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Zebrafish Meis functions to stabilize Pbx proteins and regulate hindbrain patterning

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SUMMARY

Homeodomain-containing Hox proteins regulate segmental identity in *Drosophila* in concert with two partners known as Extradenticle (Exd) and Homothorax (Hth). These partners are themselves DNA-binding, homeodomain proteins, and probably function by revealing the intrinsic specificity of Hox proteins. Vertebrate orthologs of Exd and Hth, known as Pbx and Meis (named for a myeloid ecotropic leukemia virus integration site), respectively, are encoded by multigene families and are present in multimeric complexes together with vertebrate Hox proteins. Previous results have demonstrated that the zygotically encoded Pbx4/Lazarus (Lzr) protein is required for segmentation of the zebrafish hindbrain and proper expression and function of Hox genes. We demonstrate that Meis functions in the same pathway as Pbx in zebrafish hindbrain development, as expression of a dominant-negative mutant Meis results in phenotypes that are remarkably similar to that of *lzr* mutants. Surprisingly, expression of Meis protein partially rescues the *lzr*$^{-}$ phenotype. Lzr protein levels are increased in embryos overexpressing Meis and are reduced for *lzr* mutants that cannot bind to Meis. This implies a mechanism whereby Meis rescues *lzr* mutants by stabilizing maternally encoded Lzr. Our results define two functions of Meis during zebrafish hindbrain segmentation: that of a DNA-binding partner of Pbx proteins, and that of a post-transcriptional regulator of Pbx protein levels.

Key words: Hox, Meis, Pbx, Hindbrain, Patterning, Segmentation, Rhombomere, Zebrafish

INTRODUCTION

Homeodomain-containing Hox genes are expressed in distinct, segmentally restricted domains along the A/P (anterior-posterior) axis in both vertebrates and flies (Krumlauf et al., 1993; Wilkinson, 1995). Hox loss-of-function experiments result in homeotic transformations of segment identity, consistent with a model whereby A/P identity is specified by the particular constellation of Hox genes expressed in each segment (Rijli et al., 1993; Gendron-Maguire, 1993; Horan et al., 1995; Studer, 1996). Binding site selection experiments have shown the Hox proteins to possess a rather nonspecific DNA-binding consensus, TAAT (Beachy et al., 1988; Desplan et al., 1988; Hoey and Levine, 1988; Catron et al., 1993; Ekker et al., 1991). Therefore, Hox proteins must specify differences between segments, yet paradoxically, do not seem to contain a high degree of DNA-binding selectivity. This has been explained by the existence of partners that either provide additional specificity themselves or cause conformational changes within the Hox proteins, thereby revealing hidden intrinsic DNA-binding selectivity (Mann, 1995; Mann and Chan, 1996). Mouse Pbx1 and *Drosophila* Extradenticle (Exd) are the prototypes of a growing family of Hox partners, which bind directly to Hox proteins in the nucleus and cooperatively bind DNA.

Genetic evidence from *Drosophila* has shown that Exd functions to facilitate Hox protein function (Peifer and Wieschaus, 1990; Rauskolb et al., 1993). Mutants that lack both maternal and zygotic *exd* have homeotic transformations of denticle bands consistent with loss of function of multiple Hox genes (Peifer and Wieschaus, 1990; Rieckhof et al., 1997). Biochemically, the homeodomains of Exd and its mouse ortholog Pbx1 bind directly to a tryptophan-containing peptide motif (Chan et al., 1996; Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Peltenburg and Murre, 1996), located on Hox proteins. Heterodimers of Exd/Pbx and a Hox protein directly bind to DNA such as the TGATTGAT site within the mouse *Hoxb1* enhancer (Pöpperl et al., 1995) or TGATGGATTG in the Drosophila *labial* lab48/95 enhancer (Ryoo et al., 1999).

Another homeodomain protein, Homothorax (Hth) binds directly to Exd and participates in a heterotrimeric transcription factor complex with Exd and a Hox protein. Mutations in *hth* cause phenotypes resembling loss of multiple Hox genes, demonstrating that Hth is likely to participate in most Hox functions (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al.,

1997). The Hth DNA-binding domain is required for activity as a single point mutation within the homeodomain renders it nonfunctional in ectopic expression assays (Ryoo et al., 1999). The Hth MH domain binds directly to Exd and facilitates cytoplasmic to nuclear import of the Meis-Exd complex (Abu-Shaar et al., 1999; Aspland and White, 1997; Berthelsen et al., 1999; Jaw et al., 2000; Rieckhof et al., 1997).

Murine homologs of *Hth*, known as Meis genes, were discovered on the basis of an integration site for an ecotropic murine leukemia virus, providing a connection between these genes and the regulation of cell proliferation (Moskow et al., 1995). At least four independent subfamilies of Meis genes, *Meis1*, *Meis2*, *Meis3* and *Prep1*, have been identified in mouse, human and frog (Moskow et al., 1995; Nakamura et al., 1996; Salzberg et al., 1999). Each Meis protein can bind directly to Pbx proteins implying that, like Hth, their function may be to participate in Pbx/Hox transcription complexes (Chang et al., 1997; Jacobs et al., 1999; Knoepfler et al., 1997). Consistent with this hypothesis, transcription of murine *Hoxb2* within hindbrain rhombomere 4 requires a Meis-binding element as well as a Pbx-Hox element, indicating that Meis proteins are requisite components of the Pbx-Hox complex (Maconochie, et al., 1997; Ferretti et al., 2000; Jacobs et al., 1999). Yet the transcription of mouse *Hoxb1* in rhombomere 4 is not dependent on its Meis enhancer element, indicating that not all Pbx targets require the DNA-binding activity of Meis partners (Ferretti et al., 2000).

Genetic analysis in the zebrafish has identified *lazarus* (*lzr*, also known as *pbx4*), a Pbx family gene that is expressed maternally and zygotically and is required globally for Hox gene function along the A/P axis (Pöpperl et al., 2000). In the developing hindbrain of *lzr* mutant embryos, where Hox gene expression patterns normally correspond with the boundaries of segmentally reiterated rhombomeres, segmentation is disrupted. Expression of *krox20*, *hoxb1*, *hoxa2*, *hoxb2* and *distal-less-2* (*dlx2*) are reduced and disorganized in *lzr* mutant embryos. Aberrant jaw cartilage formation and reticulospinal neuron specification in these mutants are indicative of a broad mis-specification of A/P identity both within the hindbrain and in neural crest-derived structures in the head periphery.

We report an analysis of vertebrate Meis protein function. Inhibition of Meis function by expressing dominant-negative forms of the protein mimics the *lzr*$^{-}$ phenotype, supporting the idea that, as in *Drosophila*, these genes function in a common pathway. As both dominant negative mutants lack functional DNA-binding domains, Meis is likely to function as a DNA-bound member of the heterotrimeric Pbx-Hox-Meis complex. Surprisingly, expression of wild-type Meis partially rescues the loss of zygotic Lzr protein, suggesting that Meis proteins have functions that are either partially independent of Lzr, or are dependent upon maternally encoded Lzr. We present evidence for the latter interpretation, as Meis is unable to rescue an embryo lacking both maternally and zygotically derived Lzr. Furthermore, we demonstrate that endogenous Lzr protein levels are regulated post-translationally, with domains of higher protein levels in regions which express high levels of Meis and Hox mRNA. This demonstrates that Meis proteins have two critical functions during vertebrate hindbrain patterning: that of a DNA-bound Hox partner and that of a Pbx-stabilizing activity.

MATERIALS AND METHODS

Cloning of six Meis/Prep homologs from zebrafish

A cDNA pool (kindly provided by A. Lekven) was screened using degenerate oligonucleotides based on the predicted sequence of known Meis, Prep and Homothorax proteins. Degenerate oligonucleotides were designed with aid of BLOCKS and CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) programs (http://blocks.fhcrc.org/) (Rose et al., 1998). Using primers (GCTGGCCCTGATCTTCGARAARTGYGA) and (CGTCGTCGG-GCGTTGATRAACCARTT) and Amplitaq Gold (Perkin Elmer) as a polymerase, we amplified a fragment of approximately 700 nucleotides. This product was subcloned into the TOPO T/A-4 vector (Invitrogen) and 96 resultant colonies were characterized using multiple four-base restriction enzymes (Promega). Forty-seven colonies were chosen for sequencing as harboring unique putative Meis/Prep genes: six encoded *prep1.1*; two encoded *prep1.2*, 12 encoded *meis1.1*; 10 encoded *meis2.2*; one encoded *meis3.1*; six encoded *meis4.1a*; and six encoded *meis4.1b*. *prep1.1* is identical to EST fc13f10. We acquired and sequenced clone fc13f10 (Research Genetics). *meis1.1* is identical to the ESTs fk37c10, fc02e11, fd12a02, fc84h08, fb38b03 and fc58h02. *meis1.1* is also identical to an isolate from a concurrent two-hybrid screen in our laboratory. This two-hybrid clone (library kindly provided by S. Ekker) was chosen for full-length sequence determination. *meis2.2* is identical to EST fj35d06 and is so named to avoid confusion with a non-identical *meis2* homolog (now named *meis2.1* which is itself identical to EST fc58e09). *meis3.1* is identical to an EST fk43b07, and a clone published by Vlachakis and Sagerstrom as *meis3* (Vlachakis et al., 2000). *meis4.1a* is identical to EST fc20c07 and zehl1690. *meis4.1b* is a partial cDNA identical to EST fc03d10. The sequence of all clones reported above have been deposited in GenBank with the following Accession Numbers: *meis1.1*, AF37581; *meis2.2*, AF375872; *meis4.1a*, AF376049; *meis4.1b*, AF382395; *prep1.1*, AF382393; and *prep1.2*, AF38294.

Radiation hybrid panel mapping of *meis1.1* and *meis2.2*

Primers were synthesized to amplify 3′ untranslated regions of *meis1.1* (GGATTACCTGTCACACAGTGGCCC and CATCCTCGT-CTGTCCATTGCAGTC) and *meis2.2* (TACTGGAGGACCAAGAG-TCGACGC and AGAGCGAGCTTCGATGGCGTTAAC). The radiation hybrid panel (kind gift of M. Ekker) was amplified with the following protocol: 100 ng template, 100 nM each primer, 200 μM each dNTP, 2 mM $MgCl_2$, 1.25 units Amplitaq Gold (Perkin-Elmer; Hukriede et al., 1999; Kwok et al., 1998). Forty cycles of PCR were completed: 94°C for 30 seconds; 66.5°C or 64°C for *meis1.1* and *meis2.2* respectively, for 20 seconds; 72°C for 20 seconds. Reactions were carried out in duplicate to confirm results. Results were input into the RHMAPPER software (available at http://mgchd1.nichd.nih.gov:800/zfrh/beta.cgi), which subsequently determined the map positions for both *meis1.1* and *meis2.2*.

Whole-mount RMO44 staining, in situ hybridization and genotyping

In situ hybridization was carried out essentially as described (Prince et al., 1998). Embryos for RMO44 staining were fixed in 2% trichloracetic acid in 0.1 M phosphate buffer pH 7.4 for 2 hours at room temperature. Antibodies specific for neurofilament-M, RMO44 (Zymed), were added to embryos for 4-16 hours. A 1:250 dilution of biotin-conjugated goat-anti mouse or goat-anti rabbit secondary antibodies were added, followed by avidin-biotin-horseradish peroxidase complexes (ABC) detection (Vector Laboratories). To visualize horseradish peroxidase, we incubated embryos with fluorescein-isothiocyanate-conjugated tyramide (NEN) and cleared using 50% glycerol. Genotyping and cartilage staining were performed essentially as described (Pöpperl et al., 2000).

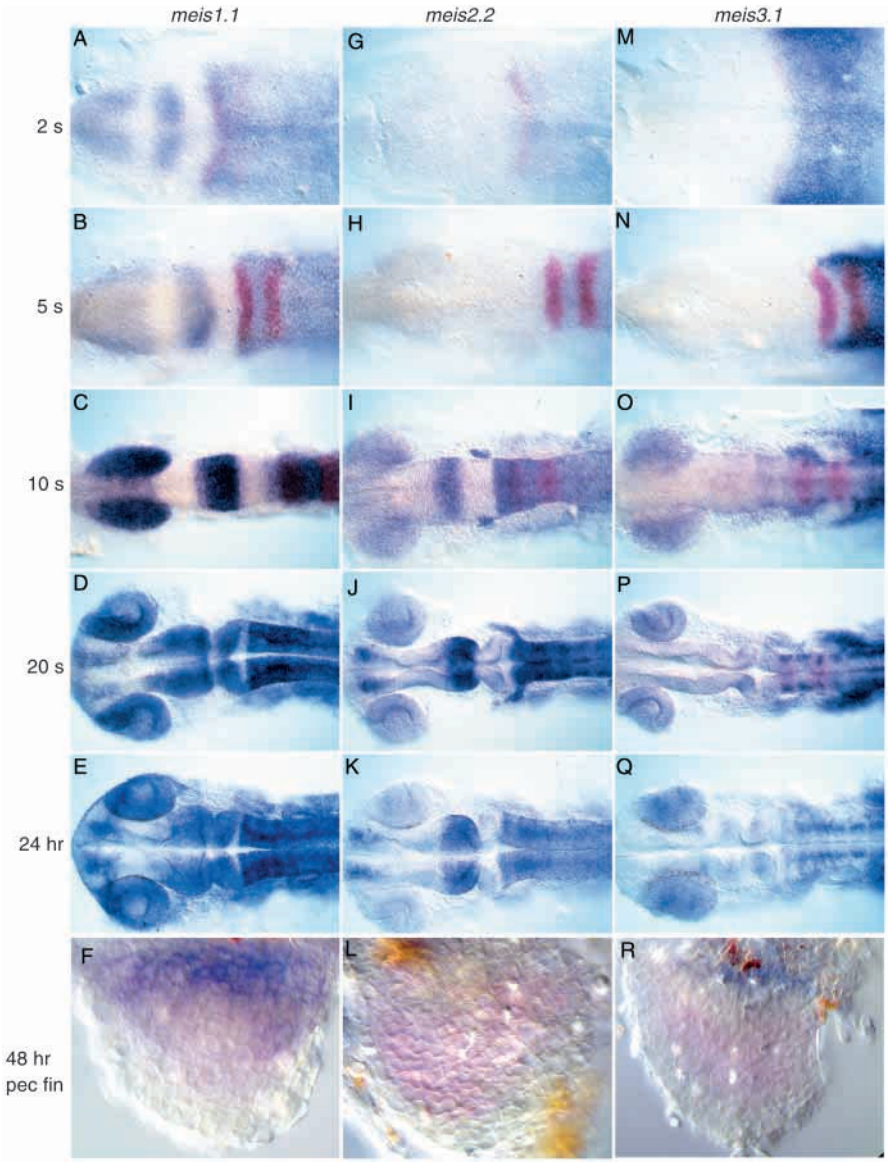

Fig. 1. Expression pattern of *meis1.1*, *meis2.2* and *meis3.1* during segmentation stages of zebrafish development. RNA in situ hybridization of *meis1.1* (A-F), *meis2.2* (G-L) and *meis3.1* (M-R) at the stages shown. *meis* expression is in blue, *krox20* in r3 and r5 is in red. (F,L,R) *meis* expression is shown in pectoral fins at 48 hours (blue) and *tbx5.1* in red is throughout the pectoral fin. Each panel is oriented such that anterior is towards the left.

DNA manipulations

The construct Pbx4/Lzr within pCS2+MT was described previously (Pöpperl et al., 2000). The construct pSP64-*hoxb2* was described previously and was a generous gift from Y. Yan (Yan et al., 1998). A deletion of the predicted PBC-A domain of Lzr was synthesized by amplifying *lzr* cDNA with the primers CACAAGATCTTGAAGCCAGCTCTCTTTCAG and CACAAGATCTTCATAGCCTGCCGTC, and subcloning the *Bgl*II cut product into pCS3-MT. This creates a fusion protein with the Myc-epitope tag (MT) fused in frame with amino acid Leu71, thus starting the Lzr-coding sequence with LKPALFSV. RNAs derived from these constructs encode proteins fused at their N termini to the Myc epitope tag, to ensure identical Kozak consensus sequences surrounding the initiator methionines and to permit subsequent quantification of protein synthesis. Full-length *meis1.1* was created by PCR amplifying with the primers GAGAAGATCTCGATGGCGCAGAGGTACGAAG and GAGAAGATCTTTACATGTAGTGCCACTGTCC, digesting with *Bgl*II and subcloning into pCS3-MT. A deletion of the predicted *meis1.1* homeodomain was constructed by amplifying with primers GAGAAGATCTCGATGGCGCAGAGGTACGAAG and GAGAAGATCTCTAGCCACTGTGCGATGTGTGTCC, digesting with *Bgl*II and subcloning into pCS3-MT. This creates a C terminus at amino acid residue 238, with new C-terminal sequence of SSGGHYSHSG. The DNA-binding mutant of *meis1.1* was constructed by mutation of Asn232 to aspartic acid with inverse PCR and Pfu-turbo-mediated replication (Stratagene) of *meis1.1* cDNA using primers GATGTTCACTTGTTGACCTTCGCTGCAGCAAGAAGAAGAATAGTGCAGC and GCTGCACTATTCTTCTTCTTGCTGCAGCGAAGGTCAACAAGTGAACATC. Constructs were confirmed by automated sequencing (ABI).

RNA synthesis and microinjection

DNAs to be transcribed were purified using Qiagen tip-500 columns. 2 μg of DNA were linearized with the appropriate restriction enzyme for 2 hours at 37°C, and subsequently treated with 100 μg/ml proteinase K (Sigma) and 0.5% SDS at 55°C for 1 hour. RNA synthesis was catalyzed using the SP6-dependent mMessage mMachine kit as recommended by manufacturer (Ambion). RNA was subsequently purified and concentrated by filtration through four YM-50 microcon columns (Amicon). Fertilized embryos were enzymatically dechorionated using a solution of 2 mg/ml proteinase E (Sigma) and subsequent washes with Fish Water (60mg Instant Ocean per liter). Injection concentration for *hoxb2* mRNA was 100-250 ng/μl; for *meis1.1WT* and *meis1.1ΔC* was 100 ng/μl; for GFP mRNA was 100 ng/μl; and for *lzrWT* and *lzrΔN* was 250 ng/μl. Injection bolus was estimated at 250 pl by reticle measurement in a droplet of mineral oil. To confirm that mRNAs did not cause a nonspecific decrease in cellular viability or differentiation, control embryos were also co-injected with a mRNA encoding GFP. High levels of fluorescence were detected in approximately 70% of embryos, regardless of which *meis* or *pbx* RNA was co-injected (data not shown).

Immunoblot analysis

Dechorionated embryos were staged according to somite number and placed in microfuge tubes. Yolk was lysed by placing 20 embryos in 167 μl of homogenization buffer (20 mM Hepes pH 7.5, 10 mM EGTA, 2.5 mM $MgCl_2$, 15 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride), mixing with microfuge pestles and centrifuging at 16,000 ***g*** for 5 minutes at 4°C. The resultant cell pellets were resuspended in of 1× SDS-PAGE sample buffer (2.5 mM EDTA, 2% SDS, 2.8 M β-mercaptoethanol, 10% glycerol, 100 mM Tris-Cl pH 6.0 and 0.01% Bromophenol Blue). Samples were boiled for 5 minutes and run on SDS-polyacrylamide gels (12.5% acrylamide, 0.1% bisacrylamide). Gels were electroblotted onto Immobilon-PVDF membranes (Millipore). Filters were probed with monoclonal 9E10 antibody (a kind gift from J. Cooper) and 1:2000 dilution of

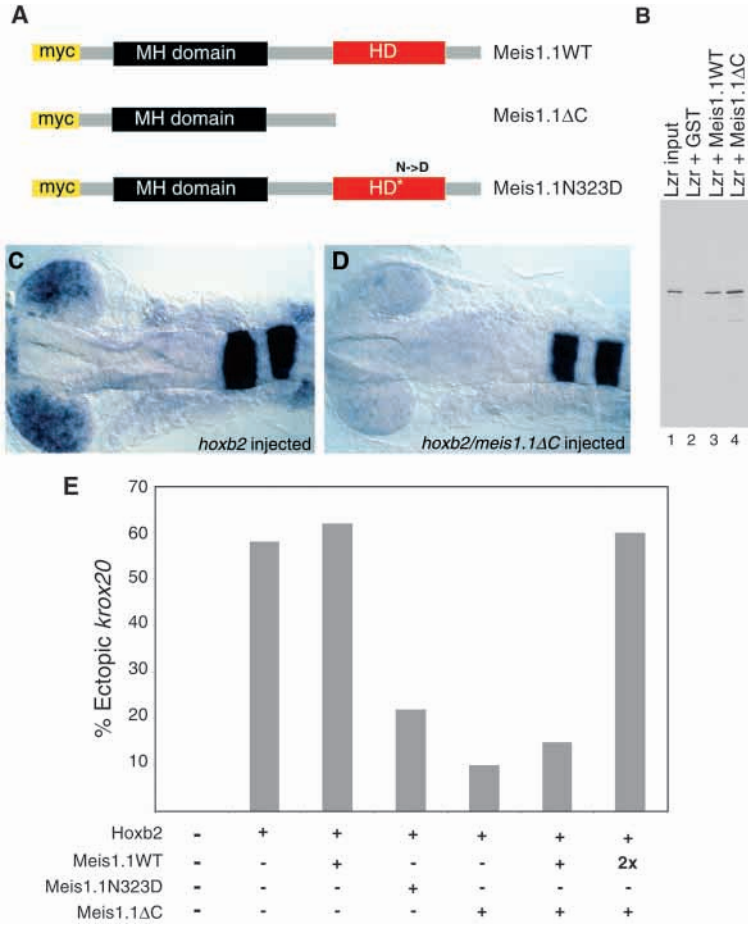

Fig. 2. Expression of dominant-negative Meis1.1 inhibits Hoxb2 function. (A) Meis1.1WT was mutated in two alternative ways to generate forms that can bind Lzr but cannot bind DNA, Meis1.1ΔC and Meis1.1N323D. (B) To confirm that deletion of the Meis C terminus does not inhibit Pbx binding and to demonstrate that Meis1.1 can bind Lzr, the proteins were synthesized in vitro and assayed for ability to bind one another. Lane 1 contains 5% of the input Lzr, while lanes 2, 3 and 4 display proteins that bind to GST (lane 2), GST-Meis1.1 (lane 3), or GST-Meis1.1ΔC (lane 4). Binding between Lzr and Meis proteins varies from 10%-30% depending on the stringency of the wash conditions (data not shown). (C,D) *hoxb2* overexpression results in ectopic expression of *krox20* within the retina of approximately 60% of injected embryos. (C) 60% embryos have expression of retinal *krox20* shown here at 20 somites. (D) 90% of embryos injected with *hoxb2* and *meis1.1ΔC* contain undetectable levels of ectopic *krox20*. (E) Quantification of embryos expressing ectopic *krox20* in the eye after injection of *hoxb2* and dominant-negative *meis* RNAs.

horseradish peroxidase-conjugated sheep-anti-mouse Fab fragment secondary (Amersham). Proteins were visualized using enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's recommendations.

Fusion protein synthesis, in vitro translation and binding analysis

GST-fusion protein synthesis and binding analysis was done essentially as described (Waskiewicz et al., 1997). Translated proteins were mixed with 100 ng of glutathione-S-transferase (GST) fusion protein and incubated in Triton-immunoprecipitation buffer (10 mM Hepes pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton, 1 mM PMSF, 1 μg/ml Aprotinin) for 2-12 hours. Unbound proteins were removed with two consecutive washes with 150 mM NaCl triton-containing buffer, two washes with 225 mM NaCl lysis buffer, and two washes with 300 mM NaCl lysis buffer. Bound proteins were separated by SDS-PAGE and visualized by autoradiography. If we washed only with 150 mM NaCl, we consistently saw a small amount (approximately 1%) of Lzr protein bound to GST alone, whereas binding to GST-Meis was approximately 30%.

9E10 and αpan-Pbx immunostaining

Embryos were fixed 3 hours with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized in acetone for 7 minutes at –20°C and blocked in PBS with 0.05% bovine serum albumin and 10% goat serum. 9E10 (Covance) and α-pan-Pbx (a kind gift from H. Pöpperl) were diluted 1:250 in blocking solution and incubated with embryos overnight at 4°C. Embryos were washed six times with PBS containing 1% DMSO, 0.5% Triton X-100. Primary antibodies were detected using 1:300 goat-anti-mouse-FITC and 1:250 goat-anti-rabbit-Alexa 594 (Molecular Probes) overnight at 4°C. Embryos were washed as above, cleared in glycerol and photographed using a Leica D5M confocal microscope.

RESULTS

Zebrafish Meis/Prep genes are expressed in the same regions of the embryo which express Hox and Pbx genes

Using two pairs of degenerate oligos and cDNA derived from 4- to 24-hour-old embryos, we isolated six zebrafish Meis gene products. Sequence analysis demonstrated that zebrafish contain at least one *Meis1* homolog (*meis1.1*), two *Meis2* homologs (*meis2.1* and *meis2.2* – *meis2.2* is described in this work, while *meis2.1* has been cloned previously and its sequence deposited in GenBank), one *Meis3* homolog (*meis3.1*, which has been described earlier (Vlachakis et al., 2000)), two *prep* homologs (*prep1.1* and *prep1.2*), and one member with two alternative splice variants of a novel Meis gene (named *meis4.1a* and *meis4.1b*). Alignment between zebrafish Meis/Prep genes and *Drosophila Hth* and Mouse Meis genes revealed similarities in the range of 48-99% and the presence of both previously identified functional domains: the MH domain, which binds to Pbx proteins, and the homeodomain, which binds DNA.

We examined the expression patterns of *meis1.1*, *meis2.2*, *meis3.1*, *prep1.1* and *prep1.2* between 10 and 24 hours of zebrafish development. Although the two *prep* genes are expressed ubiquitously during this period (data not shown), the three *meis* genes exhibit restricted patterns of expression, including domains that correspond closely with domains of Pbx and Hox function within the hindbrain. Interestingly, the *meis* genes are also expressed in anteroposterior domains outside the spinal cord and hindbrain, which might imply function in complexes without Hox proteins (Fig. 1).

At the two-somite stage (2 s; Kimmel et al., 1995), we detect *meis1.1* in three distinct domains along the anteroposterior axis: (1) within the presumptive forebrain; (2) the anterior midbrain; and (3) in the hindbrain/spinal cord from

rhombomere 3 (r3) posterior (Fig. 1A). The pattern of expression at 5 s is largely unchanged, again defining three distinct anteroposterior domains in the forebrain, midbrain and hindbrain (Fig. 1B). By 10 s, *meis1.1* expression within the hindbrain expands anteriorly to include r2, although the expression in r2 is weaker (Fig. 1C). At 10 s, expression in the forebrain is concentrated largely in the developing eye fields, although signal in the presumptive telencephalon persists. At 20 s, *meis1.1* expression broadens, with highest levels in hindbrain rhombomeres r2, r3, r4 and the developing eye (Fig. 1D). In 24-hour-old embryos, *meis1.1* is expressed broadly in the neural tube, with high levels of expression within the hindbrain, the retina and the just anterior to the midbrain-hindbrain boundary (MHB; Fig. 1E).

At early segmentation stages, at 2 s, *meis2.2* is expressed in the forebrain, in hindbrain from r4 posterior, and in the spinal cord (Fig. 1G). By 5 s, hindbrain expression expands to the anterior to include r2 (Fig. 1H). By 10 s, *meis2.2* expression delineates both presumptive eye fields, the forebrain, the presumptive tectum, two patches lateral to the MHB, and within the posterior cental nervous system from r2 in the hindbrain extending to the throughout the spinal cord (Fig. 1I). The expression in the midbrain region is anterior to and adjacent to the domain of *pax2.1*, which demarcates the MHB (data not shown). At 20 s and 24 hours, expression within the forebrain resolves to two bilaterally symmetric regions within the ventral telencephalon (Fig. 1J), the regions that also express the zebrafish *distall-less* homolog *dlx2* (data not shown). *meis2.2* expression continues at 24 hours in the tectum, hindbrain and spinal cord (Fig. 1K).

At 2 s, *meis3.1* is expressed only in the posterior region of the embryo, up to the r3/r4 boundary (Fig. 1M). This expression domain retracts to the posterior, so that by 5 s it marks the boundary between r5 and r6 (Fig. 1N), and by 10 s it is no longer expressed uniformly in the hindbrain (Fig. 1O). By 20 s and 24 hours, *meis3.1* expression is reduced in the hindbrain, although it is expressed in the spinal cord and somites at high levels (Fig. 1P,Q; data not shown). Within the hindbrain, *meis3.1* expression delineates a population of lateral cells in the center of r3, r4, r5 and r6.

We examined Meis expression in the zebrafish pectoral fin bud, as Meis genes have been implicated in the proximalization of the limb bud in the chick embryo (Capdevila et al., 1999; Mercader et al., 1999; Mercader et al., 2000). *meis1.1* is expressed throughout the developing fin bud at 25 hours (data not shown) and subsequently is restricted proximally (Fig. 1F). *meis3.1* has weak staining in the most proximal region of the fin bud at 48 hours (Fig. 1R). This defines zebrafish *meis1.1* as the most likely candidate for proximodistal patterning within the pectoral fin bud.

Zebrafish *meis2.1* was mapped by the zebrafish EST consortium to LG20. To map the position of *meis1.1* and *meis2.2* genes within the genome, we designed primers that amplify the 3′ untranslated region of each gene from zebrafish but not mouse genomic DNA. Using the LN54-Ekker panel of somatic cell hybrids, *meis1.1* primers generated a map position with a LOD score of 8.2, using RHMAPPER software, on LG13 between EST fb83c11 and Z marker Z13682 (data not shown). Using *meis2.2*-specific primers, positive signal indicated a map position on LG17 at the same approximate position as EST fb98h04 with a LOD score of 16.0 (data not shown).

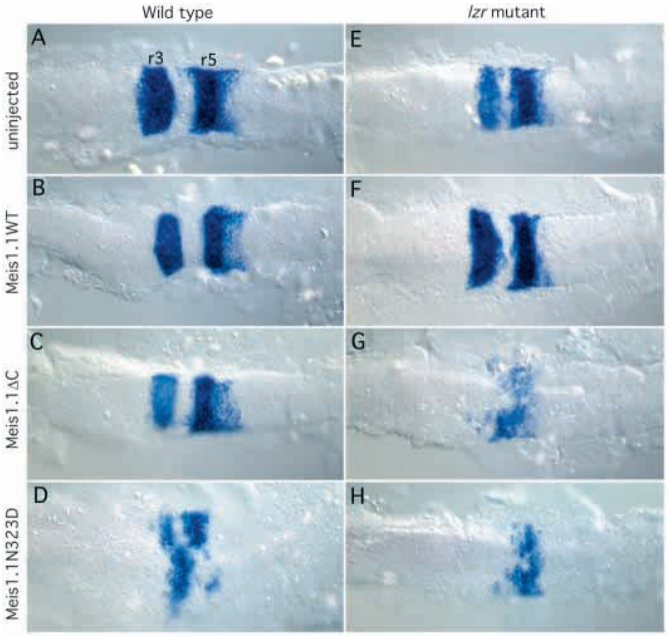

Fig. 3. Inhibition of Meis function results in reduced *krox20* expression in both wild-type and *lzr* mutant embryos, while overexpression of *meis1.1WT* partially rescues the *lzr* mutant phenotype. Each panel is oriented with anterior towards the left. (A-D) *krox20* expression (in r3 and r5) in wild-type embryos at 8-10 somites injected with mRNAs shown on the left. Note the decrease in *krox20* expression caused by Meis1.1ΔC and Meis1.1N323D in C,D, as compared with wild type in A. (E-H) *krox20* expression in *lzr*$^{-}$ embryos injected as shown. Note the increase in *krox20* expression caused by expressing Meis1.1WT, by comparing E,F. Genotypes of embryos were determined subsequent to photography using PCR-mediated dHPLC (Pöpperl et al., 2000).

Dominant-negative Meis blocks Hox function

In *Drosophila*, Hth is required for Exd nuclear localization and also participates in the DNA-bound Hox/Exd/Hth complex (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Chang et al., 1997; Jacobs et al., 1999; Ryoo et al., 1999). To test whether vertebrate Meis is similarly required in those developmental processes that are regulated by Pbx proteins, we determined the effects of expression of dominant-negative forms of Meis1.1 on zebrafish development. We tested two dominant-negative Meis mRNAs, one in which the DNA-binding homeodomain is deleted (*meis1.1ΔC*), the other in which a highly conserved residue within the homeodomain is mutated (*meis1.1N323D*; Fig. 2A). As dominant-negative proteins must bind to the same proteins as wild type, we confirmed that deletion of the C terminus of Meis1.1 did not interfere with its Pbx-binding activity. Both GST-Meis1.1WT and GST-Meis1.1ΔC bound efficiently to in vitro translated [^{35}S]-labeled Lzr (Fig. 2B), demonstrating that zebrafish Meis proteins are probably Pbx partners and that deletion of the homeodomain is a potential mechanism of creating a Pbx-binding, dominant-negative mutant.

Using these dominant-negative mutants, we first tested directly whether Meis is required for Hox gene function. In zebrafish, ectopic expression of *hoxb2* leads to activation of *krox20* expression in the developing eye (Yan et al., 1998), and we have shown that this effect is dependent on *lzr* (Pöpperl et

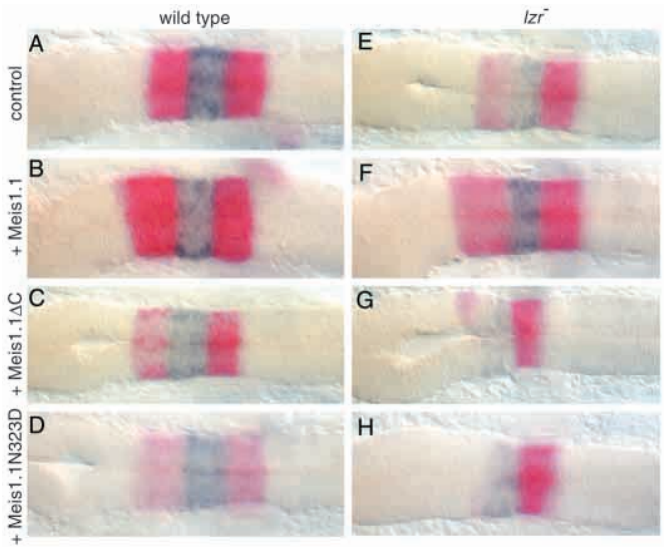

Fig. 4. Meis activity is required for normal expression of *hoxb1a*, while ectopic *meis1.1* increases *hoxb1a* expression in *lzr* mutants. Each panel is oriented with anterior towards the left. (A-D) *krox20* expression (red in r3 and r5) and *hoxb1a* expression (blue in r4) in wild-type embryos at 18-20 somites injected with mRNAs shown on the left. Note the slight the decrease in *hoxb1a* expression caused by either Meis mutant in C,D. (E-H) *krox20*, and *hoxb1a* expression in *lzr*$^{-}$ embryos which were injected with mRNAs as shown. Note the increased expression of *hoxb1a* in *lzr* mutants injected with Meis1.1WT (compare E with F). Genotypes of embryos were confirmed by PCR-mediated dHPLC (Pöpperl et al., 2000).

al., 2000). To test the role of Meis proteins in this induction, we co-injected Myc epitope-tagged wild-type or similarly tagged mutant *meis1.1* mRNAs with mRNA encoding Hoxb2 protein. Injection of *hoxb2* RNA results in 58% (*n*=111) of embryos expressing *krox20* within either left or right eye (Fig. 2C), while 0% (*n*=93) of the control uninjected embryos ectopically expressed *krox20*. *meis1.1WT* (wild type) did not cause a significant increase in *krox20*, with expression in only 62% (*n*=267) of embryos. Co-injection of *meis1.1ΔC* with *hoxb2* mRNA blocked induction of eye-specific *krox20*, with expression in only 10% (*n*=426) of embryos (Fig. 2D). Co-injection of *meis1.1N323D* had a similar effect on *hoxb2* function, with 22% (*n*=238) of embryos expressing eye-specific *krox20*.

To establish whether Meis1.1ΔC effects were caused by inhibition of the endogenous Meis protein function, *meis1.1WT* was also co-injected with same concentration of *meis1.1ΔC* mRNA as used above. Injection of equal molar amounts of *meis1.1WT* and *meis1.1ΔC* together with *hoxb2* resulted in 15% (*n*=117) of embryos expressing *krox20* in the eye. Injection of two parts *meis1.1WT* with one part *meis1.1ΔC*, plus *hoxb2* mRNA lead to 60% (*n*=313) of embryos with ectopic *krox20* expression, indicating complete rescue of the *meis1.1ΔC*-induced inhibition of *hoxb2* function (Fig. 2E). This titration effect is a strong indication that the substrates and partners of Meis1.1ΔC are identical to those of Meis1.1WT.

Reducing *meis* activity mimics *lazarus* segmentation phenotypes

If Meis is a requisite member of the Pbx-Hox-Meis complex; embryos expressing a dominant-negative Meis are expected to mimic loss of Pbx function. In zebrafish, Lzr is the primary Pbx protein that mediates Hox function during the first 24 hours of development (Pöpperl et al., 2000). At 6 s, *lzr* mutants express reduced levels of *krox20* in a disorganized pattern within r3 and r5 (compare Fig. 3A with 3E). To determine whether Meis function is necessary for normal *krox20* expression, one-cell embryos were injected with either wild-type or dominant-negative *meis1.1*. Injected embryos were grown until 6 s and examined for expression level and pattern of *krox20*. 83-88% (*n*=85 and 60) of embryos expressing either *meis1.1ΔC* or *meis1.1N323D* had reduced levels of *krox20* in comparison with control uninjected embryos or to embryos injected with wild type *meis1.1* mRNA (Fig. 3A-D). The pattern and level of *krox20* expression in these dominant-negative *meis1.1*-mutant-expressing embryos is strikingly similar to that seen in *lzr* mutants (Fig. 3E) Again, the phenotypes that are caused by *meis1.1ΔC* are likely to be those of a dominant negative mutant as we are able to suppress their effects by co-injecting twofold more wild-type *meis* mRNA (data not shown).

lzr mutants have reduced levels of Hox gene expression, probably caused by abrogation of auto- and para-regulatory loops (Pöpperl et al., 1995; Maconochie et al., 1997; Studer et al., 1998). For example, *hoxb1* expression, which in the mouse is dependent on regulatory input from itself and *Hoxa1* (Studer et al., 1998), is reduced in *lzr* mutants. To determine whether dominant-negative Meis protein could interfere with zebrafish *hoxb1a* regulation, *meis1.1ΔC*- and *meisN323D*-injected embryos were grown to 18-20 somites and examined for expression of *krox20* (expressed in r3 and r5) and *hoxb1a* (which is expressed in r4). 40% (*n*=47) of embryos injected with *meis1.1ΔC* showed a slight downregulation of *hoxb1a* (Fig. 4A-C), although in no case was the phenotype as severe as that seen in *lzr* mutant embryos. Therefore, Meis is required for maximal *hoxb1a* expression, implying that it plays a role in regulating *hoxb1a* transcription.

Dominant-negative Meis1.1 enhances the *lzr*$^{-}$ phenotype

Our observation that expression of a dominant-negative Meis protein can mimic the *lzr* mutant phenotype suggests that Meis functions in a common pathway with Lzr to mediate Hox gene function in patterning the zebrafish hindbrain. We were surprised to observe, therefore, that inhibition of Meis function caused an enhancement of the *lzr* mutant phenotype. 73% of *meis1.1ΔC*- and 60% of *meis1.1N323D*-expressing *lzr*$^{-/-}$ embryos expressed *krox20* at levels even lower than uninjected *lzr* mutants (Fig. 3G,H). Expression in r3 was completely abrogated, while r5 was significantly more disordered. At 18-20 s, expression of *krox20* remained reduced, especially in r3, in the *meis1.1ΔC*- and *meis1.1N323D*-injected *lzr* mutants (Fig. 4G,H). Expression of *hoxb1a* was also reduced in *lzr*$^{-/-}$ embryos injected with *meis1.1ΔC* or *meis1.1N323D*, indicating that it too is a target of Meis protein regulation (Fig. 4G,H). The observation that inhibition of Meis function enhances the *lzr*$^{-}$ phenotype suggests that Meis has functions that are independent of zygotically derived Lzr. Meis may interact with other Pbx proteins and/or with maternally encoded Lzr protein. We favor the latter interpretation, as phenotypes resulting from the loss of maternal and zygotic Lzr function resemble the

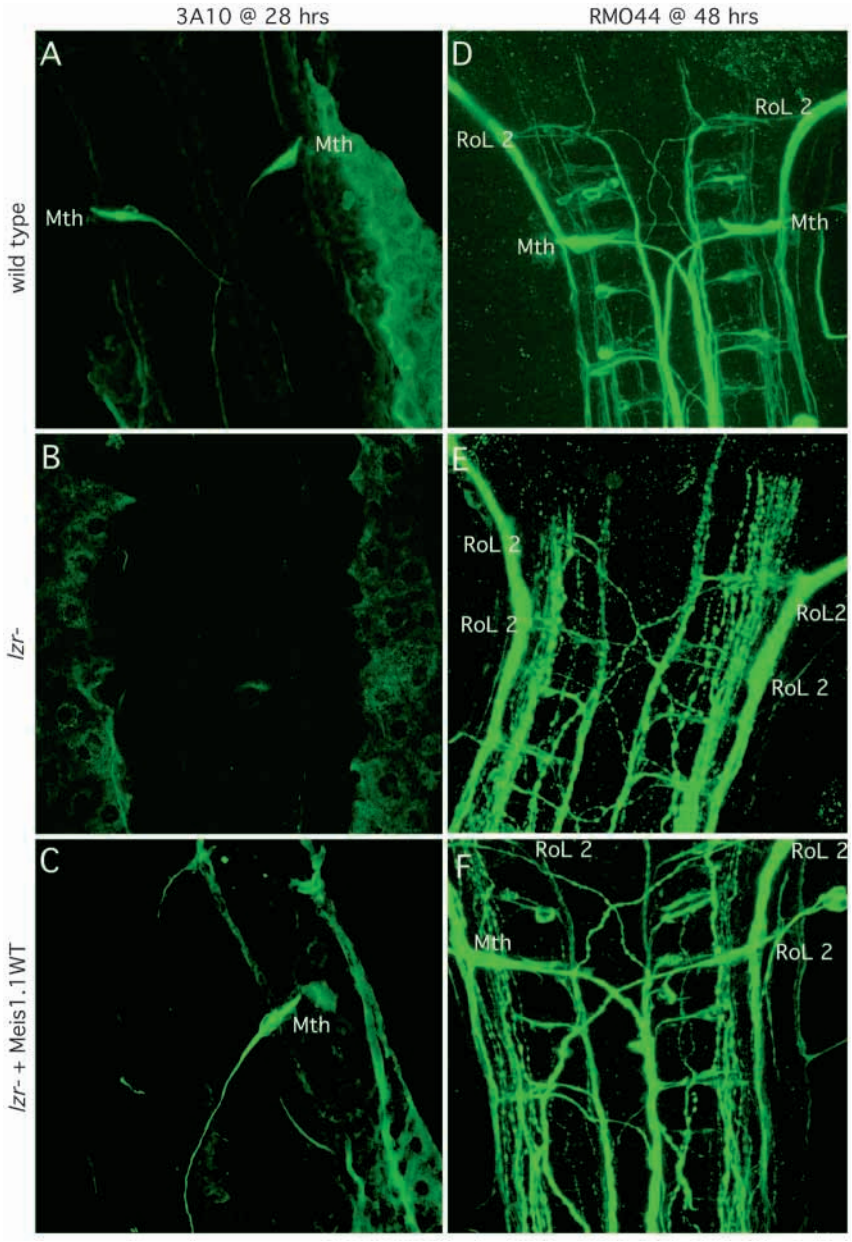

Fig. 5. Expression of *meis1.1WT* in *lzr* mutants increases the percentage of embryos with correctly specified Mauthner (labeled Mth) neurons. Each panel is oriented with anterior towards the top. (A-C) Either wild-type or *lzr*$^{-}$ embryos were injected with Meis1.1WT mRNA as shown on left. 28 hour embryos were stained with 3A10, an antibody which recognizes the Mth neuron and its axon. (D-F) 48 hour embryos, as described on the left were stained with RMO44, an antibody that recognizes the identifiable primary reticulospinal neurons of zebrafish. Neuronal cell bodies are identified and labeled as shown (RoL 2, which is normally found in r2 of a wild-type embryo; Mth, which is in r4).

phenotype of *lzr* mutants expressing *meis1.1ΔC* (A. J. W and C. B. M., unpublished).

Meis1.1 partially rescues the *lzr*$^{-}$ phenotype

Co-injection of full-length *meis1.1* mRNA (*meis1.1WT*) blocks the effects of the dominant-negative protein (Fig. 5). Interestingly, *meis1.1WT* injection into *lzr* mutant embryos caused a dramatic rescue of their visible phenotype, in terms of otic vesicle shape and rhombomere boundary formation (data not shown). Uninjected control clutches from a *lzr*$^{+/-}$ intercross contained 23% (*n*=195) mutant embryos by morphology, while Meis1.1WT-injected clutches had 15% (*n*=328) phenotypically mutant embryos. A larger than Mendelian number of Meis1.1WT-injected *lzr*$^{+/-}$ intercross embryos also displayed normal levels and organization of *krox20* at 6 s (compare Fig. 3E with 3F). To determine if any of these phenotypically normal embryos were genotypically mutant, each embryo was initially photographed and subsequently genotyped. Six out of 17 phenotypically normal embryos were genotypically *lzr* mutant, indicating that Meis1.1WT can rescue the *lzr*$^{-}$ phenotype. Although less consistent, this rescue is quite similar to that of *lzr* mutants rescued by expressing wild-type Lzr protein. The effects at 18-20 somites, in terms of increased *hoxb1a*, were equally consistent and striking (compare Fig. 4E with 4F).

We also observed a partial rescue of other phenotypes associated with loss of *lzr* function in embryos expressing wild-type Meis1.1. The primary reticulospinal neurons are a population of segmentally reiterated, individually identifiable neurons in the hindbrain that are sensitive to perturbations in Hox gene expression (Fig. 5) (Alexandre et al., 1996). In *lzr* mutants, these neurons are variably misspecified, such that the most prominent member of this class, the Mauthner (Mth) neuron, characteristic of r4, is rarely present, and is replaced by a neuron with r2 characteristics, RoL 2 (Fig. 5D,E; Pöpperl et al., 2000). The Mth neuron can be distinguished both by its large cell size and its axon, which projects in the contralateral median logitudinal fascicle (Kimmel et al., 1982). To test whether rescue of gene expression levels in *lzr* mutants expressing Meis1.1WT correlated with rescue of segment-specific cell type specification within the hindbrain, injected embryos were grown to 48 hours and examined for presence or absence of Mth neurons. The Mth neuron alone can be recognized using the monoclonal antibody 3A10 and staining at 28 hours (Fig5 A-C). Alternatively, the reticulospinal neurons can be recognized with RMO44, a monoclonal directed against Neurofilament M. To distinguish Mth neuron specification in a quantitative way, an index was defined as number of Mth neurons per embryo. Wild-type embryos have a Mth index of 2.0 (Kimmel et al., 1982). Quantification of *lzr* mutant embryos yielded a Mth index of 0.44 cells/embryo (*n*=41; Fig. 5B,E). By contrast, embryos injected with *meis1.1WT* RNA showed a demonstrable difference (P<0.001)

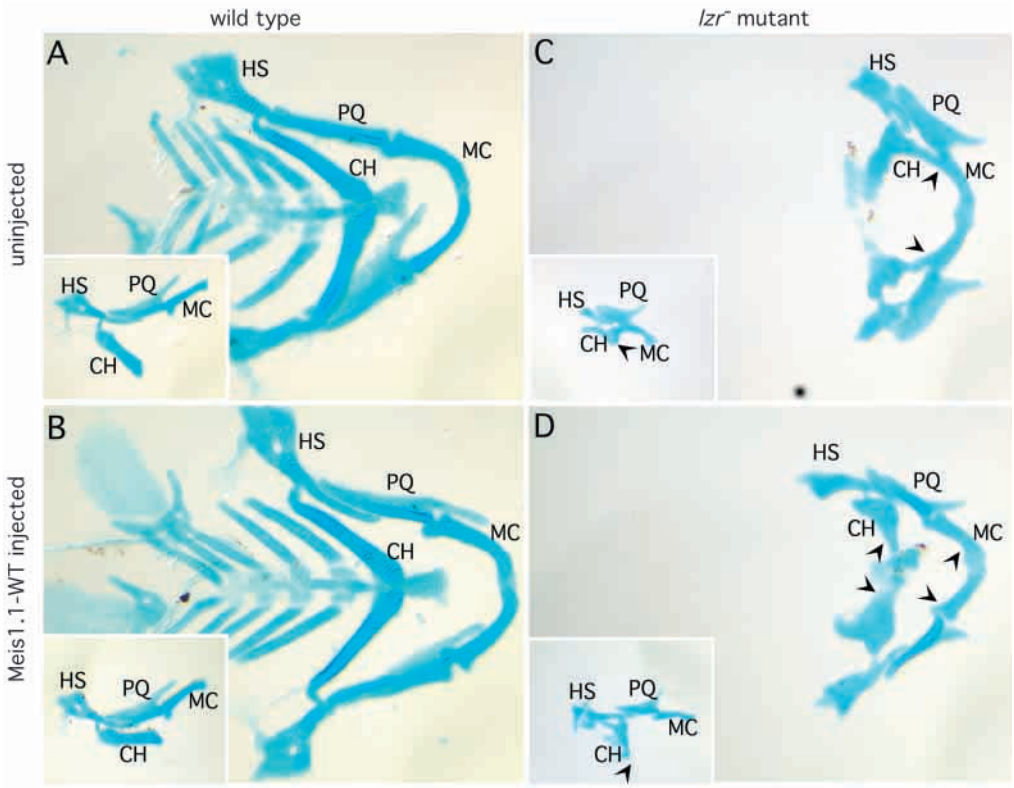

Fig. 6. Expression of *meis1.1WT* in *lzr* mutants partially rescues jaw cartilage phenotype. (A) Alcian Green-stained wild-type embryo contains normal articulation of Meckels (M), palatoquadrate (PQ), hyosymplectic (HS) and ceratohyal (CH) cartilage elements. (B) Expression of *meis1.1WT* does not affect this pattern of jaw cartilages. (C) *lzr* mutants lack caudal cartilages, and have a prominent fusion between the second-arch derived ceratohyal (CH) and first arch-derived Meckels (M) cartilage (note arrowheads demarcating fusion). (D) *lzr* mutants expressing *meis1.1WT* have separate ceratohyal and Meckels (note the arrowheads corresponding to cartilages that are fused in panel C but not in these embryos), but do not have caudal cartilages.

with a Mth index of 0.97 (n=33; Fig. 5C,F). The general organization of the hindbrain reticulospinal neurons was also partially rescued in *lzr* mutants expressing Meis1.1WT protein (compare Fig. 5E with 5F). The reticulospinal neuron rescue is evidence that Pbx function has been restored in terms of cell type specification within r4.

lzr mutants exhibit anterior-posterior fusions of neural crest-derived jaw and jaw-support cartilages in the first and second pharyngeal arches (compare Fig. 6A with 6C), fusing Meckels (M) with ceratohyal (CH) and palatoquadrate (PQ) to hyosymplectic (HS) cartilage elements. Dorsal-ventral fusions are also common (Pöpperl et al., 2000). Consistent with the ability of *meis1.1* to rescue other aspects of the *lzr*$^{-}$ phenotype, we observed a robust rescue of jaw fusions in *lzr*$^{-}$ embryos ectopically expressing *meis1.1* (Fig. 6D). Most notably, the ventral elements of the first and second arches, the M and CH cartilages are no longer fused in 15 of 17 embryos. The rescue of the *lzr*$^{-}$ jaw phenotype is only partial – first and second arch cartilages are still reduced and the more posterior gill cartilages are still missing – however the anteroposterior and dorsoventral fusions are rescued to a large degree and the jaw cartilages are more easily distinguishable.

Stabilization of Lzr by Meis1.1

The observed rescue of *lzr* mutants by ectopic Meis1.1WT could occur because of two possible mechanisms: (1) Meis may directly and independently activate Pbx targets, which has not previously been shown; or (2) Meis may increase the activity of Pbx proteins present in *lzr* mutant embryos. Such proteins exist, both in the form of maternally encoded Lzr and of another Pbx family member expressed during the first 24 hours of embryogenesis (A. J. W., C. B. M. and H. Pöpperl, unpublished). Ectopic Meis could increase this Pbx activity by a number of possible mechanisms. In the presence of extra Meis, Pbx protein could be more efficiently transported into the nucleus, could bind DNA more rapidly and stably, or could be protected from degradation. We tested these possibilities by assaying levels of Lzr protein in embryos injected with *meis* mRNA.

To examine the effect on Lzr protein in Meis1.1WT expressing embryos, we first injected Lzr RNA into embryos, ensuring equal RNA levels, and subsequently injected either *gfp* or *meis1.1WT* RNA. All constructs in this series of experiments were expressed as N-terminal fusions with the Myc epitope, to allow detection and to ensure equal translation initiation. Western blot analysis of embryos from four separate experiments demonstrate that Lzr is present in increased amounts, approximately three- to eightfold, in embryos co-expressing Meis1.1WT in comparison to control embryos (Fig. 7A, compare lanes 2 and 3; also compare lanes 5 and 6; Fig. 7B, lanes 1 and 5; Fig. 7C lanes 1 and 3). Interestingly, this effect is reciprocal: Meis protein is similarly increased in the presence of full-length Lzr (Fig. 7B, compare lanes 2 and 5).

The reciprocal stabilization of ectopically expressed Meis and Lzr proteins depends on their abilities to interact with one another. Meis1.1ΔN and LzrΔN are mutants in which the N-terminal MH and PBC domains, respectively, are deleted. The MH domain of Meis proteins and the PBC domain of Pbx proteins normally mediate the interaction between Meis and Pbx proteins, and are essential for their normal functions (Chang et al., 1997; Jacobs et al., 1999; Knoepfler et al., 1997). Expression of Meis1.1ΔN does not stabilize co-injected Lzr protein (compare lanes 1 and 7 in Fig. 7B). Similarly, expression of LzrΔN does not appreciably stabilize co-injected

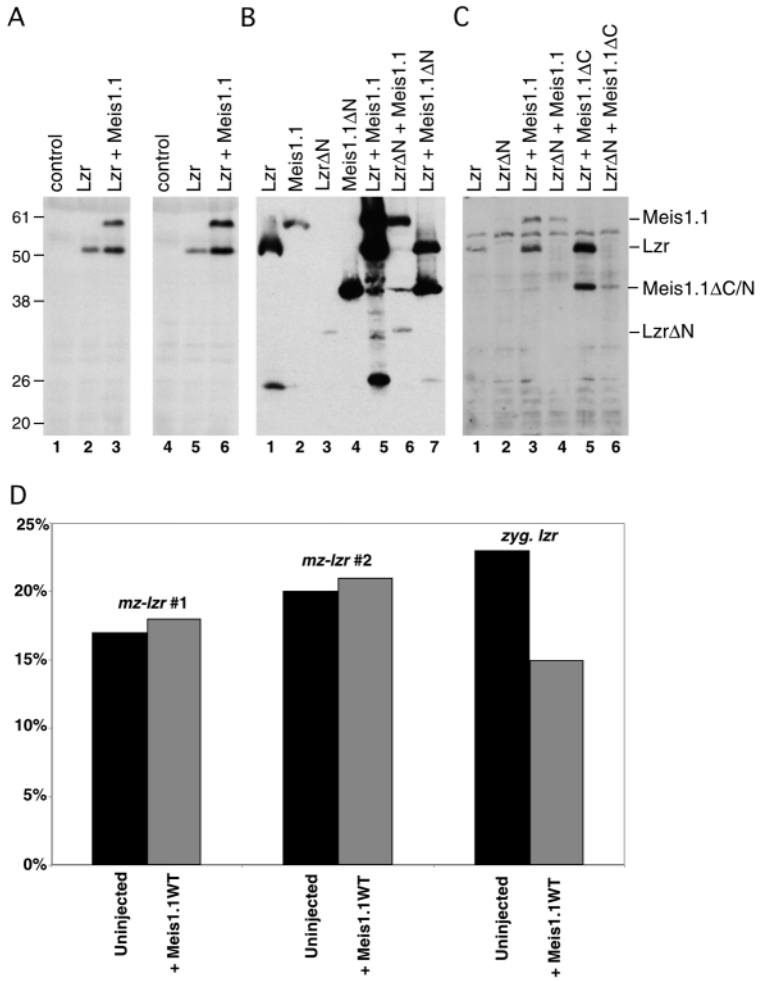

Fig. 7. Bidirectional stabilization of Meis1.1 and Lzr. (A-C) Embryos were injected with mRNAs and lysed to isolate proteins as shown. All proteins were expressed as N-terminal fusions with the Myc epitope to ensure equal rates of translation initiation and to detect proteins by immunoblot. (A) By comparing Lzr protein levels in lane 2 and 5 (Lzr alone) with levels in lane 3 and 6 (Lzr + Meis1.1WT), co-expression of Meis1.1WT increases detectable Lzr by 3.4-fold. (B) Meis1.1 levels are increased eightfold by co-expressing Lzr, in comparison to the non-binding Meis1.1ΔN (compare lanes 1, 5, and 7). Meis levels are increased similarly by co-expressed Lzr protein, but not significantly by LzrΔN (compare lanes 2, 5, and 6). (C) Meis1.1ΔC is stabilized by Lzr and can stabilize Lzr protein (compare lanes 1, 5, and 6). Apparent molecular weights in kDa are labeled and the anticipated position of each protein is indicated. (D) Expression of Meis1.1WT does not decrease the percentage of embryos from a maternal-zygotic *lzr* (*mz-lzr*) mutant clutch with abnormal *krox20*. However, the same Meis1.1WT mRNA injected into zygotic *lzr* does reduce the percentage of abnormal *krox20*, indicating the ability to rescue the phenotype associated with zygotic loss-of-function.

Meis protein (compare lanes 2 and 6 in Fig. 7B). We found that LzrΔN protein is itself highly unstable in embryos, even though it can be translated efficiently in cell-free systems (data not shown). This is consistent with the possibility that Meis binding is essential for Pbx stability.

The in vivo effects of Meis protein on Lzr stability are independent of its ability to bind DNA, as Meis1.1ΔC also increases levels of Lzr (Fig. 7C, compare lanes 1 and 5). We note, however, that Meis1.1ΔC does not rescue the *lzr*$^-$ phenotype, indicating that stabilization of Pbx protein by itself is not sufficient for rescue, and suggesting that stabilized Pbx protein requires Meis in a DNA bound complex for its activity.

These data imply that Meis1.1 may rescue the *lzr*$^-$ phenotype by stabilizing the DNA-bound Pbx-Hox complex through an interaction between the MH domain of Meis and the PBC domain of some remnant Pbx protein present in *lzr*$^-$ embryos. We asked what the source of this remnant Pbx protein in *lzr*$^-$ embryos is, by testing whether maternal Lzr protein was required for rescue. If Meis rescues zygotic *lzr*$^-$ by stabilizing maternal Lzr, we predict that Meis1.1 should not be able to rescue an embryo which is lacking both maternally and zygotically derived Lzr protein *(mz-lzr*$^-$*)*. We did not observe rescue of *mz-lzr*$^-$ embryos injected with *meis1.1WT* mRNA, as judged by *krox20* expression, while injection of the same mRNA into zygotic *lzr*$^-$ embryos does partially rescue the *krox20* phenotype (Fig. 7D). This demonstrates that Meis1.1 requires at least some Lzr protein in order to have its effects, and strongly supports a model whereby Meis functions in part by increasing Lzr protein levels. We cannot rule out the possibility that Meis also stabilizes other Pbx proteins present in the early embryo, and that stabilization of other Pbx family members may also be required for Meis to rescue zygotic *lzr*$^-$ embryos.

We asked whether corresponding differences in endogenous Pbx protein levels could be detected in situ as a result of ectopic Meis expression. In embryos mosaically expressing Myc-tagged Meis1.1WT, we observe a significant increase in Pbx protein in Meis-expressing cells compared with non-expressing cells (Fig. 8C,D). Consistent with our western blot results showing that Pbx stabilization is not dependent on DNA binding, we see a similar increase in Pbx immunoreactivity in cells expressing the non-DNA binding dominant-negative forms of Meis, Meis1.1ΔC and Meis1.1N323D (Fig. 8E-H). Both of these mutant forms are localized primarily to the nucleus, as is the Pbx protein in expressing cells. This is similar to the behavior of similar Hth mutants in the fly (Ryoo et al., 1999, Kurant et al., 2001), and is consistent with a model whereby Hth, binding to Exd via its HM domain, shifts the complex into the nucleus by revealing a nuclear import signal on Exd itself (Abu-Shaar et al., 1999; Berthelsen et al., 1999).

We have previously noted a distinct modulation of Pbx protein levels at the rhombomere 1/2 boundary in 24-hour-old zebrafish embryos in the absence of a corresponding modulation at the RNA level (Fig. 8A,B) (Pöpperl et al., 2000). The differences in Pbx immunoreactivity we observe between ectopic Meis-expressing and non-expressing cells resemble the differences we see in endogenous Pbx staining between r1 and r2. The r1/2 boundary is a prominent boundary of *meis1.1* and *meis2.2* expression (Fig. 1), as well as of the anterior-most Hox gene, *hoxa2* (Prince et al., 1998). These correlations, together with our observations of the effects of ectopically expressed Meis on Pbx stability, suggest that during normal development,

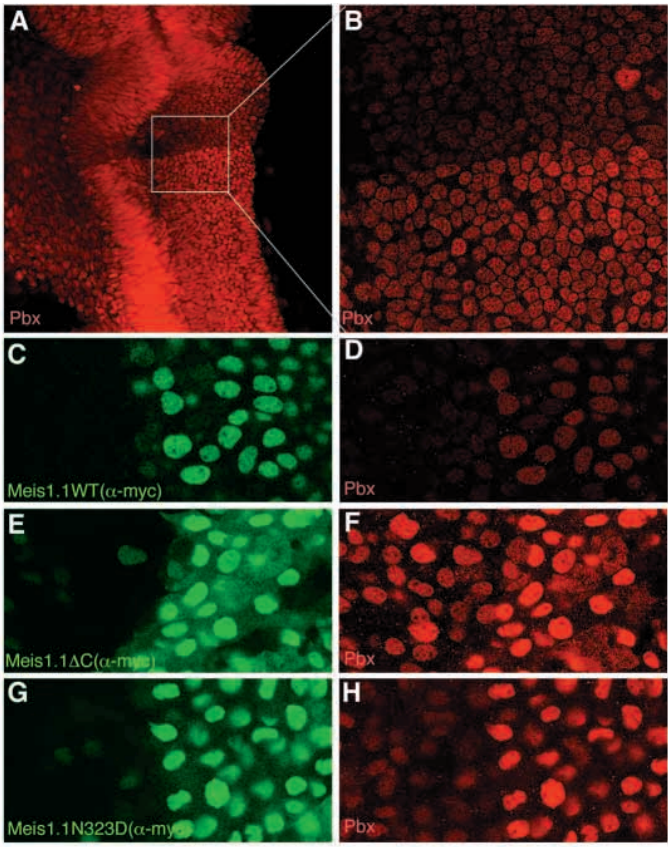

Fig. 8. Meis stabilizes endogenous nuclear Pbx protein. (A) 24 hour embryo stained with pan-Pbx antiserum, showing a prominent boundary of nuclear staining at the r1/r2 boundary with higher Pbx levels posterior to the boundary. (B) Higher magnification image of box outlined in A, demonstrating that Pbx proteins are predominantly nuclear on both sides of the boundary. (C-H) Two to four somite stage embryos expressing Meis1.1WT (C,D), Meis1.1ΔC (E,F) and Meis1.1N323D (G,H), stained with 9E10 to visualize the Myc epitope on the Meis proteins (green staining in C,E,G) and with α-pan-Pbx antibody to detect endogenous Pbx protein (red staining in D,F,H). Note that cells expressing Meis1.1WT or mutant protein exhibit stronger Pbx immunoreactivity, whereas an unrelated Myc-tagged protein did not have this effect (data not shown). Also note that all Meis forms are predominantly nuclear.

Meis and Hox proteins contribute to the increased stability of Pbx protein posterior to the r1/2 boundary.

DISCUSSION

Meis function is required for proper hindbrain segmentation

Previous research has demonstrated that Hox and Pbx proteins are crucial regulators of anteroposterior identity within the developing vertebrate central nervous system. In zebrafish, the *lazarus* mutant phenotype is caused by a point mutation that creates a premature stop codon in the Lzr/Pbx4 protein at residue 45, before any functional domains (Pöpperl et al., 2000). Ectopic overexpression assays using *hoxb2* mRNAs demonstrate that Lzr protein is required for Hoxb2 protein function. Furthermore, *lzr* mutants have reduced expression of *krox20*, *hoxb1*, *hoxa2* and *hoxb2*. These defects in gene expression can all be explained by abrogation of Hox-Pbx transcriptional activity, and demonstrate the crucial role of Hox partners, such as Pbx, during segmentation.

We have investigated the role for Meis in Pbx-dependent processes. We have demonstrated that zebrafish contains at least six Pbx partners, named either Meis or Prep on the basis of homology with similar genes in the mouse. Given that these genes are expressed in highly overlapping patterns, and have similar biochemical properties, we chose a dominant-negative approach to study Meis protein function. We designed mutations based on studies of Hth, a Meis homolog in flies, either by truncating the protein, resulting in a deletion of the DNA-binding homeodomain (Meis1.1ΔC), or by mutating a conserved DNA-binding residue in the homeodomain, again in hopes of creating a DNA-binding deficient mutant (Meis1.1N323D; Gehring et al., 1994; Ryoo et al., 1999). We observe that inhibition of Meis function blocks *hoxb2* activity in an ectopic expression assay, and causes phenotypes resembling those of *lzr* zygotic loss of function. We interpret these results as meaning that Meis is required for Pbx-dependent processes in the fish.

The phenotypes that result from expression of dominant-negative Meis are somewhat incomplete. For example, expression of mutant Meis protein suppresses but does not eliminate *hoxb2*-induced *krox20* expression in the eye (Fig. 2B,C). This can be explained in two ways: either (1) Meis proteins normally function to potentiate the transcriptional activity of Hox/Pbx complexes; or (2) dominant-negative Meis does not eliminate all Meis activity within the embryo. Although we cannot rule out the first explanation, there is precedent for the second, as expression of dominant-negative Hth does not completely eliminate expression of a Hox-dependent enhancer, *lab48/95*, whereas the same enhancer is not active in *hth*$^{-}$ mutant embryos (Ryoo et al., 1999).

Both mutant forms of Meis1.1 that we generated phenocopied the *lzr* mutant phenotype. This is consistent with a model, presented by Ryoo et al. (Ryoo et al., 1999) and supported by analysis of vertebrate Hox-dependent regulatory elements (Jacobs et al., 1999; Feretti et al., 2000), in which Meis/Hth participates in Pbx/Exd function not only by facilitating the nuclear localization of Pbx/Exd but also by participating as a required, DNA-bound component of the Hox complex. Curiously, an Hth mutant with the equivalent amino-acid change in the homeodomain as our Meis1.1N323D mutant does not act as a dominant negative (Ryoo et al., 1999), and homeodomain-deleted forms of Hth are partially functional in Hox-dependent developmental events in the fly (Kurant et al., 2001), suggesting that DNA binding may be a more important aspect of vertebrate Meis function than it is of *Drosophila* Hth function.

The converse may be true with regard to Hth and Meis function in Exd and Pbx nuclear localization. In the fly, Exd is absolutely dependent on the presence of Hth for its nuclear localization (Riekhof et al., 1997). In vertebrates, Pbx proteins may be less generally dependent on Meis proteins for their nuclear localization. Although Pbx subcellular localization appears to be regulated along the proximodistal axis of the mouse limb (González-Crespo et al., 1998), we see no evidence of modulation in the subcellular distribution of endogenous Pbx protein, in spite of zebrafish Meis genes

being expressed in sharply defined domains in the embryo (Fig. 8; Pöpperl et al., 2000, and data not shown). We do, however, see a corresponding modulation in the intensity of nuclear Pbx, consistent with a role for Meis in controlling Pbx stability (see below). We cannot rule out, however, that the ubiquitously expressed *prep* genes function to maintain nuclear Pbx localization. Although this may be the case, it does not provide a mechanism for the spatial regulation of Pbx function in the way that localized Hth expression does in the fly.

Meis target genes

Injection of *hoxb2* RNA into one-cell zebrafish embryos results in ectopic expression of *krox20* within the developing retina. Based on our previous results (Pöpperl et al., 2000) and data reported in this manuscript, we can conclude that both Hox partners, Pbx and Meis proteins, are required for the effects of ectopically expressed *hoxb2*. In addition, expression of *krox20* within rhombomere 3 and rhombomere 5 is dependent on both Pbx and Meis proteins (Fig. 3; Pöpperl et al., 2000; A. J. W. and C. B. M., unpublished). Mouse *Krox20* is expressed earlier than *Hoxb2* and directly activates *Hoxb2* transcription (Nonchev et al., 1996). Together, these observations suggest that *Hoxb2* may be a component of a positive-feedback loop that regulates *krox20*.

Intriguingly, expression of dominant-negative Meis protein reveals a subtle role for *meis* in *hoxb1a* regulation in wild-type and *lzr*$^{-}$ embryos. This is somewhat surprising, given that in the mouse, *Hoxb1* expression is independent of a Meis-binding element adjacent to the Hox/Pbx binding element (Ferretti et al., 2000). However, as Meis can bind the *Hoxb1* r4 enhancer element in vitro, this element must be functional (Ferretti et al., 2000), but mutating it may only have subtle effects on reporter expression. Given our observation of decreased *hoxb1a* in dominant-negative Meis-expressing embryos, it seems likely that Meis DNA binding contributes to *hoxb1a* expression.

meis expression patterns imply functions in addition to role as Pbx/Hox partner

Although the expression patterns of *prep* genes imply ubiquitous localization of their encoded proteins, Meis RNAs are found in highly specific and distinct patterns. Perhaps the only exception is the hindbrain, where each Meis gene is expressed from rhombomere 2 to posterior regions of the embryo at some point during segmentation stages. Within the hindbrain, this pattern coincides precisely with the expression pattern of Hox genes of paralog groups 1-4, in that no Hox genes are expressed anterior to r2.

However, Meis genes are expressed in regions of the embryo which contain no Hox genes, such as within the developing eye fields. This pattern is intriguing, given that it is also the location of *krox20* expression in *hoxb2*-injected embryos (Yan et al., 1998). That ectopically expressed Hoxb2 protein does not induce *krox20* expression throughout the embryo indicates either a requirement for an eye- and hindbrain-specific partner or the presence of a tissue-specific *krox20* inhibitor for regions outside the eye and hindbrain. The Meis genes are attractive candidates for a positive acting eye- and hindbrain-specific partner, as they are expressed strongly in these regions of the embryo and are required for Hoxb2 function. The normal function of Meis protein within the eye and the protein(s) with which it interacts remain unclear.

Both *meis1.1* and *meis2.2* are expressed in bilaterally symmetric regions of the ventral telencephalon. Recent work has shown that murine *Meis1* and *Meis2* are expressed in the posterior ganglionic eminence and lateral ganglionic eminence, respectively (Toresson et al., 2000). In both mouse and zebrafish, these are regions of expression of the *distal-less* homologs (*dlx2* in fish, and *Dlx1* and *Dlx2* in mouse) within the ventral telencephalon. We have investigated this in the zebrafish ventral telencephalon, and have shown that *meis2.2* and *dlx2* are co-expressed within this region at both 20 s and 24 hours. In *Drosophila*, *distal-less* and *homothorax* act together to induce antennal differentiation (Dong et al., 2000). Taken together, these findings may implicate Dlx2 as a Meis partner, although no biochemical evidence yet exists to support this hypothesis.

meis1.1 and *meis2.2* are also expressed at high levels within the developing midbrain. This domain is just anterior to the region that expresses *pax2.1* and is the same region which expresses the zebrafish *engrailed* homologs, *eng2* and *eng3*. Engrailed proteins share with the Hox proteins a tryptophan-containing motif that binds directly to Pbx proteins (Peltenburg and Murre, 1996; van Dijk and Murre, 1994). However, neither *lzr* loss-of-function or Meis dominant-negative expression results in defects in *pax2.1* or in midbrain morphology.

Bidirectional stabilization of Meis and Pbx proteins

Previous results from our laboratory have shown that Lzr protein is present at higher levels posterior to the r1/r2 boundary, yet its RNA is ubiquitously expressed (Pöpperl et al., 2000, Fig. 8). This indicates the existence of post-transcriptional regulation of *lzr*. We have shown that the r1/2 boundary is also a boundary of *meis1.1*, *meis2.2*,and *meis3.1* and of anterior-most Hox gene expression. This presents the intriguing possibility that Meis proteins, perhaps in concert with Hox proteins, function to regulate the levels of Pbx proteins.

A similar situation is seen in *Drosophila*, where overexpressed Hth protein can increase the intensity of immunoreactive, nuclear Exd (Jaw et al., 2000). This implies that Hth has two possible activities in addition to participating in DNA-bound Hox complexes: (1) increasing nuclear localization of Exd; and/or (2) increasing the amount of Exd protein. The first of these two activities has been demonstrated definitively using Hth deletion mutants, which fail to transport Exd to the nucleus (Abu-Shaar et al., 1999; Aspland and White, 1997; Berthelsen et al., 1999; Jaw et al., 2000; Rieckhof et al., 1997). We have extended that analysis by showing that Meis can increase Pbx protein levels in vivo, as determined both by western blot analysis and by in situ immunostaining. Thus, in addition to its role in binding DNA, Meis protein functions to stabilize Pbx protein in zebrafish.

Our observation that Meis can increase endogenous Pbx protein levels provides a mechanism for the unexpected result that over-expression of wild-type Meis can rescue the *lzr* mutant phenotype. This rescue is dependent on the presence of maternally expressed *lzr*, suggesting that Meis accomplishes this rescue by stabilizing residual maternally encoded Lzr protein that is present in *lzr*$^{-}$ embryos during segmentation stages. Importantly, however, stabilization is not sufficient for

this rescue, as the dominant negative DNA-binding defective forms of Meis, Meis1.1ΔC and Meis1.1N323D can increase Lzr levels and yet cannot rescue *lzr* mutants. Thus, Meis must not only stabilize maternal Lzr but also bind DNA in order to rescue the *lzr*$^{-}$ phenotype. Interestingly, ectopic expression of Lzr, but not of LzrΔN, increases Meis protein levels. This indicates that forming a complex between Meis and Pbx functions to stabilize both proteins rather than only one. This is supported by results from *Drosophila*, where Hth protein levels are reduced in *exd* mutants (Kurant et al., 1998).

We believe that bidirectional stabilization between Meis and Lzr occurs post-transcriptionally, as the effects that we observe involve exogenously added RNAs, indicating no role for increasing transcription. In addition, as both mRNAs are transcribed using the same vector system, with identical start sites, and are equivalently translated in reticulocyte lysate cell-free systems, we can conclude that rates of translation initiation are similar. We conclude, therefore, that Meis proteins act to stabilize Pbx proteins by a post-translational mechanism. It has been shown in vitro that protein-protein interaction between Meis and Pbx favors the formation of Hox-Pbx-Meis trimeric complexes, which are bound to DNA (Jacobs et al., 1999). As the DNA-binding activity of Meis is not required for such ternary complex formation (Berthelsen et al., 1998; Jacobs et al., 1999; Ferretti et al., 2000), we would anticipate that both wild-type Meis and DNA-binding mutant Meis promote the formation of stable DNA-bound Hox-Pbx-Meis complexes. This is consistent with our observation that both wild-type Meis and Meis1.1ΔC effectively stabilize Pbx protein within zebrafish embryos (Fig. 7).

Recently, Vlachakis et al. (Vlachakis et al., 2001) have reported a synergistic effect of expressing Meis along with Pbx and Hox proteins. They demonstrated that co-expression of Hoxb1b and Lzr proteins resulted in transformation of rhombomere 2 into a rhombomere 4-like identity, whereas co-expression of Hoxb1b, Lzr, and Meis3.1 resulted in a profound transformation of the midbrain into a hindbrain fate (Vlachakis et al., 2001). Synergism is dependent on the protein-protein interaction domains of Pbx and Meis, demonstrating that Pbx and Meis must be in a complex for this effect. As we have shown that Meis1.1 causes an increase in Pbx protein levels by three- to eightfold, it is likely that the highly conserved Meis3.1 has the same stabilizing activity. Perhaps, stabilization contributes to the strong transforming activities observed in embryos that co-express Meis, Pbx and Hox.

Our results showing that ectopically expressed Meis can stabilize Pbx protein, together with our observation of a prominent boundary of endogenous Pbx immunoreactivity that corresponds with domains of Meis and Hox expression in the hindbrain (Fig. 8), leads us to conclude that one of the normal functions of Meis is to stabilize Pbx/Hox complexes and to thereby promote Hox function during hindbrain development. As Meis can perform this stabilization function in the absence of its DNA-binding activity, we conclude that this stabilization function is separate from, and in addition to, the contribution of DNA-bound Meis to the activity of the Hox/Pbx complex.

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