Hmx4 regulates Sonic hedgehog signaling through control of retinoic acid synthesis during forebrain patterning

Patricia A. Gongal a,1, Lindsey D. March a, Vanessa L. Holly a, Laura M. Pillay a, Karyn M. Berry-Wynne a, Hiroyuki Kagechikab, Andrew J. Waskiewicz a,⁎

a Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9
b Graduate School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, 101-0062, Japan

A R T I C L E   I N F O
Article history:
Received for publication 19 December 2010
Revised 12 April 2011
Accepted 14 April 2011
Available online 22 April 2011

Keywords:
Forebrain
Retinoic acid
Sonic hedgehog
Hmx
Nkx5
Zebrafish

A B S T R A C T
Mutations in H6-homeobox (HMX) genes are linked to neural mispatterning and neural tube closure defects in humans. We demonstrate that zebrafish Hmx4 regulates the signaling of two morphogens critical for neural development, retinoic acid (RA) and Sonic hedgehog (Shh). Hmx4-depleted embryos have a strongly narrowed eye field and reduced forebrain Shh target gene expression. hmx4 morphants fail to properly transcribe the Shh signal transducer gli3, and have reduced ventral forebrain specification. Hmx4-depleted embryos also have neural tube patterning defects that phenocopy RA-deficiency. We show that Hmx4 is required for the initiation and maintenance of aldh1a2, the principal RA-synthesizing gene. Loss of RA is the primary defect in Hmx4-depleted embryos, as RA treatment rescues a number of the neural patterning defects. Surprisingly, RA treatment also rescues forebrain morphology, gli3 transcription, and Shh signaling. We propose that Hmx4 is a critical regulator of retinoic acid synthesis in a developing embryo, and that this regulation is essential for controlling Shh signaling and forebrain development.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Neural tube closure and patterning during embryogenesis are highly dependent on correct levels of retinoic acid (RA), a small lipophilic morphogen. Excess or insufficient RA levels are associated with profound phenotypes. Abnormal RA levels in the developing nervous system cause severe defects in patterning of the telencephalon, diencephalon, eye, and hindbrain, and increase the likelihood of neural tube closure defects. Outside of the nervous system, proper RA signaling is critical for the development of the heart, fin, somites, and craniofacial skeleton. RA binds to transcription factors of the retinoic acid receptor (RAR) family, which heterodimerize with retinoid X receptors (RXR). Upon ligand binding, the RAR–RXR complex exchanges a suite of corepressors for coactivator proteins, and initiates transcription of target genes (reviewed in Maden, 2002; Niederreither and Dolle, 2008). Temporally and spatially dynamic patterns of RA levels are established by the activities of RA synthesizing (aldehyde dehydrogenases, and the cytochrome p450 protein, cyp1b1) and degrading enzymes (cytochrome p450s cyp26a1, cyp26b1, and cyp26c1) (Begemann et al., 2001; Emoto et al., 2005; Grandel et al., 2002; Gu et al., 2005; Hernandez et al., 2007; Pittlik et al., 2008). Concomitant with RA-dependent anterior–posterior patterning, the neural tube is patterned dorso-ventrally by the morphogen Sonic hedgehog (Shh), which specifies ventral identity. Loss of Shh signaling is a well-established cause of severe forebrain patterning defects, including holoprosencephaly, a birth defect characterized by a lack of cerebral hemisphere separation (Belloni et al., 1996; Roessler et al., 1996). In the most severe cases, holoprosencephaly patients display cyclopia (reviewed in Ingham and Placzek, 2006). Shh ligand is secreted from the axial mesoderm and floor plate after post-translational processing (reviewed in Farzan et al., 2008) and binds its co-receptor, Patched, on target cells. This binding relieves the inhibition of Smoothened, and activates a signal cascade that regulates Gli family transcription factors, which in turn bind enhancers of target genes. Pioneering studies revealed that the single Drosophila Gli ortholog, Cubitus interruptus, is proteolytically cleaved in the absence of a hedgehog signal, and acts as a repressor of target genes (Azab-Blanc et al., 1997). Activation of the Shh signaling pathway blocks this cleavage, allowing the protein to retain an activation domain and induce the transcription of its targets. Multiple gli genes are present in vertebrates (3 in mice, and 4 in zebrafish), each with a distinct spatial and temporal expression pattern and loss-of-function phenotypes (Jacob and Briscoe, 2003; Karlstrom et al., 2003; Ke et al., 2005; Ruiz i Altaba et al., 2003; Tyurina et al., 2005). Additionally, some Gli proteins function mainly or solely as either a repressor or activator form, rather...
than undergoing a functional switch (reviewed in Jacob and Briscoe, 2003). Several Gli orthologs also show different functions between vertebrate species. For example, in mice, while Gli2 acts as the main activator, and Gli1 is dispensable, in the zebrafish forebrain, Gli1 functions as the main activator, while Gli2 mainly functions as a repressor (Bai et al., 2002; Karlstrom et al., 2003). In both mice and zebrafish, Gli3 can act as a repressor or activator of hedgehog targets depending on signaling activity, and is of particular importance for dorsal–ventral patterning of the neural tube (Bai et al., 2004; Persson et al., 2002; Tyurina et al., 2005). In Gli3 mutant mice, dorsal forebrain structures are lost, reflecting a loss of repression of dorsal Shh targets (Franz, 1994; Grove et al., 1998; Johnson, 1967; Theil et al., 1999). In contrast, gli3 zebrafish morphants have a loss of select ventral forebrain Shh target genes (Tyurina et al., 2005), and knocking Gli3 into the Gli2 locus in mice rescues select Gli2 loss-of-function phenotypes (Bai et al., 2004), demonstrating Gli3’s dual role as an activator or repressor of select target genes.

The hmx (H6 homeobox) genes (also known as the Nkx5 family) are an ancient class of homeobox transcription factors (Wang et al., 2000). Human patients with HMX1 mutations have specific eye and ear defects (Schorderet et al., 2008). In mice and humans, Hmx1 expression mid-gestation is mainly restricted to specific cell types within the retina and otic vesicle (Schorderet et al., 2008; Yoshiura et al., 1998). However, an HMX1-deficient patient has also displayed spine bifida and craniofacial anomalies (Schorderet et al., 2008), and two additional groups have reported that mice lacking hmx1 have severe neural and craniofacial defects, including exencephaly and the absence of all nasomaxilliary structures (Munroe et al., 2009; Wang and Lufkin, 2005). In fact, prior to becoming enriched in the optic and otic vesicles, Hmx1 is expressed broadly throughout the brain (Gray et al., 2004). This early expression and incidence of neural tube defects in cases of HMX1-deficiency suggests that HMX genes have an earlier role in nervous system development than previously realized. While mammals have a single HMX1-like gene, the avian and teleost evolutionary lineages have two HMX1 paralogs (in zebrafish, hmx1 and hmx4).

In addition to defects in core components of the Shh or RA pathways, mutations in transcription factors that regulate members of these pathways have also been linked to congenital forebrain defects (Geng et al., 2008; Gongal and Waskiewicz, 2008; Maurus and Harris, 2009). Here, we present evidence that hmx4, the Hmx paralog most strongly and broadly expressed during early zebrafish development, regulates both Shh and RA signaling. hmx4 morphants display phenotypes associated with the loss of both pathways, including strong narrowing of the forebrain and neural tube closure defects. Tissues dependent on RA for their development are incorrectly patterned or absent in Hmx4-depleted embryos, and we show that Hmx4 is required for the proper transcription of RA synthesis genes. Concomitantly, Shh signaling output in the forebrain is attenuated, and the transcription of the Shh signal transducer gli3 is strongly reduced. Surprisingly, forebrain morphology, Shh signaling output, and gli3 transcription can be rescued by RA treatment. Thus, this work identifies hmx4 as a novel regulator of both RA and Shh signaling, and reveals a critical functional cross-talk between these two pathways during neural tube patterning, at the level of gli3 transcription.

Materials and methods

Embryos, morpholinos and specificity controls, and statistics

Wild-type embryos were of the AB or isl1:gfp (Higashijima et al., 2000) strains, and were staged according to Kimmel et al.(1995). Morpholino oligonucleotides (MO; GeneTools) were heated to 65 °C for 10 min and allowed to cool to room temperature before microinjection into 1- to 4-cell embryos. p53 morpholino was used to suppress morpholino-induced apoptosis (Robu et al., 2007). Two MOs against hmx4 with non-overlapping target sites were used (MO1: 5′-CGGCTCTCCTTTGCTCA-TATTCTCC-3′; MO2: 5′-GGTGCTGTCTGTCCTTTTTC-3′). When injected independently, the two MOs give identical phenotypes (one neural tube, forebrain narrowing; data not shown). Embryos injected with 3 ng of a mismatch morpholino (5′-ACCCGGCTATTGCGCA-TAGTCTCA-3′) have no detectable phenotype (data not shown). We also confirmed that each MO specifically knocks down the translation of eGFP preceded by its targeting sequence (Fig. S2). Together, these data indicate the phenotype we describe is specific for the knockdown of hmx4. Embryos used for analysis were treated with 1 ng each of MO1 and MO2 plus 1 ng of p53 MO or with p52 MO alone. For statistical analyses, student t-tests, χ2 tests, or two-tailed Fisher’s exact tests were applied with α set at 0.01.

RT-PCR, in situ hybridization, and immunohistochemistry

One-step RT-PCR was performed with total RNA (RNAqueous, Ambion) and the Superscript III RT-PCR kit (Invitrogen) using hmx1, hmx4, or elongation factor 1α-specific primers (sequences available upon request). To generate probes for in situ hybridization, 600–1000 bp fragments were generated by RT-PCR and used as template for probe synthesis directly, or cloned into pCR4-Topo (Invitrogen). Probes were synthesized, purified, and in situ hybridization was performed as described (Gongal and Waskiewicz, 2008).

Pharmacological treatments

All-trans retinoic acid (RA; Sigma) and the RAR agonist Am80 (Kagechika and Shudo, 2005) were stored as a 10 mM stock in DMSO and diluted into embryo medium to 1 or 50 nM as specified. The aldehyde dehydrogenase inhibitor diethylaminobenzaldehyde (DEAB, Sigma) was stored as a 100 mM stock in DMSO and diluted in embryo medium to 10 μM. Embryos (in their chorions) were treated in the dark from 2 to 5 hpf to the time of fixation. In all experiments, controls are siblings treated with an equivalent amount of DMSO.

Real-time quantitative PCR

RNA was extracted from 18 hpf control or hmx4 morphant embryos, cDNA synthesized, and qPCR performed and analyzed as previously described (Pillay et al., 2010). Intron-spanning gli3, aldh1a2, and elongation factor 1-alpha (endogenous control) primer sequences were selected from the Universal Probe Library Assay Design Center for Zebrafish (Roche) and are available upon request. Prior to cDNA analysis, primer sets were validated as previously described (Pillay et al., 2010).

Results

Loss of Hmx4 causes severe neural defects in zebrafish

To study their role during early neural patterning, we first examined the expression of the two HMX1-related genes in zebrafish, hmx1 and hmx4. hmx1 is not detectable by in situ hybridization until mid-somitogenesis, when it is robustly expressed in the optic vesicle (Fig. 1A, C, F–G). In contrast, hmx4 transcript is present from the 1-cell stage (Fig. 1B–D), and is broadly expressed during gastrulation stages (Fig. 1H–L). After the completion of gastrulation, hmx4 becomes highly enriched in the retina (Fig. 1M–O). RT-PCR analysis reiterates that hmx4 transcript is detectable from the one-cell stage (Fig. 1E).

As the Hmx paralog expressed broadly and early in development, we examined whether hmx4 plays a role in neural patterning by performing morpholino knock-down. hmx4 morphants display a profound narrowing of the eye field (Fig. 2A–B), as well as a failure of the neural tube to close (Fig. 2C–D). We also observe small ears
(Fig. 2E–F) and the loss of pectoral fins (Fig. 2G–H). We assayed neuronal patterning by knocking down Hmx4 in the Tg(isl1:eGFP) transgenic line, which labels a subset of cranial motor neurons. We observe a strong reduction in the number of vagal motor neurons that are present in hmx4-depleted embryos (Fig. 2I–J; see also Fig. 5. K, M).

Hmx4-depleted embryos have deficient retinoic acid signaling and synthesis

Several of the phenotypes observed in hmx4 morphants (small ear, loss of fins, loss of vagal motor neurons) strongly resemble embryos with reduced retinoic acid signaling: the zebrafish aldh1a2 mutants nofin and neckless both display these phenotypes (Begemann et al., 2001; Grandel et al., 2002). To determine whether retinoic acid (RA) signaling is affected in hmx4 morphants, we examined early RA-dependent gene transcription. The gene otx2 is expressed in anterior neuroectoderm and is repressed by RA signaling (Kudoh et al., 2002). In hmx4 morphants, the domain of otx2 is strongly expanded toward the posterior of the embryo (Fig. 3A). The genes vhnf1 and meis3 are expressed in the presumptive hindbrain and require RA for their initiation (Maves and Kimmel, 2005). Both of these genes are reduced in hmx4 morphants (Fig. 3B–C). In hmx4 morphants, the domain of hoxd4a, which is expressed in r7 and the spinal cord, is strongly shortened (Fig. 3D), reminiscent of the phenotype of aldh1a2 zebrafish mutants (Grandel et al., 2002). Overall, the pattern of RA-dependent gene expression in hmx4 morphants is consistent with a reduction in RA signaling.

We hypothesized that abnormal regulation of RA levels might underlie the attenuation of RA signaling in hmx4 morphants. We therefore examined the expression of genes known to synthesize or
expression domain is RA-dependent. In signaling, and at low levels in the presumptive hindbrain, an and margin, expression domains which are independent of RA-
ring, but the low-level expression in the presumptive hindbrain is cyp26a1 expression (Fig. 4D).

We next examined the expression of the RA hydroxylases, cyp26a1, cyp26b1, and cyp26c1, which collectively function to target RA for degradation. cyp26a1 is present in the anterior neuroectoderm and margin, expression domains which are independent of RA-signaling, and at low levels in the presumptive hindbrain, an expression domain is RA-dependent. In hmx4 morphants, the anterior domain of cyp26a1 expression is slightly expanded towards the germ ring, but the low-level expression in the presumptive hindbrain is abolished (Fig. 4D), reiterating that RA signaling is attenuated. cyp26b1 and cyp26c1 are initiated during early somitogenesis, and are expressed in a rhombomere-specific pattern in the hindbrain. In hmx4 morphants, r6 and r7 expression of cyp26b1 is strongly reduced, while cyp26c1 in r3 and r4 is unaffected (Fig. 4F–G), suggesting that caudal rhombomeres, which require the highest RA levels for patterning, are most disrupted in the hmx4 morphant hindbrain. As we observe near-normal or reduced cyp26 levels in hmx4 morphants, changes in RA degradation do not explain a reduction in signaling activity. In contrast, the strong reduction in aldh1a2 expression suggests that reduced RA synthesis is responsible for the signaling deficiency.

We therefore hypothesized that the phenotypes we observe in hmx4 morphants are caused by an early loss of RA synthesis. To test this idea, we treated morphants with a low dose of exogenous RA. A 1 nM dose causes no detectable morphological phenotypes, and does not induce hmx4 expression (Fig. S2). hmx4 morphants treated with 1 nM RA show a rescue of eye field narrowing, neural tube closure and vagal motor neuron specification (Fig. 5). To quantify these rescues, we scored phenotypic frequencies (Fig. 5E, J, O). While no control embryos (n = 83) nor embryos treated with 1 nM RA (n = 82) show eye field narrowing at 48 hpf, 41% (±17%, n = 97) of hmx4 morphants display this phenotype. When hmx4 morphants are treated with 1 nM RA, this frequency decreases to 24% (±11%, n = 83), a statistically significant rescue (χ2 = 6.95, p < 0.01, df = 1). For the open neural tube phenotype, while no control embryos (n = 44) nor embryos treated with 1 nM RA (n = 48) show the phenotype at 18 hpf, 63% (±5%, n = 49) of hmx4 morphants display an open neural tube. When hmx4 morphants are treated with 1 nM RA, this frequency decreases to 34% (±16%, n = 76), a statistically significant rescue (χ2 = 10.8, p < 0.01, df = 1). Similarly, while no control embryos (n = 46) nor embryos treated with 1 nM RA (n = 48) showed any defects in the vagal motor neurons, 78% (±5%, n = 49) of hmx4 morphants display a strong reduction in this class of neurons. When hmx4 morphants are treated with 1 nM RA, the frequency of this phenotype decreases by more than half to 35% (±10%, n = 71), a statistically significant rescue (χ2 = 23.7, p < 0.001, df = 1). Together with the similarity between RA loss-of-function phenotypes and those of hmx4 morphants, these phenotypic rescues indicate that eye field narrowing, loss of vagal motor neurons and the open neural tube defect are due to reduced RA levels in hmx4 morphants.

**Hmx4-depleted embryos have deficient Sonic hedgehog signaling**

While deficiencies in RA signaling are likely to be directly related to the hindbrain neural patterning defects we observe in hmx4 morphants, the etiology of the forebrain patterning defects is less clear. Although RA rescues eye field narrowing in hmx4 morphants, aldh1a2 zebrafish mutants and DEAB-treated embryos only display an extremely mild narrowed eye field (Begemann et al., 2001 and unpublished observations, PG), which is not as severe as hmx4 morphants. We therefore hypothesized that defects in a second signaling pathway may help explain this striking phenotype. Deficiencies in Shh signaling are the most frequent cause of eye field narrowing, so we examined Shh target gene expression in 18 hpf hmx4 morphants to assess the efficacy of signaling of this pathway. ptc1, a direct Shh transcriptional target, is expressed in regions of high Shh signaling, including the ventral neural tube and somites. In hmx4 morphants, the domain of forebrain ptc1 expression is reduced (Fig. 6A–B). The Shh target nlc2.2a is expressed in the ventral telencephalon and diencephalon, and its domain of expression is also reduced in hmx4 morphants (Fig. 6C–D). These data...
suggest that Shh signaling is reduced in the forebrain of hmx4 morphants.

Loss of Shh signaling could be due to a reduction in transcription of the sonic hedgehog genes (in zebrafish, shha and/or shhb, also known as "tiggy-winkle hedgehog"), or by the loss of downstream signaling components. shha mRNA expression appears normal in the forebrain, notochord, and floorplate (Fig. 6E–H) of hmx4 morphants. Surprisingly, the expression of shhb is mildly increased in the ventral forebrain of hmx4 morphants (Fig. 6I–J). Since a reduction in shh transcription cannot explain the loss of signaling, we hypothesized that defects in other components of the pathway may underlie the signaling defect in hmx4 morphants. We first investigated the expression of smo, which is unaffected in hmx4 morphants (data not shown). Next, we examined the expression of members of the gli gene family. In zebrafish, Gli1 functions as the main activating Gli, while Gli2 acts mainly as a repressor in the forebrain, and Gli3 has important repressive and activating roles (Karlstrom et al., 2003; Tyurina et al., 2005). In hmx4 morphants, gli1 expression is upregulated and expanded dorsally in the forebrain (58% of morphants, n = 19; Fig. 6K–L), while gli2 expression is unaffected (n = 19; Fig. 6M–N). Markedly, transcription of gli3 is strongly reduced in the forebrain of hmx4 morphants (Fig. 6O–P). We quantified this result by performing qPCR, and find that compared to controls, hmx4 morphants have a 63.2% reduction in gli3 transcription (t = 27.9, p < 0.0001, df = 15, Fig. 6S). As gli1 is commonly used as a read-out to assess Shh activity, that it is expanded in hmx4 morphants while other forebrain Shh targets are reduced, is surprising. However, gli3 loss-of-function models have previously been shown to have a suite of defects that do not precisely mirror (or indeed are the converse of) the loss of the Shh ligand itself, namely an expansion of gli1 expression, as well as expansion of pax2a (Furimsky and Wallace, 2006; Tyurina et al., 2005). We therefore next examined the expression of pax2a to distinguish whether the Hmx4 loss-of-function phenotype more closely resembled a Shh or Gli3 deficiency. We find that pax2a is strongly expanded throughout the forebrain (Fig. 6Q–R), more consistent with the loss-of-function phenotype of Gli3. Thus, the reduction of gli3 transcription is likely to explain the changes in Shh target gene expression when Hmx4 is depleted.

**Retinoic acid rescues Sonic hedgehog signaling in Hmx4-depleted embryos**

Aldh1a2 mutant mice have previously been shown to have reduced Shh target gene expression, and fail to properly respond to exogenous Shh signaling (Ribes et al., 2006; Ribes et al., 2009). However, the mechanism of RA regulation of Shh-responsiveness is unknown. We wondered whether RA-deficiency in hmx4 morphants could play a causal role in disrupted Shh signaling. To test this hypothesis, we treated hmx4 morphants with a low dose of RA and examined Shh target gene expression in the forebrain (Fig. 7). 57% of hmx4 morphants show a reduction of ptc1 (40% moderately reduced, 17% strongly reduced, n = 30). This loss of expression is significantly rescued (Fisher’s exact test, p < 0.001) by RA treatment, with 50% of RA-treated morphants having moderately reduced ptc1, and 0% with a strong reduction (n = 46). 79% of hmx4 morphants have reduced nklx2.2a expression (43% with a moderate reduction, 36% with a strong reduction, n = 44), which is also significantly rescued (Fisher’s exact test, p < 0.001) by RA treatment (42% with a moderate reduction in expression, 0% with a strong reduction, n = 36). That we observe a partial restoration of forebrain Shh target gene expression in RA-treated hmx4 morphants indicates that RA can rescue morphants’ Shh signaling deficiency.

**Fig. 4.** hmx4 morphants have disrupted RA metabolism gene expression. ald1ha2 is reduced in the paraxial mesoderm of hmx4 morphants at 90% epiboly (A), and in the somites (B) and dorsal optic vesicle (C) at 18 hpf. (D) qPCR showing the significantly reduced levels of ald1ha2 transcription in hmx4 morphants (see text for statistical tests), with the levels in wild type embryos set to 1. Error bars indicate standard deviation. (E) cyp26a1 is mildly expanded towards the posterior in hmx4 morphants, and abolished in the presumptive hindbrain domain (bracket). In hmx4 morphants, the r6–7 domain of cyp26b1 is abolished (F), while the r3–r4 domain of cyp26c1 is not affected (G).
RA can act by binding and activating RAR-dependent transcription, but also binds other nuclear receptors (Schug et al., 2007), and can have transcription-independent effects (Alique et al., 2006; Aoto et al., 2008; Geissmann et al., 2003). To examine if RAR-dependent transcription could rescue Shh signaling, we treated hmx4 morphants with the RARα and RARβ agonist Am80 (Kagechika and Shudo, 2005). Like RA, Am80 treatment of hmx4 morphants also rescues eye field narrowing, indicating that the activation of RARs is sufficient to restore Shh signaling in hmx4 morphants (data not shown).

As the loss of Gli3 explains a number of the forebrain patterning defects observed in hmx4 morphants, and hmx4 forebrain defects are rescued by RA treatment, we hypothesized that RA deficiency is responsible for the reduction of gli3 transcription in hmx4 morphants. We first tested whether a moderate increase or reduction in RA signaling affects gli3 transcription levels. Treating embryos with 50 nM RA strongly expands gli3 expression (Fig. 8A–B), while treatment with the aldehyde dehydrogenase inhibitor DEAB reduces it (Fig. 8C–D). These results demonstrate that RA is sufficient, and...
endogenously required, to drive gli3 transcription in the zebrafish forebrain. This RA-dependent regulation of gli3 expression is consistent with findings in the Xenopus posterior neural plate (Franco et al., 1999) and mouse forebrain (Ribes et al., 2006), indicating this interaction is conserved between species. We next tested whether the reduction of RA signaling in hmx4 morphants is responsible for the loss of gli3. We indeed observe a rescue of gli3 expression in RA-treated hmx4 morphants (Fig. 8E-H). 85% of hmx4 morphants have reduced gli3 expression (31% with a moderate reduction, 54% with a strong reduction, n=39), which is significantly rescued (Fisher's exact test, p=0.001) by RA treatment (30% with a moderate reduction, 14% with a strong reduction, n=56). Together with the strong reduction of aldh1a2 and RA signaling in hmx4 morphants, these results strongly suggest that Hmx4 regulates gli3 transcription, Shh signaling, and forebrain patterning by regulating RA levels.

Discussion

In this work, we demonstrate that the homeobox protein Hmx4, via the transcriptional control of RA metabolism and the regulation of gli3, regulates Shh signaling output and forebrain development in zebrafish. This positions Hmx4 as a novel regulator of the Shh and RA pathways and reveals that gli3 transcriptional regulation is a critical point of cross-talk between the Shh and RA pathways. As activation of RARs is sufficient to rescue forebrain defects in hmx4 morphants, we favor a model whereby liganded RAR complexes indirectly or directly regulate gli3 expression. An examination of a reported enhancer sequence for gli3 (Paparidis et al., 2007) indeed reveals a highly conserved retinoic acid response element (RARE) of the DR5 class (unpublished observations, PG). Whether this is a bona fide functional RARE, which would suggest a direct regulation of gli3 by RAR/RXR complexes, clearly remains to be determined.

Gli3 mutants in mice and gli3 morphants in zebrafish have major forebrain patterning defects. A subset of these phenotypes resemble Shh loss-of-function (for example, the reduction of nkx2.2a) which suggests that they result from the loss of a Gli3 activation function. Others, however, are the converse phenotype of Shh loss-of-function (for example, the increase in the domain of pax2a). The gene expression changes we observe in hmx4 morphants closely resemble those seen in Gli3 knockdown models. However, loss of Gli3 has not been reported to cause severe eye field narrowing, as we see in hmx4 morphants. Therefore, while a reduction in gli3 transcription very
likely contributes to forebrain mispatterning, the etiology of the narrowed eye field phenotype is more complex. One alternative is that Hmx4 regulates one or more additional positive inputs into the Shh pathway.

hmx4 morphants have a strong reduction in aldh1a2 transcription, and show a suite of phenotypes typical of RA deficiency, including a small ear, loss of pectoral fins, and reduced vagal motor neurons. Although RA treatment can rescue the narrowed eye field, the forebrain phenotype of hmx4 morphants is far more severe than zebrafish aldh1a2 mutants or DEAB-treated embryos. Together, these observations suggest that aldh1a2-dependent RA deficiency contributes to, but is not solely responsible for the Shh signaling defect. Hmx4 may thus regulate an as-yet unidentiﬁed source of RA, perhaps one that is independent of aldehyde dehydrogenase activity, or may influence Shh signaling through a RA-independent mechanism. Overall, we favor a “multi-hit” model, where eye field narrowing is caused not only by the loss of activating Gli3, but additional Shh signaling deﬁciencies that together result in such a severe neural phenotype.

Overall, the phenotypes of RA-deﬁcient zebrafish are mild compared to other model organisms, strongly suggesting that zebrafish may indeed have an additional source of RA during embryogenesis. The zebrafish mutants neckless and nofin, which have point mutations in critical domains of the Aldh1a2 protein, as well as zebrafish embryos treated with the aldehyde dehydrogenase inhibitor DEAB have a slightly narrowed eye ﬁeld (Begemann et al., 2001; Grandel et al., 2002), although a detailed analysis of forebrain patterning has not been reported. In contrast, both aldh1a2 mouse mutants and vitamin A-deﬁcient quail have a single prosensephalic vesicle, but two lateral optic vesicles (Halilagic et al., 2003; Ribes et al., 2006). It is difﬁcult to achieve a complete absence of retinoid activity in any animal model, however, as some RA is required for the progression of pregnancy in mice, for egg-laying in birds (Dickman et al., 1997; Plack et al., 1964) and there are also several aldehyde dehydrogenase-independent mechanisms of RA synthesis (Theodosiou et al., 2010). Therefore, neither of these models, nor DEAB-treated zebrafish embryos, have a complete absence of retinoid activity. In chick, application of pharmacological retinoid receptor antagonists causes a more severe phenotype, including cyclopia and the severe loss of forebrain tissues (Schneider et al., 2001). This may represent the most severe loss of RA signaling activity in an animal model to date, and may most clearly reﬂect the importance of RA in forebrain midline development. Interestingly, retinoid receptor antagonist treated chick embryos show a loss of shh transcript. Although this phenotype may reﬂect the more severe RA signaling deﬁciency in these embryos, alternatively, it could represent a species-speciﬁc RA-Shh interaction, as shh transcription at early stages is not affected in mouse or zebrafish RA-deﬁcient models (Begemann et al., 2001; Grandel et al., 2002; Ribes et al., 2006; Ribes et al., 2009).

In mice, RA is required for the response to the Shh signal, independent of an effect on Shh transcription. Aldh1a2 mutants show reduced expression of SHH target genes, while no early changes in Shh expression are apparent (Ribes et al., 2006). However, mutants fail to appropriately activate Shh target gene expression upon exposure to
exogenous Shh protein (Ribes et al., 2009), indicating that RA is required for response to the Shh signal. One possible interpretation of these findings in light of the current work is that Gli3 underlies this requirement of RA for effective Shh signaling.

Mice lacking either Cyp26a1 or Aldh1a2 display an open neural tube (Abu-Abed et al., 2001; Ribes et al., 2006), and human mutations in both ALDH1A2 and CYP26A1 have been linked to spina bifida (Deak et al., 2005; Rat et al., 2006). These results suggest that too much or too little RA is deleterious for neural tube closure. An HMX1 mutation is associated with a case of spina bifida (Schorderet et al., 2008), and Hmx1 mouse mutants have been reported to display neural tube defects including exencephaly (Munroe et al., 2009; Wang and Lufkin, 2005). There is broad functional overlap between Hmx genes: Drosophila Hmx is normally expressed in a subset of neuronal precursors (Wang et al., 2000), but can rescue both hypothalamic and ear defects in mice mutant for Hmx2 and Hmx3 (Wang et al., 2004). That embryos depleted of Hmx4 protein show an open neural tube reflects a loss of Shh signaling, most notably a narrowed otic pit field, and we demonstrate that Hmx4, via RA, regulates the transcription of gli3, a critical Shh pathway transducer. Both Shh-deficient phenotypes and gli3 levels in Hmx4-depleted embryos can be rescued by restoration of RA, identifying a key mechanism of cross-talk between these two critical developmental signaling pathways.

Conclusions

Precise coordination of RA and Shh signaling is critical to proper neural patterning during development. We have discovered a novel role for the homeobox gene hmx4 in zebrafish, whose functional ortholog in humans has been linked to congenital neural defects. Hmx4 is required for proper transcription of the main RA synthesis gene, aldh1a2, and tissues dependent on RA are disrupted in Hmx4-depleted embryos. A subset of phenotypes in Hmx4-depleted embryos reflect a loss of Shh signaling, most notably a narrowed otic pit field, and we demonstrate that Hmx4, via RA, regulates the transcription of gli3, a critical Shh pathway transducer. Both Shh-deficient phenotypes and gli3 levels in Hmx4-depleted embryos can be rescued by restoration of RA, identifying a key mechanism of cross-talk between these two critical developmental signaling pathways.

Acknowledgments

This study was supported by NSERC. PG, LM, LP, KBW, and VH are recipients of NSERC, Alberta Ingenuity, Alberta Innovates Health Solutions, and QELI graduate scholarships, and PG is currently the recipient of Fondation de Pierre-Gilles de Gennes and Ecole Normale Supérieure postdoctoral fellowships. AJW is a Canada Research Chair. We wish to thank the members of the Waskiewicz and Charnay labs for critical comments on this work and Aleah McCorry for excellent fish husbandry.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.04.018.

References


