Xenopus Meis3 protein lies at a nexus downstream to Zic1 and Pax3 proteins, regulating multiple cell-fates during early nervous system development

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ARTICLE INFO

Article history:
Received for publication 30 January 2009
Revised 16 November 2009
Accepted 16 November 2009
Available online 24 November 2009

Keywords:
Xenopus
Meis3
Zic1
Pax3
FGF
Hindbrain
Primary neurons
Neural crest

ABSTRACT

In Xenopus embryos, XMeis3 protein activity is required for normal hindbrain formation. Our results show that XMeis3 protein knock down also causes a loss of primary neuron and neural crest cell lineages, without altering expression of Zic, Pax or Pax3 genes. Knock down or inhibition of the Pax3, Zic1 or Zic5 protein activities extinguishes embryonic expression of the XMeis3 gene, as well as triggering the loss of hindbrain, neural crest and primary neuron cell fates. Ectopic XMeis3 expression can rescue the Zic knock down phenotype. HoxD1 is an XMeis3 direct-target gene, and ectopic HoxD1 expression rescues cell fate losses in either XMeis3 or Zic protein knock down embryos. FGF3 and FGF8 are direct target genes of XMeis3 protein and their expression is lost in XMeis3 morphant embryos. In the genetic cascade controlling embryonic neural cell specification, XMeis3 lies below general-neuralizing, but upstream of FGF and regional-specific genes. Thus, XMeis3 protein is positioned at a key regulatory point, simultaneously regulating multiple neural cell fates during early vertebrate nervous system development.

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Introduction

In the developing Xenopus central nervous system (CNS), anteroposterior (A-P) pattern is established by a multi-step inductive process. In this proposed two-step model (Nieuwkoop, 1952), the initial neural inducing signal is thought to specify anterior neuroectodermal structures, such as cement gland and forebrain; this step is referred to as “activation.” The subsequent caudalizing step is called “transformation.” In this second inductive wave, anterior neuroectodermal tissue is re-specified to more posterior fates, such as midbrain, hindbrain, and spinal cord. A number of molecules have been identified that participate in the “transformation” caudalization process. Three secreted factors were shown to caudalize neural tissue in whole Xenopus embryos or explants: retinoic acid, FGF, and XWnt3a (Durston et al., 1989; Sive et al., 1990; Sharpe, 1991; Kolm and Sive, 1995; Papalopulu and Kintner, 1996; Koide et al., 2001, Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995; Holowacz and Sokol, 1999; Ribisi et al., 2000; Domingos et al., 2001; Fletcher et al., 2006; McGrew et al., 1995; McGrew et al., 1997).

XMeis3, a TALE-family homeobox protein is initially expressed 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). Studies in Xenopus and zebrafish embryos demonstrate that Meis3 protein is required for proper hindbrain formation (Dibner et al.; 2001, Dibner et al., 2004; Waskiewicz et al., 2001; Vlachakis et al., 2001). In XMeis3 morphant Xenopus embryos, posterior expansion of the forebrain occurs, with a concomitant loss of hindbrain cell fates; yet pan-neural marker expression is unaltered (Dibner et al., 2001; Dibner et al., 2004). Complementing these results, ectopic XMeis3 expression caudalizes animal cap explants or embryos by inducing posterior neural marker expression in the absence of pan-neural markers (Salzberg et al., 1999). HoxD1 is a retinoic acid (RA) direct-response gene (Kolm and Sive, 1995) that is also an XMeis3 direct-response gene (Dibner et al., 2004). Ectopically expressed HoxD1 protein acts downstream of XMeis3 protein and rescues the loss of hindbrain phenotype in XMeis3 morphant embryos (Dibner et al., 2004). RA signaling and XMeis3 protein act in concert to activate HoxD1 expression; HoxD1 is minimally expressed in the absence of either factor.

XMeis3 is a transcription factor that can induce hindbrain cell fates in a non-cell-autonomous manner. The knock down of endogenous XMeis3 activity eliminates complete hindbrain formation, despite being expressed in only r2–r4 (Dibner et al., 2001). XMeis3 caudalizing activity is absolutely dependent on the presence of FGF/MAP kinase (MAPK) signaling (Ribisi et al., 2000, Aamar and Frank, 2004).

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XMeis3 protein activates the MAPK pathway, presumably by inducing FGF3 transcription in the hindbrain (Amar and Frank, 2004). XMeis3 is required for hindbrain induction center formation in zebrafish (Maves et al., 2002, Walsh et al., 2002) and XMeis3 and FGF3 are expressed in a similar temporal and overlapping regional manner during Xenopus development (Lombardo et al., 1998). In recombinant ectoderm explants, XMeis3 expressing tissue induced mesoderm-independent convergent extension cell elongations in adjacent juxtaposed neuralized explants. Elongated explants expressed hindbrain, primary neuron and neural crest markers; while forebrain marker expression was extinguished (Amar and Frank, 2004). These experiments suggest that XMeis3 protein acts non-autonomously to establish a hindbrain induction center that regulates early A-P cell fate in the developing CNS.

Since XMeis3 protein activity is required for proper cell fate determination and morphogenesis in the hindbrain, we were curious to determine if XMeis3 knockdown affected other early cell fate types in the developing nervous system. Like the posterior CNS, neural crest and primary neuron cells form in a similar locale and time window. The signaling pathways required for posterior CNS formation, such as FGF, canonical-Wnt, and RA are also necessary for neural crest and primary neuron cell induction (Blumberg et al., 1997, Franco et al., 1999, Sharpe and Goldstone, 1997, Hardcastle et al., 2000, Villanueva et al., 2002, Shiotsugu et al., 2004).

This present study shows that in addition to the hindbrain, XMeis3 protein activity is essential for the proper formation of both primary neuron and neural crest cells types. We have elucidated the hierarchy of upstream and downstream transcription factor proteins regulating XMeis3 induction of these multiple cell types. Neural-specific Zic and Pax3 proteins lie upstream of XMeis3 to regulate its expression. Ectopic XMeis3 protein rescues the loss of hindbrain, neural crest and primary neurons in embryos depleted of Zic protein activity. The HoxD1 protein acts downstream of XMeis3 protein; its ectopic expression rescues these cell types in Zic or XMeis3 protein knocked down embryos. FGF3 and FGF8 are XMeis3 direct target genes, and their expression is reduced in XMeis3 morphant embryos. Ectopic FGF expression can also rescue the XMeis3 morphant phenotype, though partially. In the genetic cascade controlling early neural cell fates, XMeis3 lies below neuralizing, but upstream of FGF3 and FGF8 and other regional-specific genes. Thus, XMeis3 protein is located at a central regulatory point, simultaneously regulating hindbrain, neural crest and primary neuron cell fates during early vertebrate nervous system development.

Materials and methods

Xenopus embryos and explants

Ovulation, in vitro fertilization, embryo and explant culture were carried out as described (Re'em-Kalma et al., 1995). Embryos were staged according to Nieuwkoop and Faber (1967).

RNA and morpholino oligonucleotide (MO) injections

Capped sense in vitro transcribed full-length mRNAs encoding XMeis3 (Salzberg et al., 1999), Xngnr1 (Ma et al., 1996), Zic5 dominant-negative (DN) protein (Nakata et al., 2000), HoxD1 and HoxD1-antimorph, (Dibner et al., 2004), FGF8a (Fletcher et al., 2006), Pax3 and Zic1 (Monsoro-Burq et al., 2005) proteins were injected into the animal hemisphere of one blastomere in embryos at the one or two-cell stages. Zygotic FGF8a expression was driven by a CMV based expression vector (Fletcher et al., 2006). Antisense morpholino oligonucleotides (MOs) complementing the 5’ region of the XMeis3 (Dibner et al., 2001), Zic1 (Sato et al., 2005), and Pax3 (Monsoro-Burq et al., 2005) were synthesized by Gene Tools, LLC; Philomath, Oregon, U.S.A. Previously published studies using mismatch MOs showed that the XMeis3, Zic1 and Pax3 MOs all work specifically to alter cell fates in Xenopus embryos (Dibner et al., 2001, Sato et al., 2005, Monsoro-Burq et al., 2005).

In situ hybridization

Whole-mount in situ hybridization was carried out with digoxigenin labeled probes (Harland 1991). Embryos were cultured to neurula stages and subsequently fixed for in situ hybridization. Probes used were XMeis3 (Salzberg et al., 1999), Krox20 (Bradley et al., 1992), N-tubulin (Ma et al., 1996), XMyr1 (Bellefroid et al., 1996), Slug (Bonstein et al., 1998), FoxD3 (Sasai et al., 2001), Id3 (Light et al., 2005), c-Myc (Bellmeyer et al., 2003), Pax3 (Monsoro-Burq et al., 2005), FGF3 (Lombardo et al., 1998), XANF-1 (Zaraisky et al., 1995), Zic R1/Zic1 (Mizuseki et al., 1998a), Zic2 (Nakata et al., 1998), Zic5 (Nakata et al., 2000), Sox2 (Mizuseki et al., 1998a) and SoxD (Mizuseki et al., 1998b).

Semi-quantitative 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: EFla, Krox20 (Hemmatti-Briovanliou and Melton 1994), HoxB3 (Hooiveld et al., 1999), XMeis3 (Dibner et al., 2004), HoxD1 (Kolm and Sive, 1995), SoxD (Kato et al., 1999), Zic1 and Zic2 (Nakata et al., 1998), FoxD3 (Sasai et al., 2001), FGF3 and FGF8 (Domingos et al., 2001), Slug, n-tubulin, Xngnr1, NeuroD and nrp1 (Harland Lab database).

XMeis3-GR inducible protein

RNA encoding the XMeis3-GR (glucocorticoid receptor) inducible fusion protein (Dibner et al., 2004) was injected at the one-cell stage. Animal cap (AC) explants were removed at blastula stages for culture. At late blastula stages, cyclohexamide (CHX, 5 μM) was added. After 1.5 hours, dexamethasone (DEX, 1 μM) was added, at gastrula stages 10–10.25. Explants were cultured until stage 12 and total RNA was isolated for RT-PCR analysis.

Results

XMeis3 protein is required for primary neuron and neural crest formation

Our initial studies showed that n-tubulin expression in the hindbrain and spinal cord regions was inhibited by ectopic XMeis3 antimorph protein activity (Dibner et al., 2001). In XMeis3 antimorph phenotypes, HoxB9 expression was fairly normal, suggesting that the spinal cord is preserved, but primary neurogenesis is impaired (Dibner et al., 2001). To further elucidate this point, embryos were injected with the XMeis3 MO in one blastomere at the two-cell stage. In XMeis3 morphant neurula stage embryos, there was a marked reduction in primary neurogenic gene expression. Similar to the XMeis3 antimorph protein, n-tubulin expression is strongly inhibited by the XMeis3 MO (Fig. 1A). N-tubulin serves as a marker for primary neuron differentiation, and its inhibition demonstrates that initial primary neuron formation in the spinal cord and hindbrain (including the most anterior trigeminal in r2) is highly disrupted (Fig. 1A). In addition, expression of upstream pro-neural genes, such as NeuroD, Xngnr1 (not shown), and XMyr1 was highly inhibited (Fig. 1B). At later tadpole stages, we examined XMeis3 morphant embryos for expression of the tanabin/nestin and Islet-1 (Xisl-1) genes, which mark hindbrain derived cranial neurons (Hemmatti-Briovanliou et al., 1992, Bardine et al., 2009). In XMeis3 morphant embryos, Xisl-1 and tanabin/nestin expression was highly reduced in the head,
suggesting that the cranial nerve V, VII, IX and X cell fates are also eliminated (Sup. Figs. 1A, B). We further addressed expression of more upstream genes regulating neurogenesis. Gene expression of the Zic and Sox families of neuralizing transcription factors was examined in XMeis3 morphant embryos. These genes lie upstream of pro-neural genes in regulating primary neuron cell formation (Sasai, 1998, Chitnis, 1999). We found that Zic (Zic1, Zic2, Zic3 (not shown) and Zic5) and Sox (Sox2, and SoxD) family gene expression levels were unchanged in XMeis3 morphant embryos (Figs. 1E-I). These results suggest that XMeis3 could be positioned in the middle of the cascade controlling differentiation of primary neurons during early neurogenesis; XMeis3 protein could lie downstream to the more generally expressed neurogenic genes, but upstream to regionally-specific expressed pro-neural genes.

XMeis3 protein activity is required for primary neuron cell formation in embryos, thus we also investigated the effect of XMeis3 knockdown on neural crest cell formation, since both primary neurons and neural crest are induced in the neural folds-plate regions by identical signaling pathways in a similar temporal manner. In XMeis3 morphant embryos, expression of the neural crest specific Slug and FoxD3 markers was highly inhibited (Figs. 1C, D). In later tadpole stages, neural crest derived melanocyte cells are severely depleted in morphant embryos (Sup. Fig. 1C). In contrast, expression of more upstream neural crest regulators, such as Pax3, c-myc and id3 is unaltered in morphant embryos (Figs. 1J-L). Zic1 and Zic5 expression levels were also unaltered in morphant embryos (Figs. 1E, G); these proteins are required for neural crest formation in Xenopus embryos (Nakata et al., 2000; Sato et al., 2005). As a control for XMeis3 MO injection, expression of Krox20 and Xngnr1 was inhibited in 94% and 77% of embryos, respectively (Xngnr1 is not shown) (Figs. 1A–C). However, for Sox, Zic, Pax3, c-Myc and Id3, overall gene expression levels are quite normal despite morphological perturbations on the XMeis3 MO injected side.
specificity, we have performed rescue experiments (Dibner et al., 2001). As determined by both RT-PCR (not shown) and in situ hybridization to embryos, FoxD3 (Sup. Fig. 2A); Krox20 and n-tubulin (not shown) gene expression is rescued by co-injection of the XMeis3 mRNA in XMeis3 morphants.

The HoxD1 protein lies downstream of XMeis3 in regulating multiple cell fates in the developing nervous system

Our previous studies showed that HoxD1 is an XMeis3 direct-target gene (Dibner et al., 2004). We also showed that ectopic HoxD1 expression rescued hindbrain cell fates in XMeis3 morphant embryos (Dibner et al., 2004). To confirm that HoxD1 acts downstream of XMeis3 in neural crest or primary neuron cell fate formation, we injected HoxD1 antimorph encoding RNA into embryos. Ectopic HoxD1 antimorph protein expression strongly inhibits hindbrain (Dibner et al., 2004), as well as primary neuron (n-tubulin) and neural crest (Slug, FoxD3) marker expression (Fig. 2Aa–d). Confirming previously published results (Dibner et al., 2004), XMeis3 expression was unaltered in all examined HoxD1 antimorph embryos (not shown, n = 16). Similar to XMeis3 morphants, Sox2 (not shown), Pax3 and Zic1 expression levels were not reduced in HoxD1 antimorph embryos (Fig. 2Ae–f). To determine that HoxD1 is a downstream mediator of XMeis3 activity for the specification of primary neuron or neural crest cell fates, we injected HoxD1 encoding RNA into embryos knocked down for XMeis3 protein. Ectopic HoxD1 protein expression rescued primary neuron (compare Fig. 2Bb to Bc) and neural crest (not shown) cell formation in XMeis3 morphant embryos. Interestingly, neural crest marker expression was only rescued under experimental conditions that did not rescue primary neuron or hindbrain marker expression (not shown).

Fig. 2. Ectopic expression of the HoxD1 antimorph protein knockdown inhibits primary neuron and neural crest markers in addition to hindbrain markers. (A) Two-cell stage albino embryos were injected unilaterally into the animal hemisphere of one blastomere with 100 pg of RNA encoding the HoxD1 antimorph protein. All embryos are injected on the right side, viewed dorsally and are oriented anterior (top), posterior (bottom). (a) Krox20 expression is inhibited in 87% of the embryos (n = 83/95). (b) N-tubulin expression is inhibited in 97% of the embryos (n = 57/59). (c) Slug expression is inhibited in 85% of the embryos (n = 44/52). (d) FoxD3 expression is inhibited in 87% of the embryos (n = 52/60). (e) Pax3 expression is normal in 100% of the embryos (n = 16/16). (f) Zic1 expression is normal in 93% of the embryos (n = 13/14). XMeis3 expression (not shown) is normal in 100% of the embryos (n = 16/16). (B) Ectopic HoxD1 expression rescues n-tubulin expression in XMeis3 morphant embryos. The dashed red lines mark the dorsal midline. (a) Uninjected control embryo. (b) 18 ng XMeis3 MO, n-tubulin expression (right side) was inhibited in 68% (n = 22) of the embryos. (c) 18 ng XMeis3 MO, 0.8 ng HoxD1 RNA, n-tubulin expression was highly rescued (left side) in 75% (n = 12) of the embryos.
Zic proteins act upstream of XMeis3/HoxD1 to regulate multiple cell fates in the developing nervous system

XMeis3 could be located at a key regulatory point, acting downstream of neuralizing Zic proteins and acting upstream of the specific genes regulating primary neuron or neural crest cell fates. Zic proteins are excellent candidates to be acting upstream of XMeis3 in regulating neural crest, primary neuron and/or hindbrain cell fates. Ectopic expression of Zic proteins in *Xenopus* embryos and AC explants activated expression of posterior neural, primary neuron and neural crest marker genes (Mizuseki et al., 1998a, Nakata et al., 1998, Kuo et al., 1998, Nakata et al., 2000, Sasai et al., 2001). In addition, knock down of Zic protein activities, using either the Zic5 dominant-negative (DN) protein or the Zic1 MO inhibited neural crest formation in *Xenopus* embryos (Nakata et al., 2000; Sato et al., 2005).

To address Zic/XMeis3 epistasis, we examined the phenotype of Zic protein inhibition. Embryos were injected with either RNA encoding the Zic5 DN protein or the Zic1 MO. We used these two complementary inhibitory approaches, since the Zic1 MO could be a more specific tool than the Zic5 DN protein, which may inhibit activity of all endogenous Zic proteins (Nakata et al., 2000). For the Zic5 DN embryos, posterior neural marker expression was examined by both whole mount *in situ* hybridization and semi-quantitative RT-PCR in gastrula (Fig. 3C) and neurula stage embryos (Figs. 3A, B). In addition to the anticipated loss of neural crest markers (Slug and FoxD3, Figs. 3Ab, d, B), Krox20 expression in the hindbrain (Fig. 3B, Fig. 4Fb), and expression of primary neuron markers such as Xngnr1 (Fig. 3B), NeuroD (not shown), and *n-tubulin* (Figs. 3Ac, B), were also inhibited in Zic5 DN phenotype embryos. Interestingly, XMeis3 expression was also eliminated from hindbrain and anterior spinal cord in the Zic5 DN protein injected embryos (Figs. 3Aa, B). As in Zic5 morphant embryos, Zic5 DN protein expression also eliminated *HoxD1* expression in gastrula stage embryos (Fig. 3C). This is the first observation of any upstream neurogenic gene that regulates embryonic XMeis3 expression, suggesting that early expressed neuralizing Zic gene family proteins may be required for the regional-specific transcriptional activation of XMeis3 and HoxD1 genes in the embryo.

Similar to the Zic5 DN protein, Zic1 morphant embryos also have highly reduced expression of hindbrain (*Krox20*, *HoxB3*), primary neuron (*NeuroD*, *n-tubulin*) and neural crest markers (*Slug*, *FoxD3*); pan-neural marker (*SoxD*, *Nrp1*) expression is normal (Figs. 3D, 4C). Marker inhibition is dose dependent, with hindbrain and primary neuron markers being more sensitive to lower Zic1 MO concentrations than the neural crest markers, whose expression was only inhibited at the highest injected Zic1 MO levels (Fig. 3D). Similar to the Zic5 DN phenotype, gastrula stage expression of XMeis3 (not shown) and

![Fig. 3. Inhibition of Zic protein activity by ectopic expression of the Zic5 dominant-negative (DN) protein or the Zic1 MO inhibits hindbrain, primary neuron, and neural crest marker expression. (A) Two-cell stage albino embryos were injected unilaterally into the animal hemisphere of one blastomere with 200 pg of Zic5 DN protein encoding RNA. All embryos are injected on the right side, viewed dorsally and are oriented anterior (top), posterior (bottom). (a) *XMeis3* expression is inhibited in 91% of the embryos (n = 74/81). (b) *FoxD3* expression is inhibited in 84% of the embryos (n = 42/50). (B) *Krox20* expression is inhibited in 83% of the embryos (n = 91/109). (C) *Slug* expression is inhibited in 86% of the embryos (n = 37/43). (D) One-cell stage embryos were injected in the animal hemisphere with 0.4 ng of Zic5 dominant-negative protein encoding RNA. Total RNA was isolated from pools of seven neurula stage 16 embryos in each group. Semi-quantitative RT-PCR analysis was performed with the markers: *XMeis3*, *Krox20*, *n-tubulin*, *Xngnr1*, *Slug*, *FoxD3*, and *EF1α*. In all shown experiments, *EF1α* serves as a control for quantitating RNA levels in the different samples. -RT-PCR was performed on total RNA isolated from normal embryos (not shown in this experiment). (D) One-cell stage embryos were injected in the animal hemisphere with 25, 30, 35, or 40 ng of Zic1 MO. RNA was isolated from pools of ten neurula stage 18 embryos in each group. Semi-quantitative RT-PCR analysis was performed with the markers: *Krox20*, *HoxB3*, *NeuroD*, *n-tubulin*, *Slug*, *FoxD3*, *SoxD*, and *EF1α*. -RT-PCR was performed on total RNA isolated from normal embryos.
HoxD1 genes is inhibited in Zic1 morphant embryos (Fig. 4B). As a control for Zic1 MO specificity, we have repeated rescue experiments (Sato et al., 2005). Similar to neural crest markers, Krox20 and n-tubulin gene expression is rescued by co-injection of the Zic1 mRNA in Zic1 morphants (Sup. Figs. 2B, C).

To determine Zic1/XMeis3 epistasis, XMeis3 encoding RNA was ectopically expressed in Zic1 morphant embryos. The Zic1 protein knock down phenotypes are rescued by either ectopic XMeis3 (Figs. 4A-D) or HoxD1 (not shown) protein expression, however not all markers to the same extent. As expected, Nrp1 (pan-neural)
expression in normal in morphant and rescued embryos (Fig. 4C). In almost all rescue experiments, the Krox20 hindbrain marker is re-expressed at high levels (Figs. 4A, C, D). In some experiments, ectopic XMeis3 protein strongly rescues hindbrain, with strong - intermediate rescue of primary neurons (Fig. 4C). At a lower frequency, neural crest cells are rescued (Fig. 4D), with or sometimes without rescue of primary neurons. Interestingly, hindbrain is usually the "default" rescue with consistent varying degrees of rescue penetrance for primary neuron or neural crest markers. We often observe that ectopic XMeis3 activity in wild type AC explants and embryos triggers high levels of hindbrain marker (Krox20) expression that can be associated with either an increase or a decrease in neural crest or primary neuron marker expression. In embryos and AC explants expressing relatively low ectopic XMeis3 RNA levels, there is often a more efficient activation of neural crest versus hindbrain marker expression (Sup. Figs. 3A–C); neural crest markers are induced at low mRNomeis3 concentrations that do not induce ectopic Krox20 expression. However, when XMeis3 mRNA concentrations are increased in AC explants or embryos, neural crest marker expression can be suppressed with the concomitant increase of Krox20 expression (Sup. Figs. 3B–E); primary neuron marker expression may increase or decrease (Sup. Figs. 3D, E). In any given experiment, the different location and concentration of XMeis3 protein in could bias for or against specific cell types. In cases of high XMeis3 levels, the preference to induce hindbrain cell fates may be favored; at low XMeis3 levels, neural crest cell fates are favored. Thus, these experiments reveal the differential effects of XMeis3 activity in regulating posterior neural cell fates.

In a complementary manner and similar to the Zic1 MO (Fig. 3D), higher concentrations of XMeis3 MO are required to inhibit neural crest marker expression versus hindbrain or primary neurons (Sup. Fig. 3F). Like the Zic1 MO, the XMeis3 MO maximally inhibits the hindbrain marker Krox20 at the lowest concentrations, the primary neuron marker, n-tubulin is moderately affected at lower concentrations, and the neural crest marker slug is optimally affected at higher concentrations. These results reveal that the inductions of these various posterior neural cell fates possess different threshold responses to XMeis3 protein (see Discussion).

Similar results were seen for embryos co-injected with Zic5 DN and XMeis3 encoding RNAs. In Zic5 knockdown embryos, ectopic XMeis3 expression robustly rescues Krox20 (compare Fig. 4Fb to Fc) and n-tubulin (compare Fig. 4Gb to Gc–d) and slug to a lesser extent (not shown). The rescue of neural crest markers only occurs in conditions in which Krox20 or n-tubulin expression was not rescued, suggesting that XMeis3 under certain conditions can induce regional specificity on the Zic5 knockdown phenotype. In general, XMeis3 rescue of the Zic5 DN phenotype was less efficient than for the Zic1 morphant phenotype. Due to its more general inhibitory activity for all Zic proteins, the Zic5 DN tended to give a more potent phenotype that was more difficult for rescue by ectopic XMeis3 expression.

Phenotypically, Zic1 morphant and Zic5 DN embryos (not shown) also undergo significantly reduced neural convergence-extension cell movements, with a subsequent inhibition of neural folding (compare Fig. 4Ea control, to Fig. 4Eb Zic1 morphant). The ectopic expression of XMeis3 protein dramatically restores these morphogenetic cell movements in about 50% of the Zic1 morphant embryos (Fig. 4Eb Zic1 morphant to the Fig. 4Ec–d Zic1 MO/XMeis3 co-injected). This observation concurs with our previous findings, that XMeis3 protein activity is crucial to regulate correct neural convergent-extension morphogenetic cell movements in the posterior neural plate (Amar and Frank, 2004).

In the Zic1 morphant phenotype, XMeis3 (not shown) and HoxD1 (Fig. 4B) expression is inhibited. We wanted to determine if ectopic HoxD1 protein could rescue the Zic knock down phenotype. In embryos separately co-injected with the Zic5 DN protein and HoxD1 RNA, there is significant rescue of Krox20 (compare Fig. 4Fb to Fd) and n-tubulin expression (not shown); however, it was difficult to detect significant rescue of neural crest markers under these experimental conditions. Also, supporting these observations, co-injection of XMeis3 RNA rescues HoxD1 expression in Zic1 morphant embryos (Fig. 4B).

Our observations in embryos were also complemented by experiments performed in caudalized AC explants. AC explants are caudalized by the coinjection of wnt3a and noggin protein encoding RNAs (Monsoro-Burq et al., 2005). The combination of these neuralizing and caudalizing activities efficiently activates expression of the Krox20, HoxB3, FoxD3, and n-tubulin markers (Figs. 5A, B). Expression of Zic family mRNAs, such as Zic1 and Zic2 is also robustly induced in this assay (Fig. 5B). Expression of XMeis3 transcripts is also highly induced in these neuralized/caudalized AC explants (Elkouby and Frank unpublished data). When these caudalized explants are co-injected with either the Zic5 DN RNA or the Zic1 MO, there is a striking inhibition of posterior neural marker expression (Figs. 5A, B), despite the expression of the pan-neural Zic1, Zic2 and SoxD genes (Fig. 5B). Presumably, the caudalizing Zic protein activity in the untreated neuralized/caudalized explants is blocked by the Zic5 DN protein in the translational blocking Zic1 MO. Similar to whole embryos, ectopic expression of XMeis3 protein significantly restores expression of hindbrain (Krox20, HoxB3) and primary neuron markers in AC explants when Zic protein activities are knocked down (Figs. 5A, B); similar to embryos, rescue of neural crest marker expression was weak. However, XMeis3 expression did rescue Zic protein knockdown of neural crest marker expression in experiments in which the wnt3a/noggin concentration exclusively activated neural crest markers, but none of the other posterior neural markers (data not shown).

XMeis3 also lies downstream of Pax3 protein

Pax3 is another early expressed gene that was shown to interact with Zic proteins in regulating neural crest formation (Monsoro-Burq et al., 2005; Sato et al., 2005). In XMeis3 morphant embryos Pax3 expression was unaltered, suggesting that Pax3 could lie upstream to XMeis3 (Fig. 2F). To clarify if XMeis3 lies downstream to Pax3, XMeis3 expression was examined in Pax3 morphant embryos. In Pax3 morphant embryos, XMeis3 expression was strongly reduced (Fig. 6A). It was previously shown that Pax3 morphant embryos lacked neural crest cells (Monsoro-Burq et al., 2005; Sato et al., 2005). However, in addition to loss of neural crest marker expression, slug (Fig. 6B) and FoxD3 (not shown), there is also a sharp reduction in n-tubulin and Krox20 expression in Pax3 morphant embryos (Figs. 6C, D). As in XMeis3 morphants, Pax3 morphant embryos expressed fairly normal levels of the c-myc gene, which is required for neural crest specification, as well as the pan-neural expressed Sox2 gene (Figs. 6E, F). Thus, similar to Zic and Meis3 protein knockdown, Pax3 protein knockdown also eliminated hindbrain and primary neuron cell fates, suggesting that Pax3 lies upstream of XMeis3 to regulate all three of these cell fates. Unlike the Zic5 DN protein and Zic1 MO, ectopic XMeis3 expression could not rescue the Pax3 morphant phenotype (not shown). Apparently, some cue is lacking in Pax3 morphant embryos that is required for XMeis3 protein activity. As a control for Pax3 MO specificity, we have repeated rescue experiments (Monsoro-Burq et al., 2005). Krox20 and FoxD3 gene expression is rescued by co-injection of the Pax3 mRNA in Pax3 morphants (Sup. Figs. 2D, E).

These observations in embryos were complemented by experiments performed in caudalized AC explants. AC explants are caudalized by the coinjection of Pax3 and Zic1 protein encoding mRNAs (Monsoro-Burq et al., 2005; Sato et al., 2005). The combination of these two protein activities efficiently activates expression of the HoxB3, n-tubulin, and Slug markers (Fig. 5C). Previous studies had
XMeis3 protein activity is not co-injected embryos, XMeis3 MO knockdown mimics the same inhibitory effects seen downstream to neural marker expression in early neurula stage AC explants, and that XMeis3 protein cannot caudalize in the absence of FGF signaling (Ribisi et al., 2004). To determine if FGF3 and FGF8 are direct target genes acting downstream of XMeis3 protein expression, we injected RNA encoding an inducible XMeis3-GR fusion protein (Dibner et al., 2004). When AC explants were pre-treated with CHX before DEX induction of XMeis3 protein activity, FG3 and FGF8 mRNAs were still robustly induced (Fig. 8A). As a control, we show that the induction of Krox20 expression is strongly inhibited by CHX treatment. Complementing this result, we also found that FGF3 (Fig. 8B) and FGF8 (not shown) expression in early-mid neurula stage embryos was severely inhibited in XMeis3 morphant embryos. We have not seen a reciprocal effect on XMeis3 expression by manipulating FGF levels in embryos or explants. Neither ectopic expression of FGF nor its knockdown significantly modulates XMeis3 expression in explants or embryos (not shown). To determine if FGF proteins act downstream to XMeis3 protein, XMeis3 morphant embryos were co-injected with CMV-vector driving zygotic FGF8a or FGF3 expression. By examining embryo morphology or neural marker expression by whole mount in situ hybridization, we detected a significant rescue of the XMeis3 morphant phenotype. Ectopic FGF8a expression rescued neural plate folding and convergent extension in XMeis3 morphant embryos (Fig. 8C). Interestingly, expression of FGF8a strongly inhibited expanded expression of the anterior XANF-1 marker in morphants (Fig. 8D). N-tubulin expression was robustly enhanced in embryos expressing FGF8a and partially rescued in morphant embryos expressing FGF8a (Fig. 8E). FGF3 was able to inhibit and rescue XANF-1 and n-tubulin respectively, in addition to a partial rescue of the neural crest marker, FoxD3 (not shown) in XMeis3 morphants. Yet, under these experimental conditions, hindbrain markers were not significantly rescued. These results strengthen our previous observations (Ribisi et al., 2000, Aamar and Frank 2004). To determine if FG3 and FGF8 are direct target genes of the XMeis3 protein, we injected RNA encoding a pool of 10 pg Wnt3a RNA/10 pg noggin RNA and/or 0.3 ng of Zic5 DN RNA and/or 0.2 ng of XMeis3 RNA. Animal cap (AC) explants were removed at blastula stages 8–9. Explants and embryos were cultured to neurula stage 18, and total RNA was isolated from pools of sixteen AC explants in each group and five control embryos (CE). RT-PCR analysis was performed with the markers: Krox20, HoxB3, n-tubulin, FoxD3 and EF1α. -RT-PCR was performed on total RNA isolated from normal embryos. Note the strong level of inhibition of posterior neural marker expression and their rescue by XMeis3 expression. (C) One-cell stage embryos were separately injected in the animal hemisphere with a pool of 10 pg Wnt3a RNA/10 pg noggin RNA and/or 27 ng of Zic1 MO and/or 0.4 ng of XMeis3 RNA. Animal cap (AC) explants were removed at blastula stages 8–9. Explants and embryos were cultured to neurula stage 18, and total RNA was isolated from pools of eleven to eighteen AC explants in each group and five control embryos (CE). RT-PCR analysis was performed with the markers: Krox20, n-tubulin, FoxD3, Zic1, Zic2, Sox2 and EF1α. -RT-PCR was performed on total RNA isolated from normal embryos. Note the strong level of Zic1 MO inhibition of posterior neural marker expression and their rescue by XMeis3 expression. (C) One-cell stage embryos were separately injected in the animal hemisphere with a pool of 0.15 ng Zic1 RNA/0.15 ng Pax3 RNA and/or 25 ng of XMeis3 MO. Animal cap explants were removed at blastula stages 8–9. Explants and embryos were cultured to neurula stage 17, and total RNA was isolated from pools of fifteen AC explants in each group and five control embryos (CE). RT-PCR analysis was performed with the markers: Krox20, HoxB3, n-tubulin, Slug, and EF1α. -RT-PCR was performed on total RNA isolated from normal embryos. Note the strong level of XMeis3 MO inhibition of posterior marker expression in the far right row.

The Xngnr1 protein acts downstream of XMeis3 to specifically regulate primary neuron cell fates

HoxD1 lies downstream of XMeis3 to induce multiple cell fates, yet some other target proteins must lie further downstream of XMeis3/HoxD1 to regulate specific neural cell lineages. Xngnr1 is one of the earliest regionally expressed pro-neural genes regulating primary neuron formation (Ma et al., 1996). We were curious to determine if ectopic Xngnr1 protein levels could rescue primary neuron cell fates in XMeis3 morphant embryos in which n-tubulin expression is eliminated (Figs. 7A, B). Xngnr1 encoding RNA was co-injected with the XMeis3 MO. Expression of Xngnr1 encoding RNA alone induces n-tubulin expression on the injected side of normal embryos (Ma et al., 1996; Fig. 7C). In Xngnr1/ XMeis3 MO co-injected embryos, n-tubulin is expressed at high ectopic levels (Fig. 7D). Thus XMeis3 protein activity is not required for Xngnr1 protein to activate n-tubulin transcription. This effect is specific for primary neurons, since Krox20 (hindbrain) expression is not rescued in the Xngnr1 injected embryos (not shown). It appears that Xngnr1 is a stronger activator of n-tubulin expression in embryos lacking XMeis3 activity versus controls (compare Figs. 7C, D). Hence, the proper balance of XMeis3 activity in the embryo may be required to direct cells to their various cell fates, but in XMeis3 morphants, cells are driven to a specific cell-fate direction by ectopic expression of a lineage-specific downstream activator such as Xngnr1.

FGF3/8 are direct target genes acting downstream of XMeis3 protein

Our previous studies showed that XMeis3 activated FGF3 and FGF8 gene expression in early neurula stage AC explants, and that XMeis3 protein cannot caudalize in the absence of FGF signaling (Ribisi et al., 2000, Aamar and Frank 2004). To determine if FGF3 and FGF8 are direct target genes of the XMeis3 protein, we injected RNA encoding an inducible XMeis3-GR fusion protein (Dibner et al., 2004). When AC explants were pre-treated with CHX before DEX induction of XMeis3 protein activity, FG3 and FGF8 mRNAs were still robustly induced (Fig. 8A). As a control, we show that the induction of Krox20 expression is strongly inhibited by CHX treatment. Complementing this result, we also found that FGF3 (Fig. 8B) and FGF8 (not shown) expression in early-mid neurula stage embryos was severely inhibited in XMeis3 morphant embryos. We have not seen a reciprocal effect on XMeis3 expression by manipulating FGF levels in embryos or explants. Neither ectopic expression of FGF nor its knockdown significantly modulates XMeis3 expression in explants or embryos (not shown). To determine if FGF proteins act downstream to XMeis3 protein, XMeis3 morphant embryos were co-injected with CMV-vector driving zygotic FGF8a or FGF3 expression. By examining embryo morphology or neural marker expression by whole mount in situ hybridization, we detected a significant rescue of the XMeis3 morphant phenotype. Ectopic FGF8a expression rescued neural plate folding and convergent extension in XMeis3 morphant embryos (Fig. 8C). Interestingly, expression of FGF8a strongly inhibited expanded expression of the anterior XANF-1 marker in morphants (Fig. 8D). N-tubulin expression was robustly enhanced in embryos expressing FGF8a and partially rescued in morphant embryos expressing FGF8a (Fig. 8E). FGF3 was able to inhibit and rescue XANF-1 and n-tubulin respectively, in addition to a partial rescue of the neural crest marker, FoxD3 (not shown) in XMeis3 morphants. Yet, under these experimental conditions, hindbrain markers were not significantly rescued. These results strengthen our previous observations (Ribisi et al., 2000, Aamar and Frank 2004). To determine if FGF3 and FGF8 are direct target genes of the XMeis3 protein, we injected RNA encoding an inducible XMeis3-GR fusion protein (Dibner et al., 2004). When AC explants were pre-treated with CHX before DEX induction of XMeis3 protein activity, FG3 and FGF8 mRNAs were still robustly induced (Fig. 8A). As a control, we show that the induction of Krox20 expression is strongly inhibited by CHX treatment. Complementing this result, we also found that FGF3 (Fig. 8B) and FGF8 (not shown) expression in early-mid neurula stage embryos was severely inhibited in XMeis3 morphant embryos. We have not seen a reciprocal effect on XMeis3 expression by manipulating FGF levels in embryos or explants. Neither ectopic expression of FGF nor its knockdown significantly modulates XMeis3 expression in explants or embryos (not shown). To determine if FGF proteins act downstream to XMeis3 protein, XMeis3 morphant embryos were co-injected with CMV-vector driving zygotic FGF8a or FGF3 expression. By examining embryo morphology or neural marker expression by whole mount in situ hybridization, we detected a significant rescue of the XMeis3 morphant phenotype. Ectopic FGF8a expression rescued neural plate folding and convergent extension in XMeis3 morphant embryos (Fig. 8C). Interestingly, expression of FGF8a strongly inhibited expanded expression of the anterior XANF-1 marker in morphants (Fig. 8D). N-tubulin expression was robustly enhanced in embryos expressing FGF8a and partially rescued in morphant embryos expressing FGF8a (Fig. 8E). FGF3 was also able to inhibit and rescue XANF-1 and n-tubulin respectively, in addition to a partial rescue of the neural crest marker, FoxD3 (not shown) in XMeis3 morphants. Yet, under these experimental conditions, hindbrain markers were not significantly rescued. These results strengthen our previous observations (Ribisi et al., 2000, Aamar and Frank 2004).
Fig. 6. Pax3 knockdown inhibits primary neuron and hindbrain markers in addition to neural crest marker expression. Two-cell stage albino embryos were injected unilaterally into the animal hemisphere of one blastomere with 20 ng of Pax3 MO. All embryos are viewed dorsally and are oriented anterior (top), posterior (bottom). In each embryo trio panel, the control embryo is on the left, Pax3 mis-match MO injected (mm) center, and the Pax3 MO (MO) on the right. The red arrows mark the dorsal midline. (A) In the shown experiment, XMeis3 expression was inhibited in 76% of the Pax3 morphant embryos (n = 21). Expression was normal in uninjected controls (91%, n = 23) and embryos injected with a 5 bp mm Pax3 MO (90%, n = 22). In all experiments, XMeis3 expression is inhibited in 83% of the morphant embryos (n = 38). (B) In the shown experiment, Slug expression is inhibited in 95% of the morphant embryos (n = 21). Expression was normal in uninjected controls (85%, n = 20) and embryos injected with a 5-bp mm Pax3 MO (76%, n = 17). In all experiments, Slug expression is inhibited in 93% of the morphant embryos (n = 55). FoxD3 expression is inhibited in 79% of the morphant embryos (n = 33, not shown). (C) In the shown experiment, n-tubulin expression was inhibited in 78% of the embryos (n = 18) Expression was normal in uninjected controls (81%, n = 21) and embryos injected with a 5-bp mm Pax3 MO (75%, n = 24). In all experiments, n-tubulin expression is inhibited in 90% of the morphant embryos (n = 58). (D) In the shown experiment, Krox20 expression is inhibited in 95% of the morphant embryos (n = 21). Expression was normal in uninjected controls (76%, n = 21) and embryos injected with a 5-bp mm Pax3 MO (74%, n = 19). In all experiments, Krox20 expression is inhibited in 93% of the morphant embryos (n = 133). (E) In the shown experiment, c-myc expression was normal in morphant embryos (86%, n = 22). Expression was normal in uninjected controls (88%, n = 24) and embryos injected with a 5 bp mm Pax3 MO (77%, n = 22). In all experiments, c-Myc expression is normal in 92% of the Pax3 morphant embryos (n = 38). (F) Sox2 expression was normal in morphant embryos (76%, n = 23). Expression was normal in uninjected controls (91%, n = 23) and embryos injected with a 5-bp mm Pax3 MO (82%, n = 22).

Fig. 7. Ectopic Xngnr1 expression rescues n-tubulin expression in XMeis3 morphant embryos. Two-cell stage albino embryos were co-injected unilaterally into the animal hemisphere of one blastomere with 10–20 ng of XMeis3 MO and/or 0.8 ng of RNA encoding the Xngnr1 protein. All embryos are injected on the right side, viewed dorsally and are oriented anterior (top), posterior (bottom). Whole mount in situ hybridization of the n-tub marker is shown. A. N-tubulin expression is normal in 100% of the uninjected control embryos (n = 22/22). B. N-tubulin expression is highly inhibited in 92% of the XMeis3 morphant embryos (n = 11/12). As a control for XMeis3 MO activity, Krox20 expression was examined (not shown); its expression was inhibited in 90% of the embryos (n = 9/10). (C) Injection of Xngnr1 RNA induces ectopic n-tubulin expression in 87% of the control embryos (n = 20/23). (D) Injection of Xngnr1 RNA induces high ectopic n-tubulin expression levels in 100% of the XMeis3 morphant embryos (n = 18/18). Krox20 was not ectopically activated in these embryos (not shown).
that the FGF signaling pathway acts downstream to XMeis3 protein in the process of neural A→P patterning.

**Discussion**

*Meis3 protein is required for induction of primary neuron and neural crest fates*

In this study, we examined the pathway hierarchy in which XMeis3 protein regulates multiple cell fate specification in the developing nervous system (Sup. Fig. 4B—model). In neurula embryos, the knock down of XMeis3 was shown to eliminate primary neuron and neural crest cell fates in addition to the hindbrain. Furthermore, hindbrain derived neurons and neural crest derived melanocytes were also severely depleted in later stage XMeis3 morphant tadpoles. In XMeis3 morphant embryos, transcription of a battery of proneural and downstream target genes that are required for primary neuron induction, such as Xngnr1, NeuroD, XMyt1 and n-tubulin was inhibited. Ectopic Xngnr1 protein can rescue n-tubulin expression in XMeis3 morphant embryos, suggesting that it lies downstream to XMeis3 protein. In this study, we show a novel role for Zic1 and Pax3 proteins in posterior neural patterning. In addition to their role in regulating neural crest specification (Monsoro-Burq et al., 2005; Sato et al., 2005), we now show that Zic1 and Pax3 proteins have a previously undiscovered more general role in regulating both hindbrain and primary neuron cell fates (Sup. Fig. 4—model).

In XMeis3 morphant embryos, the pre-migratory neural crest markers, such as slug and FoxD3 were eliminated, whereas expression of the c-myc and id3 genes was not altered. c-myc and id3, which lie upstream to slug and FoxD3 are also expressed in more anterior neural fold regions that are not fated for neural crest. These genes are likely players in a parallel upstream XMeis3-independent component of neural crest specification. Interestingly, the expression of other upstream regulators of neural crest induction, such as Pax3 and Msx1 (not shown), is also unaltered in XMeis3 morphant embryos. Pax3
and Msx1 lie upstream of XMeis3 in regulating neural crest induction (Monsoro-Burq et al., 2005; Sato et al., 2005). These proteins are thought to interact with Zic1 to induce neural crest (Sato et al., 2005), thus supporting the observation that XMeis3 is epistatically positioned between neurogenic and cell specific genes. XMeis3 protein is positioned at a central regulatory point that is crucial for inducing primary neuron, neural crest and hindbrain cell types.

**Zic1 and Pax3 proteins lie upstream to XMeis3 in regulating multiple posterior neural cell fates**

The proteins regulating XMeis3 expression therefore give a clue as to the initial upstream pathways regulating early cell fate decisions in the nervous system. Sox and Zic family transcription factors have been associated as neuralizing proteins lying downstream of BMP signaling antagonism in the neural induction process (Sasai, 1998). Indeed Sox and Zic family genes are expressed at quite normal levels in XMeis3 morphant embryos. Zic proteins have the potential to induce posterior neural, primary neuron and neural crest cell fates in Xenopus explants (Mizuseki et al., 1998a, Nakata et al., 1998, Kuo et al., 1998, Nakata et al., 2000, Sato et al., 2005). The knockdown of Zic protein activity was also shown to inhibit neural crest formation in Xenopus embryos (Nakata et al., 2000, Sato et al., 2005). In addition to the disruption of neural crest, we show that embryos knocked down by Zic5 DN protein or the Zic1 MO lose primary neuron and hindbrain cell types. XMeis3 expression was also eliminated in these embryos. To prove that XMeis3 lies downstream of Zic protein activity, ectopic XMeis3 protein was expressed along with the Zic5 DN protein or Zic1 MO. In these embryos, the Zic protein loss-of-function phenotype was eliminated and hindbrain, primary neuron, and neural crest cell fates were rescued to varying degrees by ectopic XMeis3 protein. Additionally, the loss of neural folds phenotype typical of Zic1 morphant embryos was significantly rescued in XMeis3-expressing embryos.

Hindbrain, primary neuron and neural crest cell-types do not appear to be equivalent in their XMeis3/Zic1 epistasis. In XMeis3 morphant embryos, hindbrain genes are the most sensitive to changing XMeis3 levels, with primary neuron and neural crest cell fates typically requiring a stronger morphant phenotype (Sup. Fig. 3F). A similar observation was seen in Zic1 morphant embryos (Fig. 3D). The knockdown of Zic1 was originally shown to prevent neural crest formation (Monsoro-Burq et al., 2005; Sato et al., 2005). However, we have found that similar to XMeis3 morphants, neural crest cells are consistently lost at higher concentrations of Zic1 MO than those required to eliminate either hindbrain or primary neuron cell fates. This observation could be a direct result of residual XMeis3 expression levels present in the weaker Zic1 morphant phenotype. In AC explants and embryos, neural crest markers are only induced at very low ectopic XMeis3 expression levels that do not efficiently induce hindbrain or primary neurons (Supps. Fig. 3A–C). At typically higher XMeis3 injection levels, hindbrain cell fates are the dominant default state for XMeis3 activity, with markers like Krox20 or HoxB3, being induced in the absence or presence of neural inducers (Dibner et al., 2001). In embryos injected with very high ectopic levels of XMeis3 mRNA, we have observed a high ectopic Krox20 expression with reductions in neural crest marker expression (Sup. Figs. 3D, E). Thus, higher ectopic levels of XMeis3 preferentially drive hindbrain formation, but intermediate and lower level XMeis3 levels are permissive and required for primary neuron and neural crest formation. The results of XMeis3 rescue in Zic1 morphants also support this idea, since Krox20 was always the easiest gene to rescue followed by n-tubulin and lastly slug/FoxD3. At the time of neural caudalization, in late gastrula/early neurula stages, cells in the posterior neural plate and neural crest region are all in very close proximity. Our previous studies show that XMeis3 acts both autonomously and non-autonomously to induce hindbrain, primary neuron and neural crest cell fates (Aamar and Frank, 2004, unpublished observations). Thus the early events specifying individual cell fates downstream of XMeis3 activity are not necessarily equivalent. For example, neural crest fates appear to occur in cells with lower XMeis3 activity, whereas hindbrain fates are induced by high XMeis3 activity. XMeis3 is expressed in these earlier stages in presumptive hindbrain cells and thus this could drive their fate; whereas, in contrast, perhaps the non-autonomous “weaker” XMeis3 activity is what drives the induction of the adjacent neural crest cells that do not express XMeis3. In this manner the balance between “stronger” autonomous and “weaker” non-autonomous activities may drive differential cell fate specifications. Future experiments utilizing our recombinant exploitant assay system (Aamar and Frank, 2004) should elucidate this point.

It is still not clear if the role of Zic1 protein is to exclusively regulate XMeis3 expression levels. Alternatively, the combination of Zic1 and XMeis3 protein concentration ratios in a given cell could possibly determine differential cell fate outcomes. These genes have non-identical but partially overlapping expression patterns during development. The question of XMeis3 and Zic1 interactions in determining neural cell fates, either in a cell-autonomous or non-autonomous manner can also be addressed in embryos or explants co-expressing varying ectopic levels of each protein.

Previous studies suggested that Pax3 and Zic proteins interact to regulate neural crest formation (Monsoro-Burq et al., 2005; Sato et al., 2005). In this study, we significantly advanced this concept by showing that elimination of Pax3 or Zic protein activities prevents proper XMeis3 expression in the embryo which triggers the loss of hindbrain, primary neuron, and neural crest cell fates. Re-addition of XMeis3 to embryos knocked down for Zic, but not Pax3 activities rescues these phenotypes. Thus, upstream Pax3 and Zic proteins control XMeis3 gene expression, which then acts downstream to trigger multiple cell fate decisions in the nervous system.

**HoxD1 protein lies downstream to XMeis3 regulating posterior neural cell fate specification**

We previously showed that the HoxD1 protein was an XMeis3 direct-target gene (Dibner et al., 2004). We demonstrated that ectopic HoxD1 expression rescued hindbrain marker expression (Krox20 and HoxB3) in XMeis3 morphant embryos. Similar to XMeis3 knockdown, we show that ectopic HoxD1 antimorph protein expression also prevented primary neuron and neural crest induction. We now show that ectopic HoxD1 expression also rescues primary neuron and neural crest marker expression in XMeis3 morphant embryos. Thus HoxD1 protein is positioned downstream of XMeis3 in the cascade regulating embryonic nervous system cell fate decisions. The knockdown of the Hox paralogous group 1 (PG1) genes (Hox A1-B1-D1) in Xenopus caused severe defects in hindbrain and neural crest formation, but primary neuron cell formation was not examined in this study (McNulty et al., 2005). Knockdown of XMeis3 activity eliminates embryonic HoxD1 and HoxB1 expression (Dibner et al., 2001, Dibner et al., 2004). Presumably HoxA1 expression is also inhibited, but this still has to be examined. The HoxD1 antimorph protein and XMeis3 MO may therefore induce a similar global HOX PG1 knock down phenotype. Thus by regulating Hox PG1 gene family expression, XMeis3 can direct these early neural cell fate decisions.

**Meis3 protein integrates multiple caudalizing signaling pathways**

What still needs to be discerned is how XMeis3 or Hox PG1 proteins can simultaneously induce specificity in these different cellular regions. For example, we show that Xngnr1 lies downstream of XMeis3, where it exclusively induces primary neurons and not neural crest or hindbrain cell fates when ectopically expressed in XMeis3 morphant embryos. Therefore the question arises as to how
regional-specific expression of genes like Xngnr1 / NeuroD, slug / FoxD3, or Krox20 / HoxB3 occurs, thus enabling correct cell fate specification. The basis of these variant responses could reflect innate differential competence potentials in the cells bordering XMeis3 expressing cells. XMeis3 is expressed as early as gastrula stages in a distinct stripe mapping to the presumptive hindbrain region (Salzberg et al., 1999). XMeis3 expression is regionally restricted, yet the effects of its knockdown are widespread, altering cell fates in the hindbrain, spinal cord and neural crest regions. Many of these cells do not have overlapping XMeis3 expression domains. One possible explanation for this observation is that XMeis3 protein could induce these different cell fates in a non-cell autonomous manner (Aamar and Frank, 2004). These results suggest that XMeis3 expressing cells form a very early neural patterning induction center, responsible for specification of hindbrain, primary neuron, and neural crest cell fates. At late gastrula stages, when this center would be active, the target cells would all be clustered in fairly close proximity. XMeis3 responsive cells in the embryo may have a competence “history” of higher or lower canonical-Wnt, RA, or FGF signaling levels, which when coupled to a permissive signal emanating from XMeis3 expressing cells may enable a pre-built-in bias in cell fate decision to occur. Thus identical signaling pathways, utilizing differentially expressed Wnt or FGF ligands, or RA receptors could be utilized downstream of XMeis3 in distinct embryonic regions to caudalize cells to variant cell fates along the A–P axis.

The signaling pathways required for posterior CNS formation, such as FGF, canonical-Wnt, and RA are also necessary for neural crest and primary neuron cell fate induction (Blumberg et al., 1997, Franco et al., 1999, Sharpe and Goldstone, 1997, Hardcastle et al., 2000, Villanueva et al., 2002, Wu et al., 2005, Monsoro-Burq et al., 2005; Sato et al., 2005). How do we incorporate neural caudalizing XMeis3 protein and these signaling pathways into a working model? Our previous studies showed that XMeis3 activates FGF3 and FGF8 gene expression and that XMeis3 protein cannot induce posterior cell fates in the absence of downstream FGF signaling in Xenopus AC explants (Ribisi et al., 2000, Aamar and Frank, 2004). We now show that the FGF3 and FGF8 genes are direct targets of XMeis3 protein and that neurula stage FGF3 expression is eliminated in XMeis3 morphant embryos (Fig. 8). In this study, ectopic expression of FGF8a partially rescued the XMeis3 morphant phenotype. Ectopic expression of either FGF8a or FGF3 mRNAs inhibited the robust anterior expansion of anterior XANT-1 expression and rescued n-tubulin expression. It still needs to be determined how differing doses and timing of either FGF3 and/or FGF8 isoform activities may act downstream of XMeis3 protein to pattern the nervous system. However, these results suggest that XMeis3 protein induces downstream FGF activity that acts to regulate neural patterning. Our unpublished studies show that canonical-Wnt signaling is required for embryonic XMeis3 expression (Elkouby and Frank). Supporting this observation, in caudalized Xenopus explants, FGF acting downstream to canonical Wnt signaling (Dominos et al., 2001). RA signaling appears to additively interact with XMeis3 protein to optimize early neurula stage Hox gene expression in order to fine tune embryonic A–P patterning (Dibner et al., 2004). Thus, XMeis3 protein could act downstream to canonical Wnt signaling, upstream to FGF signaling and in concert with RA signaling to activate gene expression and thus regulate embryonic neural caudalization (Sup. Fig. 4—model).

In future experiments, we intend to address these questions of cellular responsiveness and competence. In our recombinant AC explant assay system, an XMeis3 expressing AC induces hindbrain, primary neuron (Aamar and Frank, 2004) and neural crest (unpublished) markers in juxtaposed cells neuralized by the BMP DN receptor. In this assay, we can determine if ectopic or knocked down levels of a specific signaling pathway/s can bias the direction of XMeis3 protein to induce specific types of posterior nervous system cells. This manipulation of signaling pathways in explants to modify neural cell fates may recapitulate the normal signals interacting with XMeis3 protein in the embryo.

Acknowledgments

We thank Drs. Y. Sasai, N. Sasai, J. Aruga, R.B. Fletcher, A.H. Monsoro-Burq, K.J. Liu, and T. Grammar for plasmids and MOs. D. F. was supported by grants from the Israel Science Foundation (197/05), Israel Cancer Research Fund, B. and G. Greenberg Technion Research Fund for the Advancement of Research and the F.F. Technion Research Fund. R.O. was supported by a Lady Davis Foundation postdoctoral fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.024.

References
