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The Meis3 protein and retinoid signaling interact to pattern the *Xenopus* hindbrain

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Abstract

In *Xenopus* embryos, proper hindbrain formation requires activities of both XMeis3 protein and retinoic acid (RA) signaling. In this study, we show that XMeis3 protein and RA signaling differentially interact to regulate hindbrain patterning. The knockdown of XMeis3 protein prevented RA-caudalizing activity from inducing hindbrain marker expression in both explants and embryos. In contrast, inhibition of RA signaling differentially modulated XMeis3 activity. Target genes that are jointly activated by either RA or XMeis3 activities could not be efficiently induced by XMeis3 when RA signaling was inhibited. However, transcription of an XMeis3 target gene that is not an RA target gene was hyper-induced in the absence of retinoid signaling. Target genes jointly induced by RA or XMeis3 protein were synergistically activated in the presence of both activities, while RA treatment inhibits the ability of XMeis3 to activate transcription of neural genes that are not RA targets. *HoxD1*, an RA direct-target gene was also identified as an XMeis3 direct-target gene. HoxD1 protein acts downstream of XMeis3 to induce hindbrain marker gene transcription. To pattern the hindbrain, RA requires functional XMeis3 protein activity. XMeis3 protein appears crucial for initial hindbrain induction, whereas RA signaling defines the spatial limits of hindbrain gene expression by modifying XMeis3 protein activity.

Keywords: *Xenopus laevis*; Hindbrain; XMeis3; Retinoic acid; HoxD1

Introduction

In the developing *Xenopus* central nervous system (CNS), brain pattern is established by a multistep inductive process (Nieuwkoop, 1952). In the first step, anterior forebrain tissue is induced throughout the whole brain by BMP antagonist molecules secreted from Spemann's organizer (reviewed in Doniach, 1993; Harland and Gerhart, 1997). In a second inductive wave, anteriorized neural tissue is caudalized to more posterior brain cell fates, such as the mid-hindbrain boarder and hindbrain (Doniach, 1993). Three secreted factors caudalize neural tissue in *Xenopus* embryos and explants: retinoic acid (RA), fibroblast growth factor (FGF), and Xwnt3a (Cox and Hemmati-Brivanlou, 1995; Domingos et al., 2001; Durston et al., 1989; Godsave et al., 1998; Holowacz and Sokol, 1999; Kenkgaku and Okamoto, 1995; Kolm and Sive, 1995; Lamb and Harland, 1995; McGrew et al., 1995, 1997; Papalopulu and Kintner, 1996; Ribisi et al., 2000; Ruiz i Altaba and Jessell, 1991; Sharpe, 1991; Sive et al., 1990). These three caudalizing pathways interact to regulate A–P pattern formation in the nervous system; however, these molecules are not equivalent in their caudalizing activities (Kolm et al., 1997; review in Gamse and Sive, 2000).

RA and its metabolites, the retinoids, are required for cell differentiation in many systems. Deficiencies in retinoid metabolism are associated with severe defects during vertebrate embryonic development; similarly, administra-

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tion of excess RA during early vertebrate embryogenesis alters pattern formation in the limb buds and in the developing nervous system. Vertebrate neural development is particularly sensitive to RA signaling. Ectopic application of RA during *Xenopus* gastrulation results in the formation of embryos with severe anterior truncations (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991; Sharpe, 1991; Sive et al., 1990). Ectopic RA treatment differentially alters cell fate along the A–P axis in two ways; at low concentrations, hindbrain development is perturbed, and at higher concentrations, anterior neural tissues like forebrain and cement gland are lost (Godsave et al., 1998). Ectopic RA causes a loss of anterior hindbrain, with a subsequent expansion of posterior hindbrain tissue (Chen et al., 2001; Godsave et al., 1998). In *Xenopus* embryos expressing RA signaling antagonist or RA synthesis inhibitor molecules, posterior hindbrain tissue is perturbed (Blumberg et al., 1997; Hollemann et al., 1998; Kolm et al., 1997; Van der Wees et al., 1998). Forebrain and anterior hindbrain tissues are often expanded as a result of RA activity antagonism (Blumberg et al., 1997; Hollemann et al., 1998; Koide et al., 2001; Kolm et al., 1997).

It is still not clear how RA mechanistically acts to pattern the developing CNS. Little is known about proteins, which interact with the RA pathway to mediate its caudalizing effects. RA activates expression of many Hox genes, which play a role in CNS A–P patterning decisions (reviewed in Lufkin, 1997). In *Xenopus*, two early expressed labial Hox genes, *HoxA1* and *HoxD1*, were shown to be RA direct-target genes, but their role in CNS A–P pattern formation is still unclear (Kolm and Sive, 1995). One major question regarding posteriorization by the RA pathways is, "What are the roles of transcription factors, which may respond to or mediate the effects of caudalizers like RA?"

One candidate protein that regulates hindbrain formation and could interact with RA signaling in the CNS is the *Xenopus* Meis3 (XMeis3) homeobox transcription factor (Dibner et al., 2001; Salzberg et al., 1999). *XMeis3* expression is initially detected as a stripe in the presumptive neuroectoderm as early as mid-gastrula stages, where it becomes localized in rhombomeres two, three, and four, and the anterior spinal cord (Salzberg et al., 1999). Genetic and knockdown studies in *Xenopus* and zebrafish embryos have demonstrated a strong requirement for the Meis3 protein in proper hindbrain formation (Dibner et al., 2001; Vlachakis et al., 2001; Waskiewicz et al., 2001). Knockdown of the XMeis3 protein in *Xenopus* embryos causes a posterior expansion of the forebrain with a concomitant loss of hindbrain cell fates; pan-neural marker expression was unaltered in these embryos (Dibner et al., 2001). Complementing these results, ectopic XMeis3 expression in animal cap explants or embryos activates expression of posterior neural markers without inducing pan-neural marker expression (Salzberg et al., 1999). Thus, XMeis3 like RA appears to be exclusively involved in neural patterning, since it does not induce neural tissue.

In this study, we show that XMeis3 protein and RA signaling differentially interact to regulate hindbrain pattern. Knockdown of XMeis3 activity prevents the ability of RA to pattern the hindbrain in embryos and explants, whereas antagonism of RA activity prevents XMeis3 transcriptional activation of RA target genes in explants and embryos. RA target gene transcription is synergistically activated in the presence of RA/XMeis3 activities, while RA treatment inhibits the ability of XMeis3 to activate transcription of a non-RA target hindbrain gene. In the absence of RA activity, this gene undergoes super-induction by XMeis3 in embryos. These results suggest that the extent of RA signaling in a given cell can differentially modify XMeis3 caudalizing activity. When RA signaling is high, a biased expression favoring RA target genes is observed, but when RA signaling is low, RA target genes are suppressed and non-RA target gene expression is enhanced. *HoxD1* is an RA direct-response gene (Kolm and Sive, 1995), which we now show is also an XMeis3 direct-response gene. HoxD1 and XMeis3 proteins appear to interact to activate hindbrain gene expression. Thus, combined RA and XMeis3 activity interactions establish correct A–P pattern in the developing *Xenopus* hindbrain.

Materials and methods

Xenopus embryos, explants, and inducing factors

Ovulation, in vitro fertilization, embryo culture, and dissections were carried out as described (Re'em-Kalma et al., 1995). Embryos were staged according to Nieuwkoop and Faber (1967). Explants removed at blastula stages or whole embryos were treated with all-trans retinoic acid (0.01–1.0 μM) at early gastrula stages 10+/10.25. Embryos and explants were typically cultured in RA to stages 15–18.

RNA, DNA, and morpholino oligonucleotide injections

Different concentrations of capped sense in vitro transcribed full-length mRNA, *XMeis3* (Salzberg et al., 1999), *XMeis3*-AM, (Dibner et al., 2001), *XCYP26* (Hollemann et al., 1998), *RALDH2* (Chen et al., 2001), *Xpbx1* (Maeda et al., 2002), *HoxD1*, and *HoxD1*-Eng (Kolm and Sive, unpublished) were injected into the animal hemisphere of one blastomere in embryos at the one- or two-cell stages. Plasmids containing an *rpt3-Luciferase* reporter gene construct (in pGL3 basic-Promega) or an *rpt3-CAT* reporter gene construct (driven by a minimal adenovirus major late promoter; Kolm and Sive, unpublished) were injected at a concentration of 50 pg/embryo. The *rpt3* enhancer element binds labial and pbx proteins to drive gene expression in the mouse *HoxB1* gene (Chan et al., 1996; Popperl et al., 1995). A mutant nonresponsive version of the reporter construct was also injected in parallel (Chan et al., 1996). Extract preparation and luciferase assays were performed using the Luciferase Assay System (Promega) and activity

was measured by integrating total light emission over 30 s using a Berthold luminometer. Luciferase activity was normalized to total protein concentration. Antisense morpholino oligonucleotides (MOs) complementing the 5′ region of the XMeis3 mRNA (Dibner et al., 2001) were purchased from Gene Tools, LLC (Philomath, Oregon, USA) (www@gene-tools.com). In two-cell stage embryos, one blastomere was injected with 7.5 ng in a 5-nl volume. The uninjected side serves as an internal control in all experiments.

XMeis3-GR inducible protein

A full-length *XMeis3* fragment was generated by PFU DNA polymerase. This fragment was cloned 5′ to the amino terminus of the hormone-binding domain of the human glucocorticoid receptor (hGR) in the pCS2 vector. RNA encoding the XMeis3-GR fusion protein was injected at the one-cell stage. Animal cap explants were removed at late blastula stages for culture. Dexamethasone (10 μM) was added at stages 10.25–10.5. Cyclohexamide (5 μM) was added 30 min before the dexamethasone treatment. In these experiments, explants were cultured until stages 12.5–13.0 and total RNA was isolated for RT-PCR analysis.

In situ hybridization

Whole-mount in situ hybridization was carried out with digoxigenin-labeled probes (Harland, 1991; Hemmati-Brivanlou et al., 1990). Embryos were cultured until early to late neurula stages and subsequently fixed for in situ hybridization. The uninjected side serves as an internal control in all experiments. In some embryos, for lineage tracing analysis, 50 pg of RNA encoding the β-galactosidase protein (β-gal; Smith and Harland, 1991) and various injected RNAs were co-injected unilaterally at the two-cell stage. Embryos were stained for β-gal activity and fixed for whole-mount in situ hybridization as described (Bonstein et al., 1998). Perturbations of gene expression were always observed on the co-injected side. Probes used were *HoxD1* (Kolm et al., 1997), *HoxB3* (Godsave et al., 1998), and *Krox20* (Bradley et al., 1992).

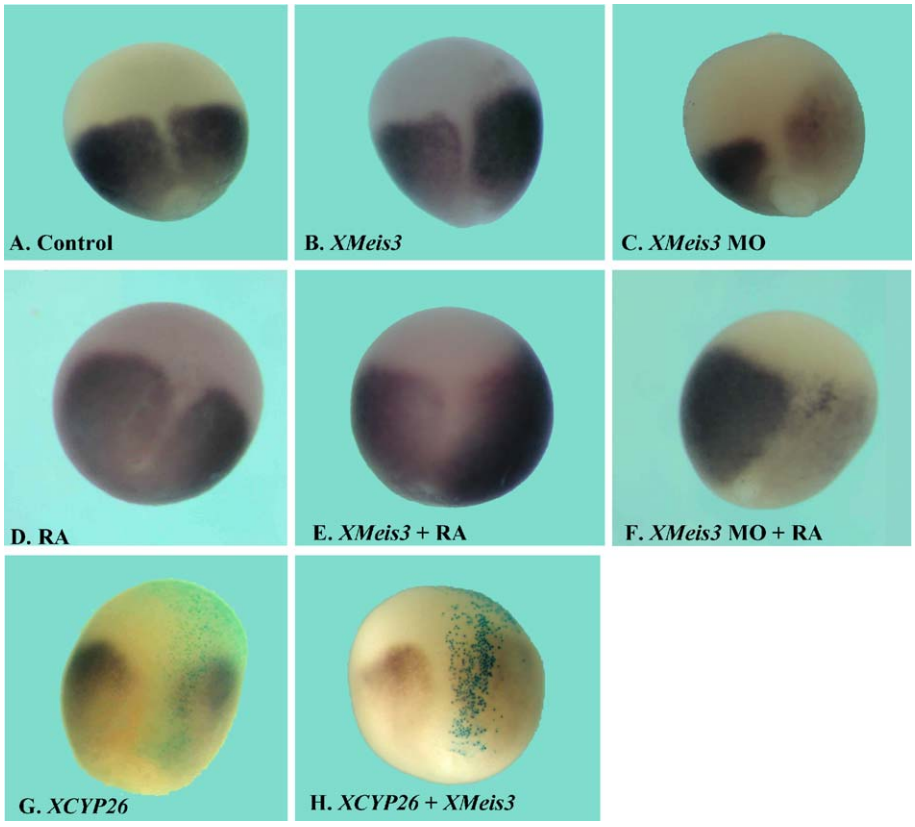

Fig. 1. XMeis3 and RA cooperatively regulate *HoxD1* expression. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere. All embryos are injected on the right side, viewed dorsally, and are oriented anterior (top), posterior (bottom). (A) Control (uninjected). (B) 0.8 ng *XMeis3* RNA, *HoxD1* expression is increased in 67% of the embryos ($n = 30/45$). (C) 7.5 ng of *XMeis3* MO; 50 pg β-gal RNA, *HoxD1* expression is reduced in 52% of the embryos ($n = 11/21$). (D) 0.1 μM RA (uninjected), *HoxD1* expression is anteriorly expanded in 98% of the embryos ($n = 61/62$). (E) 0.8 ng *XMeis3* RNA; 0.1 μM RA, *HoxD1* expression is highly increased in 71% of the embryos ($n = 27/38$). (F) 7.5 ng *XMeis3* MO; 0.1 μM RA, *HoxD1* expression is reduced in 66% of the embryos ($n = 21/32$). (G) 0.8 ng *XCYP26* RNA; 50 pg β-gal RNA, *HoxD1* expression is reduced in 70% of the embryos ($n = 16/23$). (H) 0.8 ng *XCYP26* RNA; 0.8 ng *XMeis3* RNA; 50 pg β-gal RNA, *HoxD1* expression is reduced in 90% of the embryos ($n = 18/20$).

RT-PCR analysis

RT-PCR was performed as described (Wilson and Melton, 1994), except that random hexamers (100 ng/reaction) were used for reverse transcription. Primers used for PCR were *EF1α*, *Krox20*, *HoxB9* (Hemmati-Brivanlou and Melton, 1994), *HoxD1*, *RARα2.2* (Kolm et al., 1997), *HoxB1*, *B3*, and *B4* (Hooiveld et al., 1999).

Results

Functional XMeis3 protein is required for RA caudalizing activity

To determine the epistatic relationship between XMeis3 protein and neural caudalizing pathways, RA activity was examined in embryos knocked down by *XMeis3*-MO expression. Embryos at the two-cell stage were injected with *XMeis3*-MO in one blastomere, treated with RA at early gastrula stages, and grown to neurula stages for neural marker expression analysis. In RA-treated embryos, *HoxD1* and *HoxB3* expression patterns were anteriorized versus controls (compare Figs. 1A and 2A to Figs. 1D and 2D). RA also induced an anteriorly fused single r5-like stripe of *Krox20* expression (compare Figs. 3A to D). However, RA treatment could not rescue the effects of hindbrain elimination by the *XMeis3*-MO, as seen by the loss of *HoxD1* (compare Figs. 1C to F), *HoxB3* (compare Figs. 2C to F) and *Krox20* (compare Figs. 3C to F) expression. In fact, the joint effect of RA and the *XMeis3*-MO seemed to enhance the hindbrain-loss phenotype. Thus, the anterior expansion and expression of posterior hindbrain markers in RA-treated embryos cannot occur in the absence of XMeis3 protein activity.

A complementary observation was detected for hindbrain marker expression in animal cap explants expressing the XMeis3 antimorph protein (XMeis3-AM). *XMeis3*-AM encoding RNA was injected into one-cell embryos and animal caps were removed at blastula stages for RA treatment. In XMeis3-AM/RA-treated animal cap explants, there was a large decrease in expression of RA-activated hind-

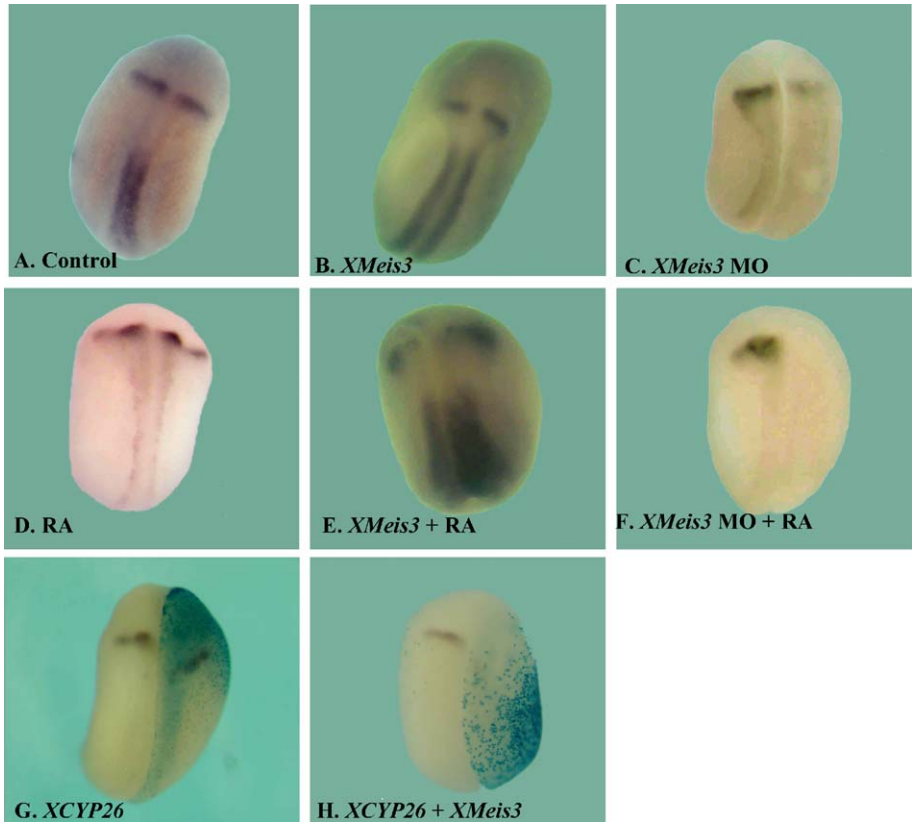

Fig. 2. XMeis3 and RA cooperatively regulate *HoxB3* expression. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere. All embryos are injected on the right side, viewed dorsally, and are oriented anterior (top), posterior (bottom). (A) Control (uninjected; *HoxB3* and *HoxB9* staining). (B) 0.8 ng *XMeis3* RNA (*HoxB3* and *HoxB9* staining) *HoxB3* expression is increased in 44% of the embryos (n = 18/41). (C) 7.5 ng of *XMeis3* MO (only *HoxB3*), *HoxB3* expression is reduced in 73% of the embryos (n = 25/34). (D) 0.1 μM RA (uninjected; only *HoxB3*) *HoxB3* expression is anteriorly expanded in 94% of the embryos (n = 33/35). (E) 0.8 ng *XMeis3* RNA; 0.1 μM RA (*HoxB3* and *HoxB9* staining), *HoxB3* expression is highly increased in 67% of the embryos (n = 22/33). (F) 7.5 ng *XMeis3* MO; 0.1 μM RA (only *HoxB3*), *HoxB3* expression is highly reduced in 92% of the embryos (n = 34/37). (G) 0.8 ng *XCYP26* RNA; 50 pg β-gal RNA (only *HoxB3*), *HoxB3* expression is posteriorized in 68% of the embryos (n = 13/19). (H) 0.8 ng *XCYP26* RNA; 0.8 ng *XMeis3* RNA; 50 pg β-gal RNA (only *HoxB3*), *HoxB3* expression is reduced in 82% of the embryos (n = 23/28).

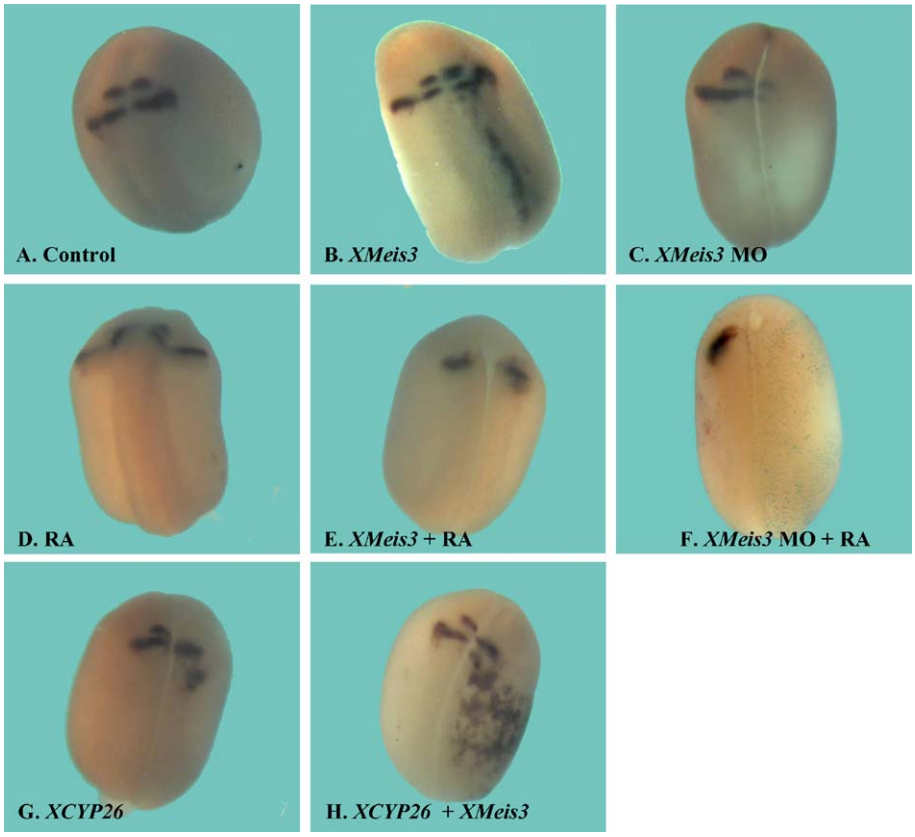

Fig. 3. RA modulates XMeis3 activation of *Krox20* expression. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere. All embryos are injected on the right side, viewed dorsally, and are oriented anterior (top), posterior (bottom). (A) Control (uninjected). (B) 0.8 ng *XMeis3* RNA, *Krox20* expression is increased in 70% of the embryos (n = 62/89). (C) 7.5 ng of *XMeis3* MO, *Krox20* expression is decreased in 68% of the embryos (n = 32/47). (D) 0.1 μM RA (uninjected), *Krox20* expression stripes are fused in 98% of the embryos (81/83). (E) 0.8 ng *XMeis3* RNA; 0.1 μM RA, ectopic *Krox20* expression is decreased in 78% of the embryos (n = 62/80). (F) 7.5 ng *XMeis3* MO; 0.1 μM RA; 50 pg β-gal RNA, *Krox20* expression is decreased in 89% of the embryos (n = 49/55). (G) 0.8 ng *XCYP26* RNA, *Krox20* expression is posteriorized in 89% of the embryos (17/19). (H) 0.8 ng *XCYP26* RNA; 0.8 ng *XMeis3* RNA, *Krox20* expression is highly expressed in 68% of the embryos (13/19).

brain markers, such as *HoxD1*, *RARα2.2*, *HoxB1*, *HoxB3*, and *HoxB4*, in comparison to explants solely treated with RA (Fig. 4A). Thus, inhibition of XMeis3 activity by the XMeis3-AM protein significantly inhibited RA caudalizing activity in animal cap explants.

Retinoid signaling simultaneously synergizes and antagonizes XMeis3 caudalizing activity

To further determine how XMeis3 protein interacts with RA signaling pathways, we treated embryos expressing the XMeis3 protein with RA. Embryos at the two-cell stage were injected with *XMeis3* encoding RNA in one blastomere. At early gastrula stage, embryos were treated with RA. Whole-mount in situ hybridization of treated embryos showed that the combination of XMeis3 and RA maximally activated expression of the RA target genes *HoxD1* and *HoxB3* versus embryos only expressing XMeis3 or treated with RA (compare Figs. 1E and 2E to (Figs. 1A and 2A, 1B and 2B, and 1D and 2D)).

Similar to the *HoxB3* and *HoxD1* genes, XMeis3 ectopically spread *Krox20* expression on the injected side (compare Figs. 3B to A); however, in sharp contrast to the *HoxB3* and *HoxD1* genes, RA treatment did not enhance but alleviated this effect (compare Figs. 3B to E). RA treatment reduced the XMeis3-induced *Krox20* posterior spread to a fused stripe in the expanded r5 region, similar to the non-injected RA-treated side of the embryo, which resembles the non-XMeis3-injected RA-treated embryos (compare Figs. 3E to D). Unlike the *HoxB3* and *HoxD1* genes, *Krox20* is not an RA target gene; XMeis3, but not RA, activates its expression in animal cap explants. Thus, in addition to caudalizing the hindbrain, RA also limits the regional spread of XMeis3-induced *Krox20* expansion along the A–P axis, suggesting that RA and XMeis3 may differentially interact to establish pattern in the CNS. Common XMeis3/RA target genes are expressed synergistically in the presence of both activators. However, in the case of a non-RA XMeis3 target gene, like *Krox20*, increasing RA levels inhibits its transcriptional activation by XMeis3 protein.

To extend these studies, we also examined the ability of XMeis3 to activate posterior neural marker gene expression in animal cap explants treated with RA. XMeis3-injected

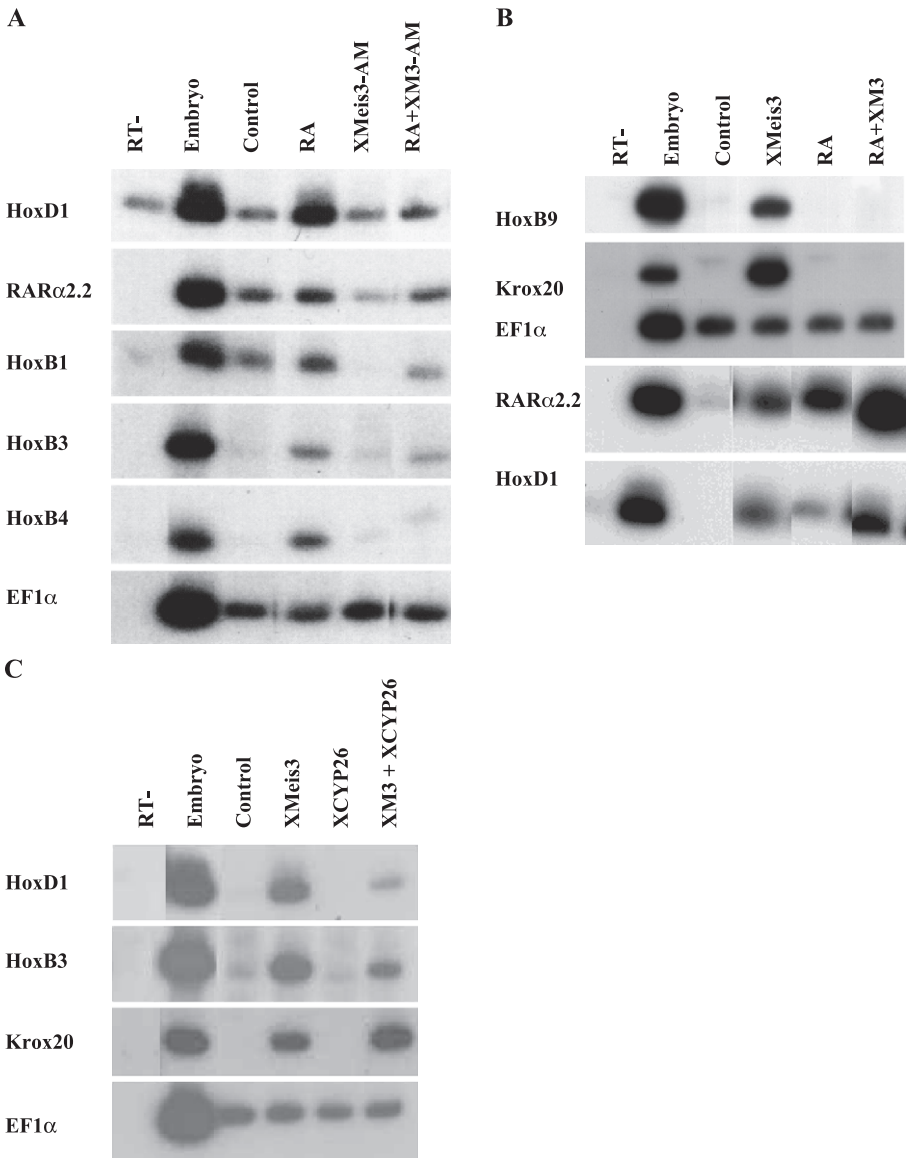

Fig. 4. XMeis3 and RA interactions in animal cap explants. (A) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of *XMeis3*-antimorph (AM) encoding RNA. Eighteen animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8–9) and treated with RA (1.0 μM) at stage 10.25. Explants from each group were grown to stage 18 and total RNA was isolated. RT-PCR analysis was performed with the markers *HoxD1*, *RARα2.2*, *HoxB1*, *HoxB3*, and *HoxB4*. *EF1α* served as a control for quantitating RNA levels in the different samples. For controls, RT-PCR and -RT-PCR was performed on total RNA isolated from normal embryos. (B) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of *XMeis3* encoding RNA. Eighteen animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8–9) and treated with RA (0.1 μM). Explants from each group were grown to stage 18 and total RNA was isolated. RT-PCR analysis was performed with the markers: HoxB9, *Krox20*, *RARα2.2*, and *HoxD1*. *EF1α* served as a control for quantitating RNA levels in the different samples. For controls, RT-PCR and -RT-PCR was performed on total RNA isolated from normal embryos. (C) One-cell stage embryos were injected in the animal hemisphere with either 1.6 ng of *XMeis3* or *XCYP26* encoding RNAs. Eighteen animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8–9). Explants from each group were grown to stage 18 and total RNA was isolated. RT-PCR analysis was performed with the markers: *Krox20*, *HoxB3*, and *HoxD1*. *EF1α* served as a control for quantitating RNA levels in the different samples. For controls, RT-PCR and -RT-PCR was performed on total RNA isolated from normal embryos.

explants typically expressed high levels of *Krox20*, *HoxB9*, *HoxD1*, and *RARα2.2* mRNAs in comparison to uninjected control animal cap explants (Fig. 4B). RA treatment of animal cap explants activated expression of the RA target genes, *HoxD1* and *RARα2.2*; however, RA treatment of animal cap explants strongly inhibited the XMeis3-induced transcription of non-RA target genes, like *Krox20* and *HoxB9* (Fig. 4B). In contrast, the combination of RA treatment and ectopic XMeis3 expression synergistically activated transcription of the *HoxD1* and *RARα2.2* RA-responsive genes (Fig. 4B). These results were observed over a 100-fold concentration range of RA (0.01–1.0 μM; not shown). In parallel, experiments were also performed in animal caps ectopically expressing the RA activating en-

zyme, RALDH2 (Chen et al., 2001) in the presence of the retinoid substrate, all-trans-retinal (ATR). When explants were activated in the presence of ATR (1.5 μM) and RALDH2, XMeis3-induced gene expression patterns were modified. Similar to RA explant treatment, XMeis3 induction of *Krox20* expression was inhibited in the presence of ATR and RALDH2 (not shown). Expression of RA inducible genes, such as *RARα2.2* and *HoxB3*, was synergistically stimulated by the combination of XMeis3, ATR, and RALDH2 (not shown). Thus, like in whole embryos, XMeis3 and RA signaling interact to simultaneously modulate A–P-specific hindbrain marker gene expression in explants.

XMeis3/RA response genes require RA signaling for activation by XMeis3

To further investigate a functional interaction between XMeis3 protein and retinoid signaling, XMeis3 protein was co-expressed with the RA hydroxylase, XCYP26 (Hollemann et al., 1998). XCYP26 antagonizes RA signaling by inhibiting endogenous retinoid biosynthesis. Embryos were co-injected in one blastomere at the two-cell stage with *XMeis3* and *XCYP26* encoding RNAs. The co-expression of XMeis3 and XCYP26 led to a dramatic posterior and lateral expansion of *Krox20* expression levels (Fig. 3H), in comparison to embryos expressing either *XMeis3* or *XCYP26* RNAs alone (Figs. 3B, G). This observation again supports the idea that expression of XMeis3/non-RA target genes may be negatively regulated by RA signaling. We also examined expression of the two RA target genes (*HoxB3* and *HoxD1*), which are also induced by ectopic XMeis3. In *XMeis3*-expressing embryos, *HoxD1* expression is expanded on the injected side (Figs. 1B); a slight but reproducible expansion of *HoxB3* is also observed (Fig. 2B). In *XCYP26*-expressing embryos, there is a reduction and posterior shift in *HoxD1* and *HoxB3* expression (Figs. 1G and 2G). In *XMeis3*/*XCYP26* co-expressing embryos, there is an even stronger inhibition of *HoxD1* and *HoxB3* expression levels in comparison to the effects of *XCYP26* alone (Figs. 1H and 2H). Thus, while inhibition of RA signaling by XCYP26 protein stimulated the ability of XMeis3 to activate *Krox20* expression, XMeis3/RA target genes were poorly expressed in the same embryos. Experiments in animal cap explants also supported these observations. The XMeis3 and XCYP26 proteins were co-expressed in animal cap explants (Fig. 4C). As in embryos, XMeis3 activity was modified in a differential manner. Ectopic XCYP26 expression inhibited XMeis3 activation of RA-responsive genes, such as *HoxD1* and *HoxB3* by at least fourfold; in contrast, XMeis3 activation of *Krox20* expression was stimulated twofold by RA antagonism (Fig. 4C).

These results suggest that XMeis3 protein activity is differentially regulated by RA signaling. High or low levels of RA signaling can modulate XMeis3 protein activity in the embryo. In cells with low RA activity, a *Krox20*-like pathway of gene expression is favored and RA target gene expression is repressed; however, at high RA levels, XMeis3/RA target gene expression is maximal and expression of non-RA target genes like *Krox20* is inhibited.

RA signaling modifies XMeis3 expression along the A–P axis

To determine if RA signaling can regulate *XMeis3* expression, two-cell stage embryos were injected in one blastomere with either *XCYP26*- or *RALDH2*-encoding RNAs. Ectopic XCYP26 activity did not eliminate *XMeis3* mRNA levels in the hindbrain, but caused a posterior shift in its expression (Fig. 5A). Previous studies in embryos expressing ectopic XCYP26 levels have shown an identical posterior shift in hindbrain gene expression for markers such as *Krox20*, *Pax6*, and *HoxB3* (Hollemann et al., 1998). The typical gap in *XMeis3* expression between the posterior

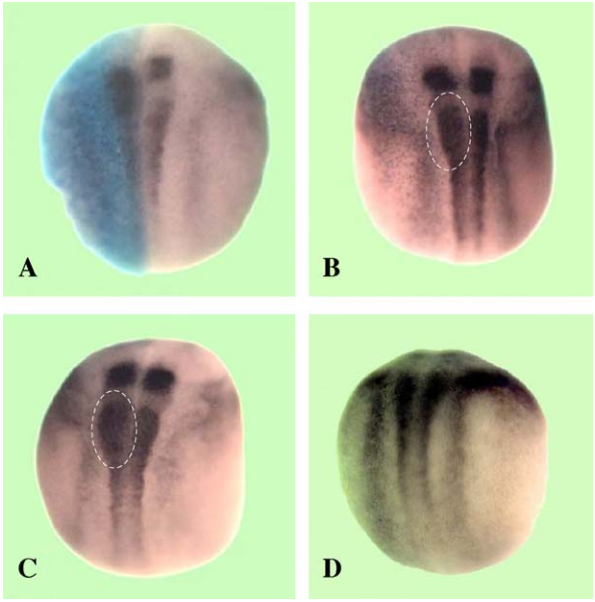

Fig. 5. RA signaling modifies *XMeis3* expression along the A–P axis. Two-cell albino embryos were injected unilaterally into the animal hemisphere with *XCYP26* or *RALDH2* encoding RNAs. The injected side is on the left as marked by β-gal staining (blue) and all embryos are viewed dorsally with anterior on top and posterior at the bottom. (A) 0.5 ng of *XCYP26* RNA, *XMeis3* expression is shifted posteriorly in the hindbrain, and inhibited in the spinal cord in 100% of the embryos (86/86). The gap between hindbrain and spinal cord expression was narrowed or lost in 82% of the embryos (70/86). (B) 2.0 ng of *RALDH2* RNA, *XMeis3* expression is slightly shifted anteriorly, laterally expanded in the anterior spinal cord (as indicated with the dashed line) in 68% of the embryos (37/54). Expression in the hindbrain was not increased. (C) 2.0 ng of *RALDH2* RNA and treatment with 500 nm all-trans-retinal (ATR) at stage 10.5. Similar phenotypes described in B were observed, including no increase in hindbrain expression, a slight anterior shift, and a lateral expansion of *XMeis3* expression in 74% (35/48) of the embryos. (D) 18 nM RA treatment at gastrula stage 10.5. *XMeis3* expression was strongly shifted anteriorly and increased in the spinal cord in 100% (76/76) of the embryos.

hindbrain and the anterior spinal cord was lost in *XCYP26*-expressing embryos (Fig. 5A). This expression pattern is nearly identical to the expression pattern of *XMeis3* in mild *XMeis3*-MO knockdown phenotypes (Dibner et al., 2001). Also, in mild XMeis3 knockdown phenotypes, hindbrain *Krox20* expression shifted posteriorly (Dibner et al., 2001). These observations and the differential effects of *XMeis3*/*XCYP26* co-injection on neural marker expression highly suggest that antagonism of RA signaling does not likely mediate its effects on XMeis3 activity by simply turning off gene transcription.

RALDH2-injected embryos were treated with ATR at early gastrula stages. RALDH2, RALDH2/ATR, or RA treatments led to an anterior expansion of *XMeis3* expression (Figs. 5B–D). A similar anterior expansion was also shown for *Krox20* expression in the hindbrain of RALDH2/ATR-

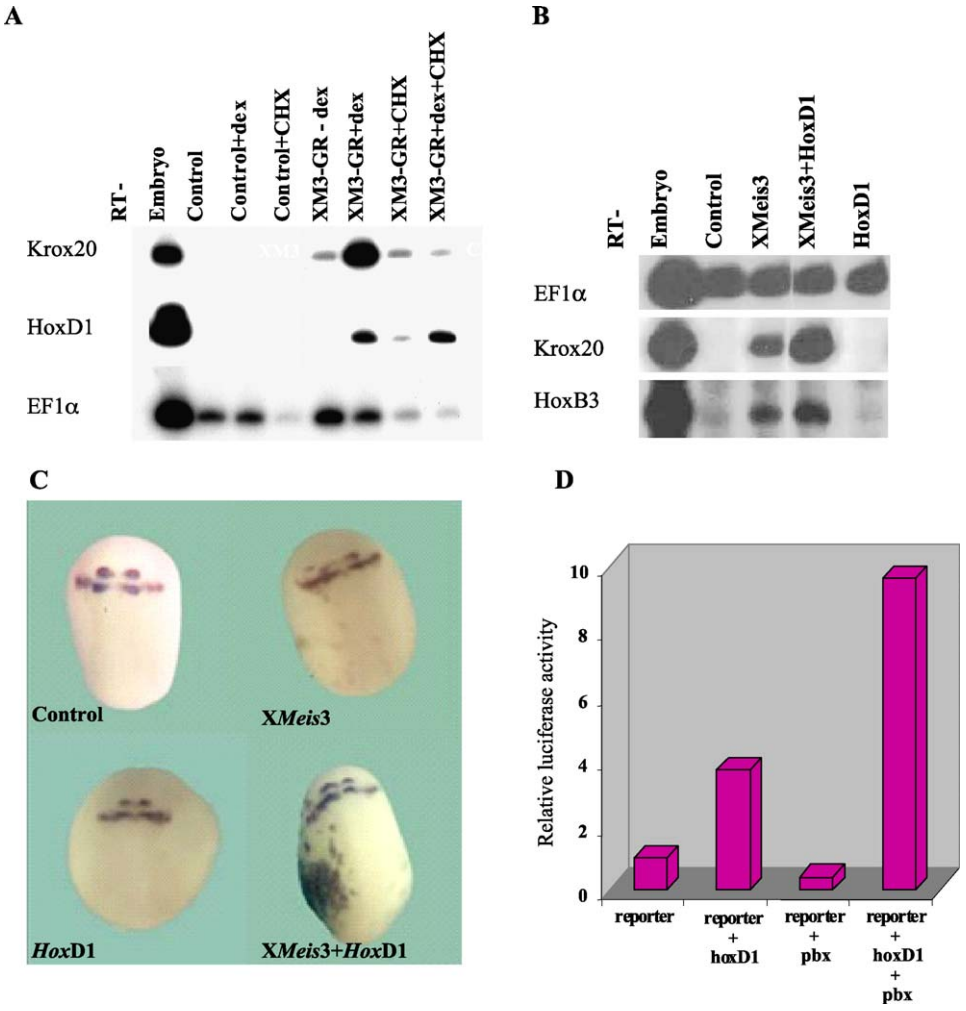

Fig. 6. *HoxD1* is an *XMeis3* direct-target gene that interacts with *XMeis3* to activate hindbrain marker expression. (A) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of inducible *XMeis3-GR* encoding RNAs. Fifty-four and seventy-two animal cap explants were respectively removed from uninjected and injected groups of blastula embryos (stage 8–9). Eighteen explants from each group were grown in cyclohexamide and/or dexamethasone (see Materials and methods) to stage 12.5 and total RNA was isolated. RT-PCR analysis was performed with the markers: *Krox20* and *HoxD1*. *EF1α* served as a control for quantitating RNA levels in the different samples. For controls, RT-PCR and -RT-PCR was performed on total RNA isolated from normal embryos. (B) One-cell stage embryos were injected in the animal hemisphere with either 1.6 ng of *XMeis3* or *HoxD1* encoding RNAs. Eighteen animal cap explants were removed from uninjected and injected groups of blastula embryos (stages 8–9). Explants from each group were grown to stage 18 and total RNA was isolated. RT-PCR analysis was performed with the markers: *Krox20* and *HoxB3*. *EF1α* served as a control for quantitating RNA levels in the different samples. For controls, RT-PCR and -RT-PCR was performed on total RNA isolated from normal embryos. (C) Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere with RNAs encoding XMeis3 or HoxD1 proteins. In situ hybridization to *Krox20* was performed in neurula stage embryos. All embryos are injected on the left side, viewed dorsally, and are oriented anterior (top), posterior (bottom). Upper left panel: control embryo. Upper right panel: *XMeis3* injected (0.8 ng), 70% of the embryos had expanded *Krox20* expression (62/89). *HoxD1* injected (0.8 ng), none of the embryos had expanded *Krox20* expression (0/27). *XMeis3* and *HoxD1* co-injected (0.8 ng), 68% of the embryos had ectopic *Krox20* expression (28/41). (D) Embryos were co-injected with the *rpt3-luc* or *rpt3-CAT* reporter constructs (see Materials and methods) along with RNAs encoding HoxD1, Xpbx1, or XMeis3 proteins. One representative experiment is shown. At early-mid gastrula stages, 10 embryos were lysed per injection group and luciferase activity was assayed. The bar graph describes relative luciferase activity in each sample, with the control embryos expressing only the *rpt3-luc* reporter taken as 1. A mutant version of the *rpt3-luc* reporter did not activate luc transcription (not shown) in injected embryos.

treated embryos (Chen et al., 2001). *XMeis3* expression levels expanded laterally in the spinal cord, but in the hindbrain, no increased *XMeis3* mRNA levels were detected (Figs. 5B–D). Our previous studies have shown that RA cannot efficiently induce *XMeis3* expression in animal cap explants. Together, these results suggest that RA is not a major mediator of *XMeis3* transcriptional activation per se, but that XMeis3 protein and the RA signaling cascade intermesh to regulate A–P pattern in the hindbrain.

HoxD1 is an XMeis3 direct-target gene

To reveal the pathways downstream of XMeis3 protein, experiments were performed to identify XMeis3 direct-target genes. An XMeis3 growth hormone receptor fusion protein (XMeis3-GR) was constructed that could be activated only in the presence of dexamethasone (dex). In injected animal cap explants, inducible XMeis3-GR protein activated posterior neural marker expression in the presence of dex (Fig. 6A, data not shown). Uninjected dex-treated animal caps or animal caps expressing the XMeis3-GR in the absence of dex did not express posterior neural markers (Fig. 6A). To identify XMeis3 direct-response genes, blastula stage animal caps expressing the XMeis3-GR were pretreated with cyclohexamide (chx; see Materials and methods) before the addition of dex. Explants were grown to late gastrula/early neurula stages in the presence of dex/chx, and RNA was isolated for RT-PCR analysis. We examined a wide array of posterior markers normally induced by XMeis3 protein in animal cap explants such as various Hox genes (not shown) as

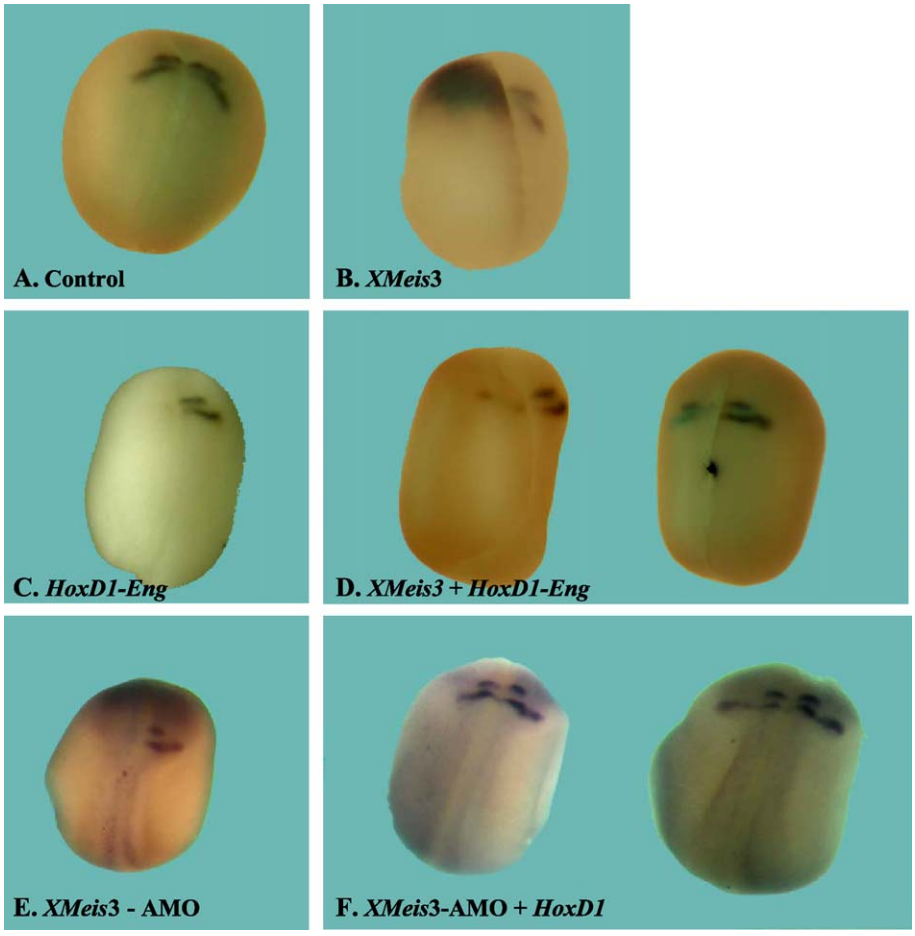

Fig. 7. HoxD1 protein acts downstream of XMeis3. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere with RNAs encoding XMeis3 and HoxD1-Eng (antimorph) proteins or *XMeis3*-MO and RNA encoding HoxD1 protein. In situ hybridization to *Krox20* was performed in neurula stage embryos. All embryos are injected on the left side, viewed dorsally, and are oriented anterior (top), posterior (bottom). (A) Control (uninjected). (B) 0.8 ng *XMeis3* RNA, *Krox20* expression was increased in 81% of the embryos (9/11). (C) 0.1 ng *HoxD1*-Eng RNA, *Krox20* expression was lost in 90% of the embryos (20/22). (D) 0.8 ng *XMeis3* RNA, 0.1 ng *HoxD1*-Eng RNA RNA (two representative embryos), ectopic *Krox20* expression was inhibited in 93% of the embryos (13/14). (E) 18 ng *XMeis3*-MO, *Krox20* expression was highly inhibited in 75% of the embryos (39/52), Krox20 expression was fairly normal on both sides in 13.5% of the embryos (7/52). (F) 18 ng *XMeis3* MO, 0.8 ng *HoxD1* RNA (two representative embryos), *Krox20* expression was fairly normal in 58% of the embryos (14/24). *Krox20* expression was highly inhibited in only 21% of the embryos (5/24). Embryos solely expressing HoxD1 RNA resembled uninjected controls (see Fig. 6C). Similar results were observed for HoxB3 expression (not shown).

well as *Krox20*. Only one mRNA was detected, whose level was not inhibited by chx, and this was the *HoxD1* gene (Fig. 6A). Interestingly, *HoxD1* is also an RA direct-response gene (Kolm and Sive, 1995). This raises an interesting point of potential pathway interaction, because both RA and XMeis3 activities were required for the correct induction of *HoxD1* expression in embryos and explants (Figs. 1C, G).

To elucidate XMeis3 and HoxD1 interactions, experiments were performed in which XMeis3 and *HoxD1* encoding RNAs were co-injected into one-cell embryos and animal caps were removed at blastula stages for culture until neurula stages. In animal cap explants, HoxD1 alone could not induce *Krox20* or *HoxB3* expression (Fig. 6B), but it significantly enhanced the ability of XMeis3 to activate their transcription (Fig. 6B). As in explants, ectopic HoxD1 levels did not activate *Krox20* expression in embryos (Fig. 6C), yet in HoxD1/XMeis3 co-injected embryos, *Krox20* expression was ectopically induced, in comparison to embryos injected with XMeis3 alone, especially when XMeis3 induction of ectopic *Krox20* expression was intermediate (Fig. 6C). To further investigate XMeis3 and HoxD1 interactions, mRNAs encoding these proteins were co-expressed with a reporter construct driven by the mouse *HoxB1-rpt3* enhancer element (see Materials and methods). In other studies, the *rpt3* element was shown to bind labial/pbx proteins (Chan et al., 1996; Popperl et al., 1995). We show that the HoxD1 protein alone moderately stimulated the reporter construct, and pbx had negative effects (Fig. 6D). When co-injected, pbx and HoxD1 proteins highly activated the mouse *HoxB1 rpt3* enhancer element (Fig. 6D). XMeis3 protein did not significantly activate trandscription of this reporter, alone or in combination with pbx or HoxD1 proteins (not shown).

Embryos were co-injected with RNAs encoding XMeis3 and HoxD1-antimorph (Engrailed domain) proteins (Kolm and Sive, unpublished). The injection of the HoxD1-antimorph protein eliminated *Krox20* expression in the hindbrain (Fig. 7C). Overexpression of XMeis3 typically expanded *Krox20* expression (compare Figs. 7A to B), but in co-injected embryos, XMeis3 activity failed to stimulate *Krox20* expression and most of the embryos failed to express significant *Krox20* levels in r3/r5 on the injected side (Fig. 7D). *HoxB3* expression was inhibited in a similar manner (not shown). In the same HoxD1-antimorph-expressing embryos, *XMeis3* expression was not severely reduced, but in some cases, shifted posteriorly by one or two rhombomeres (not shown). Ruling out nonspecific toxic effects, co-injection of *pbx* and *HoxD1* encoding RNAs rescued hindbrain formation in embryos expressing the HoxD1-antimorph protein (Kolm and Sive, unpublished). While ectopic XMeis3 protein expression cannot rescue the HoxD1-antimorph phenotype, ectopic *HoxD1* expression can efficiently rescue *Krox20* (Figs. 7E, F) and *HoxB3* (not shown) expression in embryos knocked down by the *XMeis3*-MO. These results strongly suggest that HoxD1 acts downstream of XMeis3 protein in hindbrain formation.

Discussion

In this study, we examined the interaction of the neural caudalizing activities of XMeis3 protein and RA. Little is known as to how transcription factors interact with signaling pathways to pattern the early CNS. Previous studies have shown that either XMeis3 or RA can pattern ectoderm in the absence of neural induction and both XMeis3 protein and RA activities are required for proper hindbrain formation. XMeis3 is expressed in a single stripe of presumptive hindbrain cells in mid-gastrula stage embryos and its knockdown leads to a loss of the whole hindbrain with a concomitant expansion of the forebrain. Embryos depleted of RA activity typically lose the posterior hindbrain and gain some anterior neural structures. It was suggested that RA might act in two ways to pattern the *Xenopus* CNS. RA can induce a posterior hindbrain, while also inhibiting anterior CNS structures such as forebrain. Because of these similarities in XMeis3 and RA activities, we determined the potential epistatic relationship between XMeis3 protein and RA signaling activities.

XMeis3 protein acts upstream of RA signaling in regulating hindbrain formation. In XMeis3 knockdown embryos or explants, RA caudalizing activity was diminished; RA could not rescue hindbrain cell fates nor induce hindbrain marker expression in XMeis3 knockdown embryos. In animal cap explants, XMeis3 knockdown significantly inhibited the ability of RA to activate the expression of a panel of Hox genes. These results suggest that XMeis3 is required to initially induce the hindbrain and that RA endows a distinct A–P character to it.

Further experiments demonstrated that RA greatly modulates the activity of XMeis3 protein to turn on expression of posterior neural markers. Ectopic XMeis3 activity induces expression of a wide range of posterior neural markers in embryos and animal cap explants. Ectopic XMeis3 expression in the presence of high RA levels inhibits non-RA target gene expression in explants and embryos (*HoxB9*, *Krox20*). However, in the same explants and embryos, RA target genes, such as *RARα2.2*, *HoxB3*, and *HoxD1*, are expressed at maximal levels by the combination of XMeis3 and RA. In support of this observation, in the absence of RA signaling, RA target genes are weakly expressed in embryos and explants, even in the presence of ectopic XMeis3 activity. However, *Krox20* gene expression is maximally induced by XMeis3 in the absence of RA signaling.

These results suggest that the presence or absence of RA signaling can bias the direction of XMeis3 protein activity in the embryo. In cells with low RA activity, a *Krox20*-like pathway of gene expression is observed with RA target gene expression being repressed; however, at high RA levels, XMeis3/RA target gene expression is optimal and expression of genes like *Krox20* is inhibited. In the hindbrain, a correct spatial and temporal balance of RA signaling is most likely required to enable proper A–P pattern formation by endogenous XMeis3 protein. The RA/XMeis3 interactions that

dictate *Krox20* expression levels suggest that XMeis3 may be responsible for the absolute levels of *Krox20* transcripts, whereas RA signaling may dictate the regional localization in the hindbrain. Inhibition of XMeis3 protein activity by strong areas of retinoid signaling may localize *Krox20* expression to specific hindbrain regions (r3 and r5). Ectopic XCYP26 levels disrupt this delicate balance, leading to expansion of XMeis3-induced *Krox20* expression throughout the CNS. This bimodal regulation of XMeis3 activity should enable differential gene expression in the hindbrain as determined by higher or lower areas of RA signaling.

In chick embryos, RA antagonism induced upregulation of *Krox20* expression, presumably by spreading r5 caudally (Dupe and Lumsden, 2001). These *Krox20* positive cells excluded expression of *HoxB1* (Dupe and Lumsden, 2001). Other studies showed that forced ectopic *Krox20* expression inhibited *HoxB1* expression in r4; in these embryos, odd-numbered rhombomeric identity was superimposed on even-numbered rhombomeres (Giudicelli et al., 2001). It was suggested that RA signaling defines the r3/4 boarder between *Krox20* and *HoxB1* expressing cells (Dupe and Lumsden, 2001). XMeis3 is expressed in both even and odd-numbered rhombomeres (r2–r3–r4) and its activity is required for formation of the whole hindbrain. Our results show that XMeis3 can induce *Krox20* expression in a nonautonomous manner (Aamar and Frank, 2004). Therefore, to build the r3/r4 border and to distinguish odd and even numbered rhombomeres, RA suppression of XMeis3's ability to activate *Krox20* expression outside of r3 and r5 could be crucial for defining even numbered rhombomeres. Presumably, this RA activity would be attenuated in odd-numbered rhombomeres such as r3 and r5, thus enabling XMeis3 activation of *Krox20* gene expression. In this manner, RA signaling may modify XMeis3 activity to properly pattern the hindbrain. In areas of high RA signaling, XMeis3 maximizes activation of RA response gene expression while minimizing its activation of a non-RA target gene, such as *Krox20*.

HoxD1 is an XMeis3 direct-response gene, being transcriptionally activated by XMeis3 in the presence of chx. Interestingly, *HoxD1* was also shown to be an RA direct-response gene (Kolm and Sive, 1995). HoxD1 and XMeis3 proteins appear to jointly function to activate high levels of *Krox20* and *HoxB3* gene transcription in embryos and explants. Supporting this observation, ectopic expression of the HoxD1 antimorph protein strongly inhibited XMeis3 caudalizing activity in embryos. Unlike RA inhibition by XCYP26 protein, which only altered the A–P expression pattern of hindbrain-expressed genes like *Krox20* (Hollemann et al., 1998), knockdown of HoxD1 activity, like knockdown of XMeis3 activity, caused a loss of *Krox20* and *HoxB3* expression. These results suggest that HoxD1 protein is not working as an antagonistic arm of the RA signaling pathway to repress XMeis3 activation of *Krox20* expression. In contrast, it appears that the activation of *HoxD1* by XMeis3 is a prerequisite for proper hindbrain formation. In HoxD1 knockdown embryos, the ability of ectopic XMeis3 protein to rescue *Krox20* expression is inhibited, yet ectopic HoxD1 expression can rescue hindbrain marker expression in XMeis3 knockdown embryos. In HoxD1 knockdown embryos, the *XMeis3* expression pattern is fairly normal, but in XMeis3 knockdown embryos, *HoxD1* expression is strongly inhibited. These results strongly suggest that HoxD1 protein acts downstream of XMeis3 in regulating hindbrain formation. XMeis3 activity and RA signaling are necessary for the optimal expression of *HoxD1* mRNA, suggesting that both are required for proper spatial and temporal regulation of *HoxD1* transcription.

While XMeis3 directly regulates *HoxD1* transcription, our results suggest the two proteins could interact to induce gene expression, since functional HoxD1 protein is required for proper XMeis3 hindbrain inducing activity and co-injection of both proteins stimulated transcriptional activation of *Krox20* and *HoxB3* in explants and embryos. We have shown that *Xenopus* PBX and HoxD1 proteins jointly activate the mouse *HoxB1* promoter element (*rpt3*), but co-injection of XMeis3 had negligible effects in activating this reporter construct. Whether XMeis3 physically interacts with PBX or HoxD1 proteins to bind DNA and activate transcription of certain target genes is still an open question. XMeis3/HoxD1 ectopic activation of *HoxB3* and *Krox20* expression in embryos and explants could occur if XMeis3 was inducing a prepattern in non-neural regions that allows the HoxD1 protein to activate posterior neural gene expression in these regions. The *rpt3-HoxB1* enhancer element is r4-specific and may not be the optimal target for activation by co-expression of XMeis3 with HoxD1/PBX proteins. Additional investigation of XMeis3/HoxD1/PBX interactions on r3- or r5-specific promoters should clarify this question. In future experiments, we hope to determine how XMeis3 and HoxD1 proteins act to form the *Xenopus* hindbrain. Unraveling the molecular pathways in which RA signaling bimodally modulates XMeis3 protein activity should enable a better understanding of how transcription factors and signaling pathways mesh to pattern the early vertebrate CNS.

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