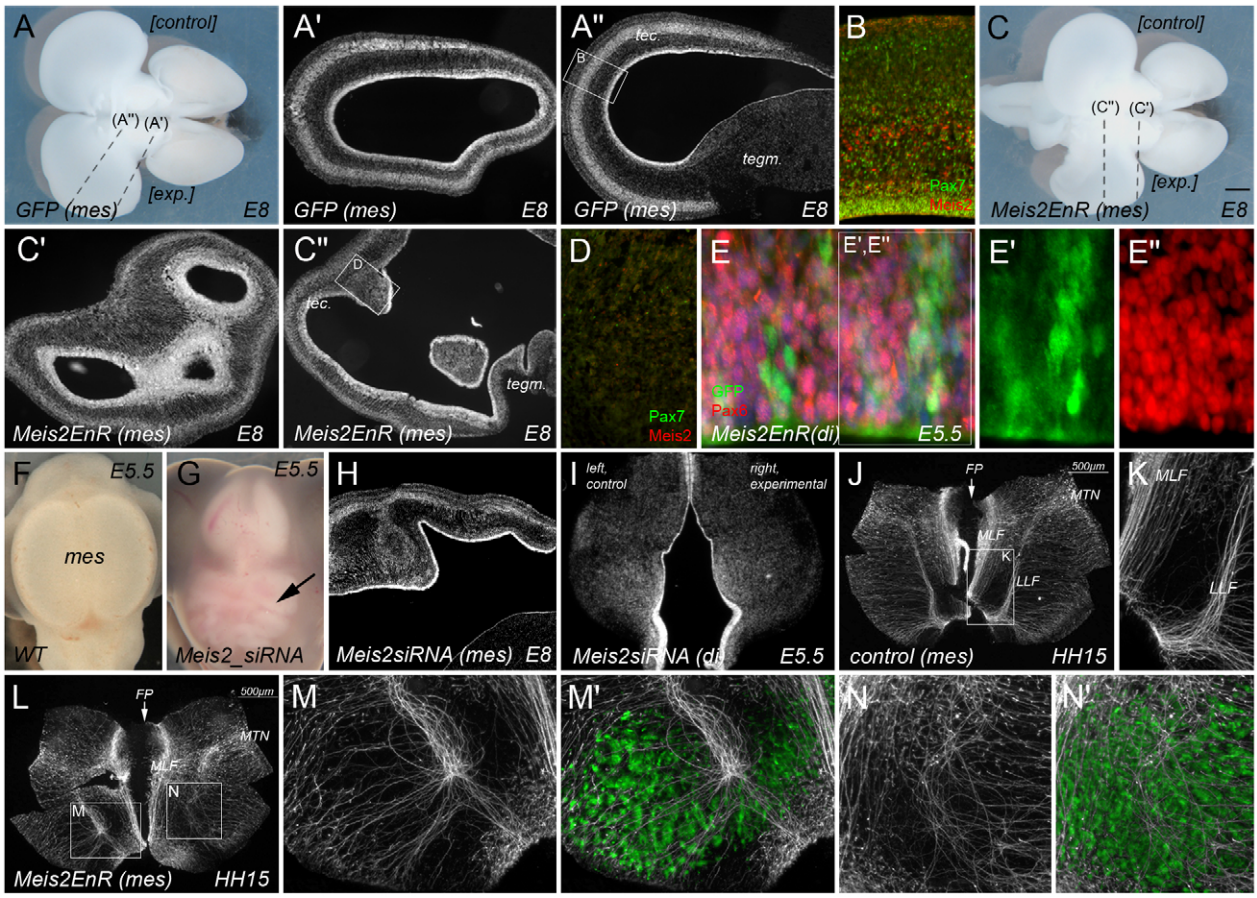

Fig. 3. Brain morphology following transfection of *Meis2EnR*, *Meis2*-specific RNAi targeting vectors or *GFP*. (**A**) Chick E8 brain transfected with *GFP* into the right tectal anlage. (**A′**,**A″**) Coronal sections of the right optic tectum of the brain shown in A at the sites indicated by dashed lines and stained with DAPI to visualize cell nuclei. (**B**) Immunohistochemical staining of the boxed region in A″ with antibodies specific for Pax7 (green) and Meis2 (red). (**C**) E8 brain transfected with *Meis2EnR* together with *GFP* into the right tectal anlage. Control: left, unelectroporated control side; exp.: right, electroporated side. Scale bar: 1 mm. (**C′**,**C″**) Frozen sections through the right optic tectum of the brain shown in C at the planes indicated. (**D**) Immunohistochemical staining of the boxed region in C″ with antibodies specific for Pax7 (green) and Meis2 (red). Loss of the characteristic laminar organization is accompanied by loss of expression of the tectal markers Pax7 and Meis2. (**E-E″**) Section through an E5.5 chick diencephalic vesicle transfected 4 days previously with *Meis2EnR* together with *GFP* and stained with antibodies against Pax6 (red, E,E″), GFP (green, E,E′) and counterstained with DAPI (blue). Most GFP-Meis2EnR-expressing neuroepithelial cells retain expression of the diencephalic marker Pax6. (**F**,**G**) E5.5 chick heads viewed from the top. (F) Wild-type control. (G) Embryo following transfection of a *Meis2*-specific RNAi targeting vector. The arrow points to the aberrantly folded dorsal midbrain following RNAi-mediated knockdown of *Meis2* expression. (**H**) Frozen sections through an E8 optic tectum 7 days after siRNA-mediated *Meis2* knockdown, stained with DAPI. (**I**) Coronal section through an E5.5 diencephalon transfected with *Meis2* siRNA, stained with DAPI. (**J-N′**) Disrupted axon trajectories in the midbrain following transfection of *Meis2EnR*. Open-book preparation of E2.5 (HH15) chick midbrains stained for neurofilament (white) and GFP (green). (J,K) Medial longitudinal fascicle (MLF) and lateral longitudinal fascicle (LLF) in an open-book preparation of a non-electroporated control midbrain. (K) Higher magnification of the boxed area in J. (L) Example of a midbrain transfected with *Meis2EnR*. (M-N′) Higher magnifications of the boxed areas in L. In M′ and N′, the region of *Meis2EnR* misexpression is visualized by fluorescence of the co-electroporated GFP. FP, floor plate; MTN, mesencephalic trigeminal nucleus.

development of ectopic tectal structures (Araki and Nakamura, 1999; Crossley et al., 1996; Funahashi et al., 1999; Okafuji et al., 1999; Ristoratore et al., 1999). To investigate whether *Meis2* is part of this molecular network at the MHB, we targeted *Meis2* expression to different regions of the neural tube and assessed the expression of genes that are essential for MHB organizer activity at different times following transfection (Fig. 5; Table 1). Forced *Meis2* expression in the diencephalic vesicle failed to induce expression of the MHB marker genes *En1* or *Pax2* (Fig. 5A,A′,C,D). We also never detected upregulation of *Fgf8*, a key molecule of MHB organizer function, at times up to 4 days following *Meis2HA* transfection (Fig. 5B,E,E′,H,J). Notably, from 48 hours post-transfection onwards, the diencephalic vesicles exhibited ectopic enlargements indicative of the beginnings of a diencephalic-to-mesencephalic transformation, yet these morphological alterations were not accompanied by ectopic *Fgf8* expression (Fig. 5E-K). Furthermore, *Meis2* transfection into the diencephalic vesicle did not induce *Pax3* or *Pax7*, which also contribute to MHB maintenance and function (see Fig. S4 in the supplementary material) (Matsunaga et al., 2001). Conversely, expression of *Fgf8*, *Otx2*, *En1*, *Pax2*, *Pax3*, *Pax5*, *Pax7*, *Wnt1* and *Wnt3a* was normal following targeted misexpression of the dominant-negative *Meis2EnR* in the MHB territory (Table 2; see Fig. S4 in the supplementary material). In contrast to other known factors, which can induce ectopic midbrain development in the diencephalon, *Meis2* thus triggers the diencephalic-to-mesencephalic fate change without inducing a secondary MHB organizer.

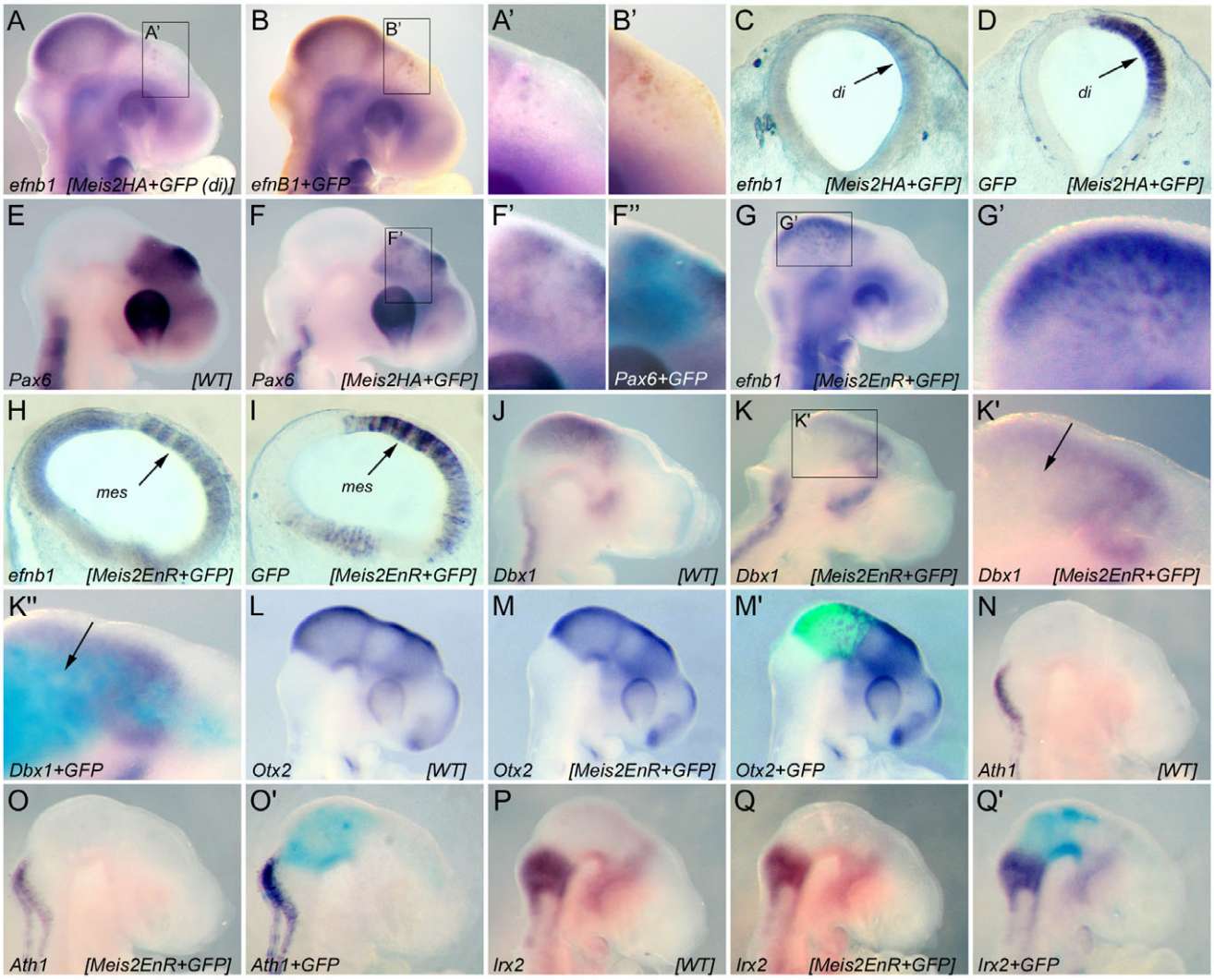

Fig. 4. Regulation of mid-hindbrain patterning genes by *Meis2*. (**A-B'**) Targeted transfection of *Meis2HA* together with *GFP* (brown) into the diencephalic vesicle induces ectopic *Efnb1* transcripts (blue) within 24 hours. (A',B') Higher magnifications of the boxed areas in A,B. (**C**,**D**) Induction of *Efnb1* expression by *Meis2HA* on a coronal section through the diencephalic vesicle. (**E-F''**) *Pax6* transcripts in an HH15 wild-type chick embryo (E) and following targeted expression of *Meis2* in the diencephalon. (F-F''). (F') Higher magnification of the boxed area in F. (**G**,**G'**) Downregulation of *Efnb1* expression by *Meis2EnR* in the HH15-17 midbrain. (**H**,**I**) Repression of *Efnb1* expression by *Meis2EnR* on a coronal section through the mesencephalic vesicle. (**J**) *Dbx1* expression in an HH15 wild-type chick embryo. (**K-K''**) Targeted expression of *Meis2EnR* in the mesencephalic vesicle represses *Dbx1*. (**L-M'**) *Otx2* transcripts in a wild-type HH15 chick embryo (L) and following forced expression of *Meis2EnR* and *GFP* in the mesencephalic vesicle (M). (M') The domain of cells transfected with *Meis2EnR* and *GFP* in the embryo shown in M as visualized by GFP fluorescence. (**N-Q'**) *Ath1* (N-O') and *Irx2* (P-Q') expression in a wild-type HH15 chick embryo (N,P) and following forced expression of *Meis2EnR* and *GFP* in the mesencephalic vesicle (O,O',Q,Q'). In F'',K'',O',Q', the domain of *Meis2EnR/GFP* misexpression is visualized in turquoise by in situ hybridization with a *GFP*-specific probe, and in B,B' in brown with an antibody directed against GFP. The arrows (C,D,H,I,K',K'') point to transfected regions of the neural tube in the respective specimens.

Meis2 directly interacts with Otx2 in vivo and in vitro

Our observation that ectopic tectal development upon *Meis2* transfection was restricted to the anterior neural tube and thus to regions of the neural tube that express *Otx2* (see Fig. S2 in the supplementary material) raises the possibility that both proteins cooperate in tectal development. As a first test of this idea, we performed pull-down experiments with a GST-tagged form of Meis2a using protein extracts of the HH15-18 (24- to 36-somite stages) chick mesencephalic alar plate. Meis2-GST, but not GST alone, readily precipitated Otx2 (Fig. 6A). Notably, complex formation also occurred when the experiment was performed in the presence of DNaseI (Fig. 6B). To test which protein domain(s) of Meis2 was involved in complex formation, we performed pull-down experiments with truncated forms of Meis2 fused to GST. Fusion proteins that lack the homeodomain (Meis2ΔHD$_{[1-190]}$) failed to precipitate Otx2, whereas precipitation of Otx2 was reduced when GST fusion proteins that lack the N-terminus including the MEINOX domain (Meis2ΔN$_{[199-400]}$) were used (compare Fig. 6A with 6C). Meis2-Otx2 interaction therefore involves the three-dimensional structure of the entire Meis2 polypeptide chain. The homeodomain of Meis2 appears to be essential for complex formation, whereas the N-terminal protein,

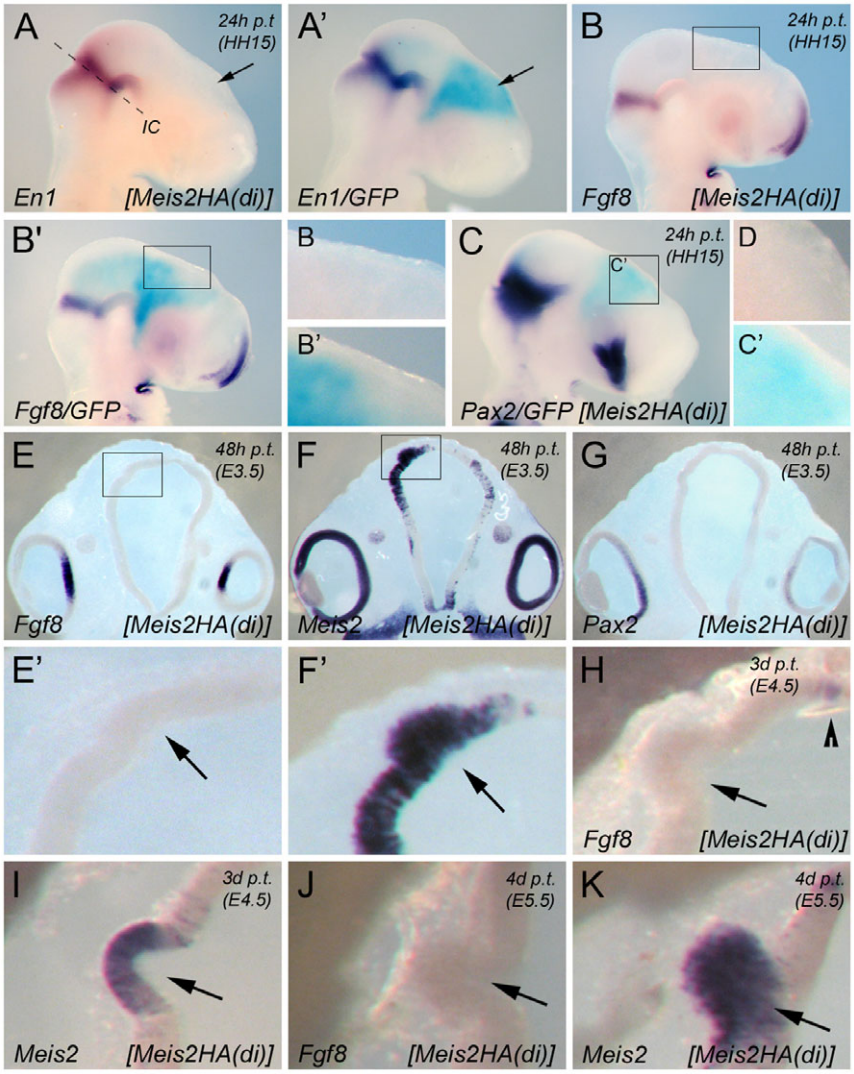

Fig. 5. Meis2 is not involved in MHB activity and maintenance. (**A-C'**) Expression of *En1* (A,A'), *Fgf8* (B,B') or *Pax2* (C,C') 24 hours after misexpression of *Meis2HA* together with *GFP*. In A',B',C', transgene expression is visualized in turquoise. Arrows in A,A' point to the domain transfected with *Meis2HA* and *GFP*. (**D**) High magnification of a non-electroporated chick embryo stained for *Pax2*. (**E-K**) Expression of *Fgf8* (E,E',H,J), *Meis2* (F,F',I,K) or *Pax2* (G) at different times after electroporation of *Meis2HA* into the diencephalic vesicle. Times post-transfection (p.t.) are indicated in the upper right corner. (E',F') Higher magnifications of the boxed areas in E,F. The arrows in E'-K point to the ectopic bulges that form as a consequence of *Meis2HA* misexpression; these express *Meis2* uniformly but lack *Fgf8*. The arrowhead in H points to a domain of endogenous *Fgf8* expression in the dorsal diencephalon, presumably equivalent to domain F12 of Crossley and colleagues (Crossley et al., 2001).

including the MEINOX domain, might serve to stabilize the complex. In support of this, we found that EnR fusion constructs lacking the N-terminus including the MEINOX domain or the homeodomain failed to inhibit *Efnb1* expression or tectal development upon transfection into the midbrain vesicle (data not shown).

Next, we investigated whether Meis2-Otx2 interaction was direct or required the presence of additional mesencephalic proteins. To this end, GST pull-down experiments were performed with Meis2-GST and ^{35}S-radiolabeled Otx2 produced in vitro by coupled transcription-translation. Meis2-GST, but not GST alone, precipitated Otx2 (Fig. 6D, left lanes). Again, complex formation was not disrupted by prior treatment with DNaseI (Fig. 6H). Next, truncated forms of Otx2 were tested for their ability to associate with Meis2-GST (Fig. 6D-G). Of these polypeptides, only those that contained 18 amino acids N-terminal of the homeodomain (corresponding to the sequence RKQRRERTTFTRAQLDVL) precipitated with Meis2. Notably, the eh1 (engrailed homology region 1) motif, which mediates binding of Otx2 to the Groucho repressor protein Tle4 (Grg4) (see below), was dispensable for Otx2-Meis2 complex formation (Heimbucher et al., 2007). In summary, these results demonstrate that Meis2 and Otx2 can directly interact in the absence of DNA via an 18 amino acid motif in Otx2.

Meis2 competes with the Groucho co-repressor Tle4 for binding to Otx2 and restores Otx2 transcriptional activator function

Neuroepithelial cells of the tectal anlage co-express Meis2 and Otx2 (Fig. 7A,A'). To investigate whether Otx2-Meis2-containing protein complexes exist in the tectal anlage in vivo, we performed co-immunoprecipitation experiments from protein extracts of native HH14-18 (20- to 36-somite stage) chick tecta with an antibody directed against the N-terminus of Meis2. As expected for transcriptional regulators, both proteins were predominantly found in the nuclear fractions. Upon immunoprecipitation, Otx2 was enriched in the precipitates when the Meis2-specific antibody was used, but not with an unrelated antibody (Fig. 7B). Both transcription factors are therefore not only able to interact in vitro, but also form higher order protein complexes in the mesencephalic alar plate in vivo.

Because we had previously observed that *Meis2* functions as a transcriptional activator in an in vitro reporter assay (see Fig. S1 in the supplementary material), we speculated that binding of Meis2 to Otx2 might augment Otx2 transactivation activity. To test this, we assessed whether Meis2 was able to modulate the activity of an Otx2-dependent reporter in vitro. In this assay, the basal activity of a Gal4-responsive promoter is enhanced after co-transfection of an

Table 1. Expression of *Efnb1* is induced in the diencephalon 24 hours after *Meis2HA* transfection

	Marker gene					
Transfection	*Efnb1*	*Fgf8*	*En1*	*Pax2*	*Pax3*	*Pax7*
Meis2HA	10/12	0/5	0/5	0/4	0/5	0/6
Control	0/14	0/9	0/7	n.d.	n.d.	n.d.

Number of embryos with induced MHB marker gene expression following transfection/total number of transfected embryos. Of the genes analyzed, only *Efnb1* expression was consistently upregulated upon *Meis2HA* misexpression.
n.d., not determined.

Otx2-Gal4 fusion protein (Fig. 7C) (Heimbucher et al., 2007). Meis2 alone (not fused to Gal4) was not able to activate the reporter. Yet, contrary to our initial expectation, Meis2 co-transfection with Otx2-Gal4 also did not elevate reporter activity above the level seen after transfection of Otx2-Gal4 alone (Fig. 7C).

Otx2 can function as transcriptional activator or repressor depending on the cellular context. Binding to the Groucho co-repressor protein Tle4 is sufficient to attenuate Otx2 transactivation activity and leads to effective repression of an Otx2-dependent promoter (Heimbucher et al., 2007; Puelles et al., 2004). Binding of Tle4 to Otx2 was mapped to the eh1 domain, an evolutionary conserved motif located C-terminal to the Otx2 homeodomain (Heimbucher et al., 2007). Although the Meis2-binding motif identified in the present study is separated from the eh1 region by the entire homeodomain, the Otx2 homeodomain, like the related Bicoid homeodomain, adopts a three-helical global fold in the non-DNA-bound state, which brings the amino acids N-terminal of helix 1 into close proximity of those C-terminal of helix 3 (Baird-Titus et al., 2006) (NCBI Structure MMDB ID #42063). We therefore speculated that Meis2 binding to Otx2 might interfere with the ability of Otx2 to interact with Tle4. To test this, we monitored the activity of the Gal4-dependent reporter in the presence of Otx2-Gal4 alone, following transfection of Otx2-Gal4 together with Tle4 and upon co-transfection of increasing amounts of Meis2 (Fig. 7D). As reported previously, Tle4 alone effectively repressed transactivation by Otx2-Gal4 (Fig. 7D, bar 2) (Heimbucher et al., 2007). Interestingly, co-transfection of the *Meis2*-expressing plasmid restored reporter activity in a concentration-dependent manner (Fig. 7D, bars 3 and 4). To verify that this effect required Meis2 binding to Otx2, a Gal4 fusion to a truncated form of Otx2, Otx2Δ1-100-Gal4, was used. This protein, although lacking the entire N-terminus including the 18 amino acid Meis2-binding motif, can still activate the Gal4-dependent reporter and interact with Tle4 (Fig. 7E, bars 1 and 2) (Heimbucher et al., 2007). In contrast to full-length Otx2-Gal4, co-transfection of *Meis2* with Otx2Δ1-100-Gal4 did not restore reporter activity, underscoring the importance of the 18 amino acid motif (Fig. 7E, bars 3 and 4). Direct interaction with Otx2 is therefore indispensable for the ability of Meis2 to interfere with Otx2-Tle4-mediated repression. In support of this, we found that Otx2 lacking the N-terminus including the 18 amino acid motif was ineffective in inducing ectopic midbrain structures in the metencephalic vesicle (data not shown).

To test whether Meis2 is also capable of binding to Tle4, we performed pull-down experiments with Meis2-GST together with ^{35}S-radiolabeled Tle4 and compared the efficiency of the precipitation with that of Meis2-GST together with ^{35}S-radiolabeled Otx2 under identical experimental conditions (Fig. 7F). Relative to the amount of radiolabeled protein added into the reactions (input, 'in'), only a minor fraction of Tle4 could be detected in the precipitates, whereas Otx2 was highly enriched by the Meis2-GST fusion protein (compare 'in' and PD in Fig. 7F, top and bottom panels). Meis2 thus binds robustly to Otx2, but only weakly, if at all, to Tle4. It is tempting to speculate that the weak binding of Meis2 to Tle4 might reflect a transient contact between the two proteins, which might take place when Meis2 releases Otx2 from Tle4-mediated repression.

DISCUSSION

Here we show that the TALE-homeodomain protein Meis2 is a key regulator of tectal development. In contrast to other known genes involved in tectal development, *Meis2* initiates tectal fate specification without inducing a secondary MHB organizer. Instead, Meis2 binds to Otx2 in the absence of DNA, competes with the co-repressor Tle4 for binding to Otx2 and thereby restores Otx2 transcriptional activator function. As discussed below, these results suggest a model in which the balance between a co-repressor and a co-activator, which compete for binding to Otx2 in the mesencephalic vesicle, provides spatial and temporal control over the onset of tectal development. Our data thus argue for a novel, potentially DNA-independent function of TALE-homeodomain proteins: the controlled assembly and disassembly of transcription regulator complexes.

Meis2 acts as a key regulator of tectal fate downstream of the MHB organizer

Tectum development is induced when an ectopic *Fgf8* source is generated in the prosencephalon through transplantation of an ectopic MHB organizer or implantation of Fgf8-releasing beads into the lateral wall of the diencephalon (Crossley et al., 1996; Martinez et al., 1991). In addition to Fgf8, several transcription factors can trigger tectal development upon misexpression, including Otx2, Pax2/5, En1/2 and Pax3/7 (Araki and Nakamura, 1999; Funahashi et al., 1999; Katahira et al., 2000; Matsunaga et al., 2001; Okafuji et al., 1999; Ristoratore et al., 1999). Unlike Meis2, expression of these proteins is not specific for the tectal anlage. Moreover, each of these proteins participates in the interdependent, positive maintenance loop at the MHB organizer and, consequently, induces ectopic expression of MHB marker genes, including *Fgf8*, when misexpressed. These molecules therefore evoke tectal development indirectly through formation of an ectopic MHB organizer. *Meis2*, by contrast, is unique as it can initiate tectal development without participating in MHB organizer function or maintenance. As we have recently shown, endogenous *Meis2* expression is repressed when metencephalic development is experimentally induced through activation of the Ras-MAP kinase pathway in the mesencephalon and is upregulated

Table 2. Reduced expression of *Efnb1* and *Dbx1* in the midbrain 24 hours after *Meis2EnR* transfection

	Marker gene										
Transfection	*EfnB1*	*Dbx1*	*Otx2*	*Fgf8*	*En1*	*Pax2*	*Pax3*	*Pax5*	*Pax7*	*Wnt1*	*Wnt3a*
Meis2EnR	22/28	8/9	0/7	0/4	0/12	0/4	0/19	0/11	0/23	0/6	0/5
Control-*EnR*	0/13	0/4	0/3	n.d.	0/6	n.d.	0/3	0/5	0/3	0/3	0/3

Number of embryos with reduced MHB marker gene expression following transfection/total number of transfected embryos. Of the genes analyzed, only *Efnb1* and *Dbx1* were repressed upon *Meis2EnR* transfection.
n.d., not determined.

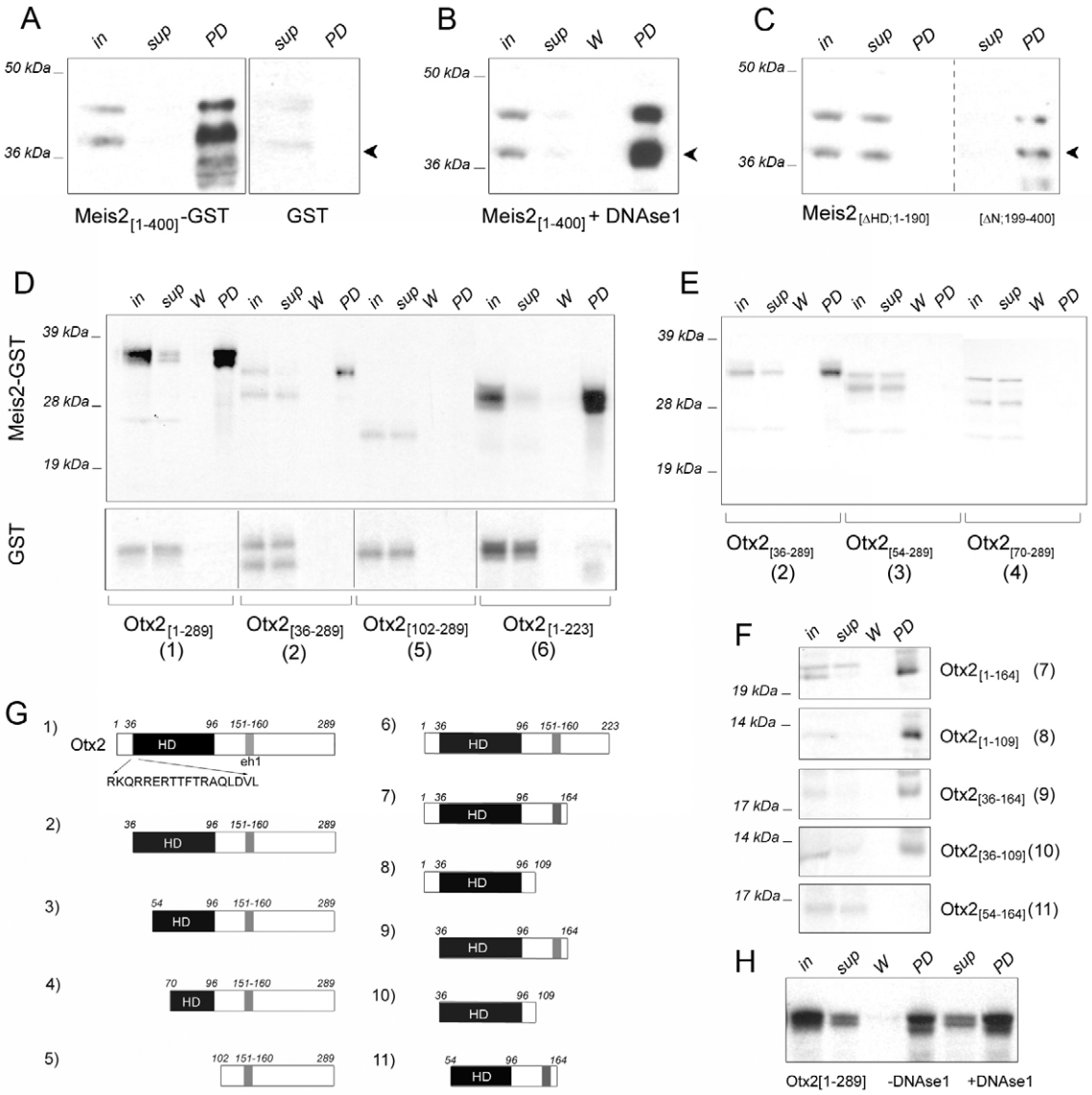

Fig. 6. Meis2 and Otx2 directly interact in vivo and in vitro. (**A-C**) GST pull-down experiments from chick tectal extracts. (A) Full-length Meis2 fused to GST (Meis2$_{[1-400]}$), but not GST alone, precipitates Otx2. (B) Meis2-Otx2 complex formation in the presence of DNaseI. (C) Meis2 lacking the homeodomain (Meis2$_{[1-190]}$) fails to enrich Otx2 in the precipitates, whereas precipitation of Otx2 decreases when Meis2 lacking the N-terminus including the MEINOX domain (Meis2$_{[199-400]}$) is used. (**D-F**) GST pull-down experiments with Meis2$_{[1-400]}$-GST and ^{35}S-radiolabeled Otx2 protein produced in vitro. Full-length Otx2 (Otx2$_{[1-289]}$) or an N-terminally truncated protein lacking the first 35 amino acids (Otx2$_{[36-289]}$), but not Otx2 lacking amino acids 1-53 (Otx2$_{[54-289]}$), readily precipitate with Meis2, indicating that amino acids 36-53 mediate Meis2 binding. Upper panels in D,E,F show pull-down experiments with Meis2-GST. The lower panel in D shows control experiments with GST. (**G**) Structure of the Otx2 deletion constructs tested. HD, homeodomain; eh1, engrailed homology region 1 motif. (**H**) Meis2-Otx2 complex formation in the presence of DNaseI in vitro. Loading control 'in' corresponds to ~1/15th of the protein amount used for the pull-down experiments. in, input; sup, supernatant; W, final wash; PD, pellet of the pull-down experiment. The blots shown in C, D and E were cut to remove marker or empty lanes.

concomitantly to the metencephalic-to-mesencephalic fate change that occurs when Ras-MAP kinase signaling is blocked in rhombomere 1 (Vennemann et al., 2008). *Meis2* expression must therefore be directly or indirectly under control of the MHB organizer. Notably, a single, transient transfection of *Meis2* in the diencephalic alar plate at the 10- to 11-somite stage was sufficient to initiate long-term expression of endogenous *Meis2* in transfected cells (Fig. 5; see Fig. S3 in the supplementary material). *Meis2*, once induced, can therefore stabilize its own expression. Together, these results suggest a model in which regulation of tectal development by signals from the MHB is mediated via induction and subsequent auto-maintenance of *Meis2* expression.

Meis2 restores Otx2 transcriptional activator function in the tectal anlage by competing with Groucho/Tle co-repressors

Meis family proteins act as co-factors of other transcriptional regulators (Moens and Selleri, 2006). To date, Meis-interacting proteins have been isolated from non-neuronal tissue and the posterior hindbrain, yet Meis co-factors in the developing anterior brain have remained elusive. We performed GST pull-down experiments from tectal extracts or with in vitro translated proteins as well as co-immunoprecipitation experiments with native tectal proteins to demonstrate direct interaction of Meis2 and Otx2 during

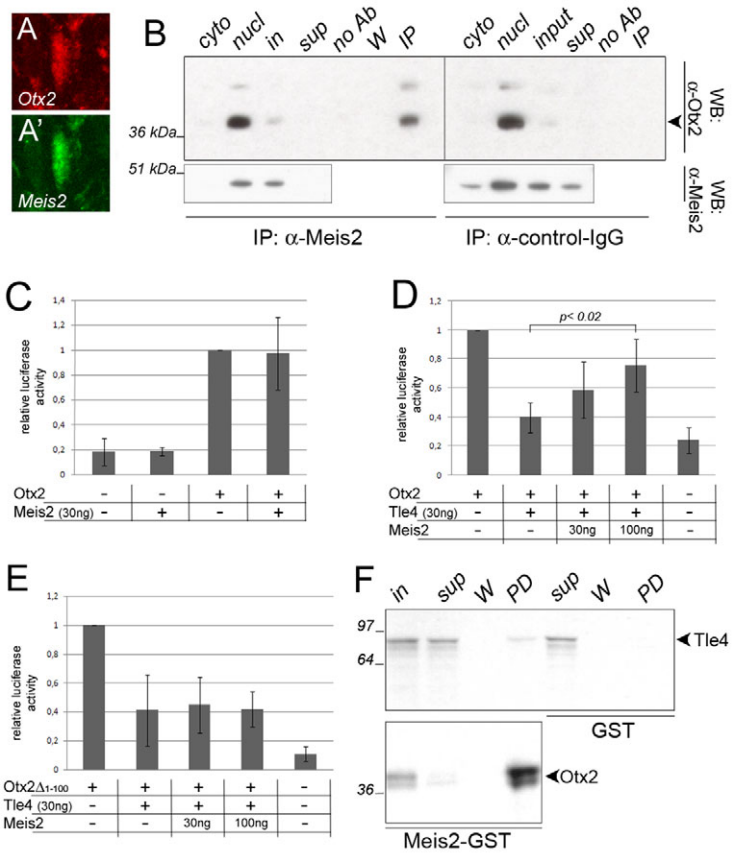

Fig. 7. Otx2 and Meis2 form higher order complexes in the tectal anlage in vivo and Meis competes with Tle4 for binding to Otx2. (**A**,**A′**) Immunoreactivity of Otx2 (A) and Meis2 (A′) in chick neuroepithelial cells of the E3 tectal anlage. (**B**) Co-immunoprecipitation of Otx2 with a Meis2-specific antibody from HH14-16 tectal extracts. Left, precipitation with the Meis2-specific antibody; right, unrelated control antibody. Upper panel, immunodetection of Otx2; lower panel, immunodetection of Meis2. Because the Meis-specific antibody used for precipitation is identical to that used for immunodetection, Meis2 proteins in the precipitates are not shown. The polyclonal anti-Otx2 antibody recognizes a major band at 38 kDa (arrowhead) and a minor band at 43 kDa. (**C-E**) Transient reporter assays in mouse NIH 3T3 cells. Gal4-dependent reporter plGC-luc and constitutive reference reporter phRG-TK were co-transfected with the expression plasmids and at the concentrations listed. Empty vector was co-transfected if needed to adjust for varying DNA concentrations. Luciferase reporter values were normalized to phRG-TK activity and are shown relative to plGC-luc activity in the presence of 10 ng pMC-Gal4Otx2 or pMC-Gal4Otx2Δ1-100, respectively. Mean and s.d. of three to five independent experiments are given. (**F**) GST pull-down experiments with $Meis2_{[1-400]}$-GST and ^{35}S-radiolabeled labeled Tle4 or Otx2 produced in vitro by coupled transcription-translation. The loading control 'in' corresponds to ~1/15th of the protein amount used for the pull-down experiments. cyto, cytoplasmic fraction; nucl, nuclear fraction; in, input; sup, supernatant; W, final wash; no Ab, protein G-agarose beads without primary antibody; IP, immunopellet.

early midbrain development. Using deletion constructs of Otx2, we find that complex formation requires a short motif N-terminal of the Otx2 homeodomain. The region of the Otx2 polypeptide chain that contacts Meis2 thus differs from the tryptophan-containing hexapeptide that mediates cooperative DNA binding of Hox or myogenic bHLH proteins with TALE-homeodomain proteins (Knoepfler et al., 1999; Lu and Kamps, 1996). Meis family proteins can therefore interact with different protein motifs present in a variety of transcription factors.

Employing an Otx2-dependent reporter assay, we provide evidence that Meis2 competes with the co-repressor Tle4 for binding to Otx2. *Tle4* expression begins in the anterior primitive streak (thus preceding that of *Meis2*), is later strong in the anterior neural tube and decreases after the 20- to 25-somite stage (Sugiyama et al., 2000; Van Hateren et al., 2005). Tle4 binding to Otx2 was previously shown be required for the ability of Otx2 to repress *Fgf8* anterior to the MHB, an important step in the formation and stabilization of the MHB organizer (Heimbucher et al., 2007). Overexpression of *Tle4* in the mesencephalic vesicle, in turn, disrupts normal development and lamination of the optic tecta (Sugiyama et al., 2000; Sugiyama and Nakamura, 2003). Together with the results presented here, these data might allow us to reconstruct the probable temporal sequence of tectal fate specification in the embryo. In the anterior neural plate and anterior neural tube at early somite stages, *Tle4* is co-expressed with *Otx2* but *Meis2* is missing. In the absence of Meis2, Otx2 and Tle4 can interact, prevent precocious tectal differentiation and inhibit *Fgf8* expression anterior to the organizer, which stabilizes the MHB signaling center. *Meis2* expression in the mesencephalic alar plate begins at HH11-12 (13-16 somites) and is strong from the 20- to 22-somite stage onwards, at which time the MHB organizer is established (Fig. 1). Meis2 competes with Tle4 for binding to Otx2 in the tectal anlage, releases Otx2 from Groucho-mediated repression and thereby allows tectal development to commence.

If correct, two predictions can be drawn from this model. First, loss of *Tle4* from the diencephalic vesicle (where *Tle4* is co-expressed with *Otx2* at early somite stages) should lead to de-repression of tectal genes. Second, precocious and ectopic expression of *Meis2* in the MHB territory may destabilize the *Fgf8* expression domain through premature restoration of Otx2 transcriptional activator function. Indeed, as previously demonstrated, transfection of a putative dominant-negative form of *Tle4* – a truncation comprising only the first 203 amino acids of the protein – into the diencephalic vesicle causes widespread ectopic induction of *En2* transcripts (Sugiyama et al., 2000). In addition, when we ectopically introduced *Meis2* into the MHB region at the 4- to 6-somite stage, small ectopic patches of *Fgf8* transcripts anterior to the normal *Fgf8* expression domain at the MHB were visible (see Fig. S8 in the supplementary material). These ectopic patches of *Fgf8* expression might correspond to cells that have escaped *Fgf8* downregulation by Otx2-Tle4 during the period of MHB organizer formation owing to the precocious inactivation of the Otx2-Tle4 complex by *Meis2HA*.

Meis2 had to be transfected in excess to *Tle4* in order to restore *Otx2* transactivation in the Otx2-dependent reporter assays. This observation is consistent with the fact that between the 24- and 44-somite stages, *Meis2* transcripts are abundant in the dorsal midbrain, whereas *Tle4* expression is barely detectable (see Fig. S7 in the supplementary material). Tle4 thus appears to bind to Otx2 with higher affinity than does Meis2, which might allow for tight control over the tectum-inducing activity of Otx2. Recently, the spatial-temporal windows of Otx2 control over head, brain and body

development were defined by Tamoxifen-induced deletion of *Otx2* (Fossat et al., 2006). Interestingly, *Otx2* deletion at E10.5-12.5 resulted in a mesencephalic-to-metencephalic fate change without shifting the molecular MHB. Hence, the interaction of Otx2 with Meis2 in the tectal anlage reported here occurs at a similar developmental stage to that at which Otx2 is required for mesencephalic fate determination but not MHB organizer positioning.

Possible targets of Otx2/Meis2 include Efnb1 and Dbx1, both of which carry several potential consensus Bicoid- and Meis-binding sites upstream of their transcriptional start sites. Direct regulation of a midbrain-specific regulatory element of the *EphA8* gene by Meis2 has also been demonstrated in mice (Shim et al., 2007). However, because Meis2 binding to Otx2 does not require either protein to be bound to DNA, Meis2-Otx2 interaction and restoration of Otx2 transcriptional activator function might in fact take place before both proteins have contacted the regulatory elements of downstream genes. Regulation of gene expression by putative transcription factors independent of DNA binding is not unprecedented. For instance, several Hox proteins can modulate gene expression by inhibiting the activity of CBP histone acetyltransferases (HATs) without forming DNA-binding complexes with CBP HAT (Shen et al., 2001). Meis family members might therefore affect gene expression by multiple, DNA-dependent and -independent mechanisms. This view is supported by the fact that despite the identification of Meis proteins as transcriptional co-factors, few direct target genes of these proteins have been reported to date.

In summary, the results reported here strongly suggest that Tle4 and Meis2 compete for binding to Otx2 in the mesencephalic vesicle and that the balance between these proteins provides spatial and temporal control over the onset of tectal differentiation. Formation of spatially and temporally distinct higher order protein complexes involving Meis proteins and known regulators of neural patterning or fate determination might serve as a simple, yet versatile, mechanism to subdivide broad territories into smaller functional units during brain development.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/19/3311/DC1

References

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A. and Brulet, P. (1995). Forebrain and midbrain regions are deleted in Otx2–/– mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**, 3279-3290.

Araki, I. and Nakamura, H. (1999). Engrailed defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. *Development* **126**, 5127-5135.

Azcoitia, V., Aracil, M., Martinez, A. and Torres, M. (2005). The homeodomain protein Meis1 is essential for definitive hematopoiesis and vascular patterning in the mouse embryo. *Dev. Biol.* **280**, 307-320.

Baird-Titus, J. M., Clark-Baldwin, K., Dave, V., Caperelli, C. A., Ma, J. and Rance, M. (2006). The solution structure of the native K50 Bicoid homeodomain bound to the consensus TAATCC DNA-binding site. *J. Mol. Biol.* **356**, 1137-1151.

Ben Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M. and Zoghbi, H. Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. *Nature* **390**, 169-172.

Berkes, C. A., Bergstrom, D. A., Penn, B. H., Seaver, K. J., Knoepfler, P. S. and Tapscott, S. J. (2004). Pbx marks genes for activation by MyoD indicating a role for a homeodomain protein in establishing myogenic potential. *Mol. Cell* **14**, 465-477.

Bessa, J., Tavares, M. J., Santos, J., Kikuta, H., Laplante, M., Becker, T. S., Gomez-Skarmeta, J. L. and Casares, F. (2008). meis1 regulates cyclin D1 and c-myc expression, and controls the proliferation of the multipotent cells in the early developing zebrafish eye. *Development* **135**, 799-803.

Braisted, J. E., McLaughlin, T., Wang, H. U., Friedman, G. C., Anderson, D. J. and O'Leary, D. D. (1997). Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. *Dev. Biol.* **191**, 14-28.

Capdevila, J., Tsukui, T., Rodriquez, E. C., Zappavigna, V. and Izpisua Belmonte, J. C. (1999). Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell* **4**, 839-849.

Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G. and Cleary, M. L. (1997). Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. *Mol. Cell. Biol.* **17**, 5679-5687.

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

Crossley, P. H., Martinez, S., Ohkubo, Y. and Rubenstein, J. L. (2001). Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. *Neuroscience* **108**, 183-206.

Dibner, C., Elias, S. and Frank, D. (2001). XMeis3 protein activity is required for proper hindbrain patterning in Xenopus laevis embryos. *Development* **128**, 3415-3426.

Eberhard, D., Jimenez, G., Heavey, B. and Busslinger, M. (2000). Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J.* **19**, 2292-2303.

Finkelstein, R. and Perrimon, N. (1990). The orthodenticle gene is regulated by bicoid and torso and specifies Drosophila head development. *Nature* **346**, 485-488.

Fossat, N., Chatelain, G., Brun, G. and Lamonerie, T. (2006). Temporal and spatial delineation of mouse Otx2 functions by conditional self-knockout. *EMBO Rep.* **7**, 824-830.

Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H. (1999). Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. *Dev. Growth Differ.* **41**, 59-72.

Hamburger, V. and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. 1951. *Dev. Dyn.* **195**, 231-272.

Heimbucher, T., Murko, C., Bajoghli, B., Aghaallaei, N., Huber, A., Stebegg, R., Eberhard, D., Fink, M., Simeone, A. and Czerny, T. (2007). Gbx2 and Otx2 interact with the WD40 domain of Groucho/Tle corepressors. *Mol. Cell. Biol.* **27**, 340-351.

Heine, P., Dohle, E., Bumsted-O'Brien, K., Engelkamp, D. and Schulte, D. (2008). Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development. *Development* **135**, 805-811.

Hisa, T., Spence, S. E., Rachel, R. A., Fujita, M., Nakamura, T., Ward, J. M., Devor-Henneman, D. E., Saiki, Y., Kutsuna, H., Tessarollo, L. et al. (2004). Hematopoietic, angiogenic and eye defects in Meis1 mutant animals. *EMBO J.* **23**, 450-459.

Huang, H., Rastegar, M., Bodner, C., Goh, S. L., Rambaldi, I. and Featherstone, M. (2005). MEIS C termini harbor transcriptional activation domains that respond to cell signaling. *J. Biol. Chem.* **280**, 10119-10127.

Katahira, T., Sato, T., Sugiyama, S., Okafuji, T., Araki, I., Funahashi, J. and Nakamura, H. (2000). Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum. *Mech. Dev.* **91**, 43-52.

Knoepfler, P. S., Bergstrom, D. A., Uetsuki, T., Dac-Korytko, I., Sun, Y. H., Wright, W. E., Tapscott, S. J. and Kamps, M. P. (1999). A conserved motif N-terminal to the DNA-binding domains of myogenic bHLH transcription factors mediates cooperative DNA binding with pbx-Meis1/Prep1. *Nucleic Acids Res.* **27**, 3752-3761.

LaVail, J. H. and Cowan, W. M. (1971). The development of the chick optic tectum. I. Normal morphology and cytoarchitectonic development. *Brain Res.* **28**, 391-419.

Lin, J. C. and Cepko, C. L. (1998). Granule cell raphes and parasagittal domains of Purkinje cells: complementary patterns in the developing chick cerebellum. *J. Neurosci.* **18**, 9342-9353.

Lu, Q. and Kamps, M. P. (1996). Structural determinants within Pbx1 that mediate cooperative DNA binding with pentapeptide-containing Hox proteins: proposal for a model of a Pbx1-Hox-DNA complex. *Mol. Cell. Biol.* **16**, 1632-1640.

Martinez, S., Wassef, M. and Alvarado-Mallart, R. M. (1991). Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene en. *Neuron* **6**, 971-981.

Matsumoto, K., Nishihara, S., Kamimura, M., Shiraishi, T., Otoguro, T., Uehara, M., Maeda, Y., Ogura, K., Lumsden, A. and Ogura, T. (2004). The prepattern transcription factor Irx2, a target of the FGF8/MAP kinase cascade, is involved in cerebellum formation. *Nat. Neurosci.* **7**, 605-612.

Matsunaga, E., Araki, I. and Nakamura, H. (2001). Role of Pax3/7 in the tectum regionalization. *Development* **128**, 4069-4077.
Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martinez, C. and Torres, M. (1999). Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature* **402**, 425-429.
Moens, C. B. and Selleri, L. (2006). Hox cofactors in vertebrate development. *Dev. Biol.* **291**, 193-206.
Okafuji, T., Funahashi, J. and Nakamura, H. (1999). Roles of Pax-2 in initiation of the chick tectal development. *Brain Res. Dev. Brain Res.* **116**, 41-49.
Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037-2048.
Ristoratore, F., Carl, M., Deschet, K., Richard-Parpaillon, L., Boujard, D., Wittbrodt, J., Chourrout, D., Bourrat, F. and Joly, J. S. (1999). The midbrain-hindbrain boundary genetic cascade is activated ectopically in the diencephalon in response to the widespread expression of one of its components, the medaka gene Ol-eng2. *Development* **126**, 3769-3779.
Schulte, D. and Cepko, C. L. (2000). Two homeobox genes define the domain of EphA3 expression in the developing chick retina. *Development* **127**, 5033-5045.
Shen, W. F., Montgomery, J. C., Rozenfeld, S., Moskow, J. J., Lawrence, H. J., Buchberg, A. M. and Largman, C. (1997). AbdB-like Hox proteins stabilize DNA binding by the Meis1 homeodomain proteins. *Mol. Cell. Biol.* **17**, 6448-6458.
Shen, W. F., Krishnan, K., Lawrence, H. J. and Largman, C. (2001). The HOX homeodomain proteins block CBP histone acetyltransferase activity. *Mol. Cell. Biol.* **21**, 7509-7522.
Shim, S., Kim, Y., Shin, J., Kim, J. and Park, S. (2007). Regulation of EphA8 gene expression by TALE homeobox transcription factors during development of the mesencephalon. *Mol. Cell. Biol.* **27**, 1614-1630.
Simeone, A. (2002). Towards the comprehension of genetic mechanisms controlling brain morphogenesis. *Trends Neurosci.* **25**, 119-121.
Sugiyama, S. and Nakamura, H. (2003). The role of Grg4 in tectal laminar formation. *Development* **130**, 451-462.
Sugiyama, S., Funahashi, J. and Nakamura, H. (2000). Antagonizing activity of chick Grg4 against tectum-organizing activity. *Dev. Biol.* **221**, 168-180.
Swift, G. H., Liu, Y., Rose, S. D., Bischof, L. J., Steelman, S., Buchberg, A. M., Wright, C. V. and MacDonald, R. J. (1998). An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2). *Mol. Cell. Biol.* **18**, 5109-5120.
Van Hateren, N., Belsham, A., Randall, V. and Borycki, A. G. (2005). Expression of avian Groucho-related genes (Grgs) during embryonic development. *Gene Expr. Patterns* **5**, 817-823.
Vennemann, A., Agoston, Z. and Schulte, D. (2008). Differential and dose-dependent regulation of gene expression at the mid-hindbrain boundary by Ras-MAP kinase signaling. *Brain Res.* **1206**, 33-43.
Vlachakis, N., Choe, S. K. and Sagerstrom, C. G. (2001). Meis3 synergizes with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* **128**, 1299-1312.
Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat. Rev. Neurosci.* **2**, 99-108.
Zhang, X., Friedman, A., Heaney, S., Purcell, P. and Maas, R. L. (2002). Meis homeoproteins directly regulate Pax6 during vertebrate lens morphogenesis. *Genes Dev.* **16**, 2097-2107.