Developmental Biology 355 (2011) 55–64

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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 Kagechika [b], 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

ARTICLE INFO

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

ABSTRACT

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.

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 (Aza-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

* Corresponding author. Fax: +1 780 492 9234.
E-mail address: aw@ualberta.ca (A.J. Waskiewicz).
[1] Present Address: Institut de Biologie de l'Ecole Normale Superieure, U1024 INSERM, UMR 8197 CNRS, École Normale Supérieure, Paris cedex 05, 75230, France.

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.04.018

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 spina 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′-CGCGTCCTCCTTGCTCA-TATTCTCC-3′; MO2: 5′-GGGTCCGTGTCTGTGCCTCTTTTTC-3′). When injected independently, the two MOs give identical phenotypes (open neural tube, forebrain narrowing; data not shown). Embryos injected with 3 ng of a mismatch morpholino (5′-AGCGGCCTCATTGCGCA-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 *p53* MO alone. For statistical analyses, student t-tests, χ^2 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 1*α-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).

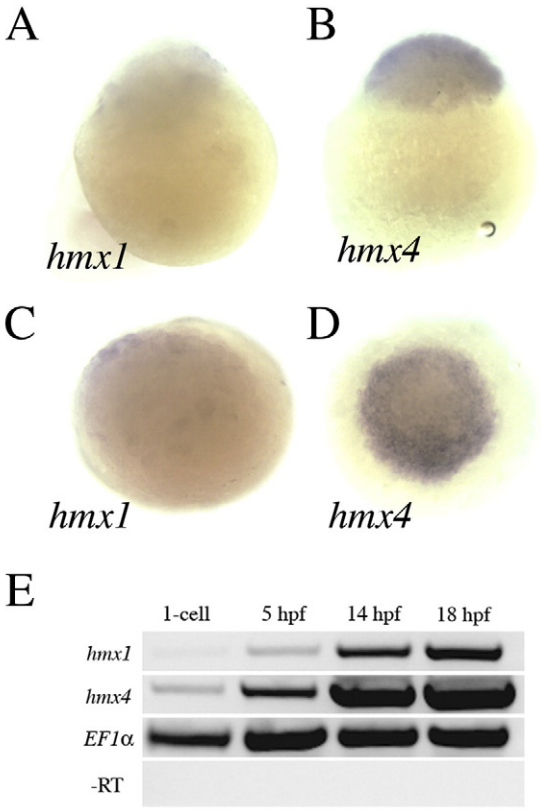
Results

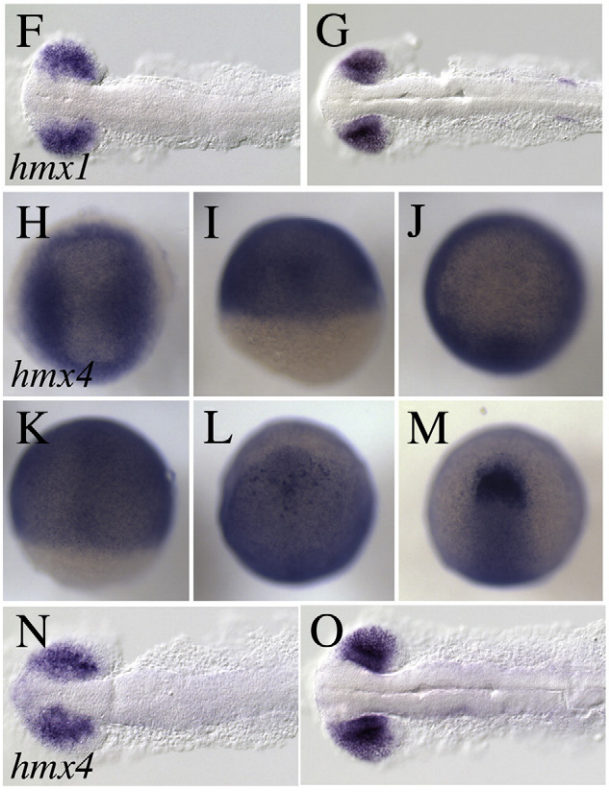
Loss of Hmx4 causes severe neural defects in zebrafish

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).





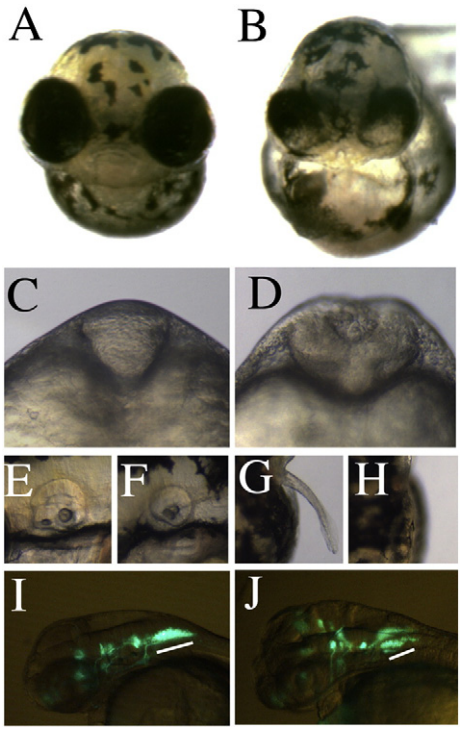


Fig. 2. Hmx4 deficient embryos have a narrowed eye field and open neural tube. (A–B) *hmx4* morphants have a strongly narrowed eye field, shown at 3 dpf. (C–D) The neural tube fails to close in *hmx4* morphants, shown at 18 hpf. *hmx4* morphants have (E–F) small otic vesicles, (G–H) a loss of pectoral fins, and (I–J) a reduction in vagal motor neurons (underlined in white), assessed in the Tg(*isl1:gfp*) line at 3 dpf.

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 neurectoderm 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

Fig. 1. *hmx1* and *hmx4* expression during early zebrafish development. *hmx1* is not detectable in 1-cell embryos by in situ hybridization (lateral view in A; dorsal view in C), while robust expression of *hmx4* is detected (lateral view in B; dorsal view in D). *hmx1* transcript can be weakly detected by RT-PCR at 5 hpf, and becomes robust at 14–18 hpf (E), when it is strongly expressed in the eye and otic vesicle (F–G). *hmx4* transcript can be detected at the 1-cell stage by RT-PCR, and is robustly expressed from 0 to 18 hpf (E). *hmx4* transcript is broadly distributed from 0 to 10 hpf, and is slightly enriched at the dorsal midline during gastrulation (K). After gastrulation is complete, *hmx4* is strongly expressed in the eye field (M), and at 14–18 hpf, in retina progenitors (N–O).

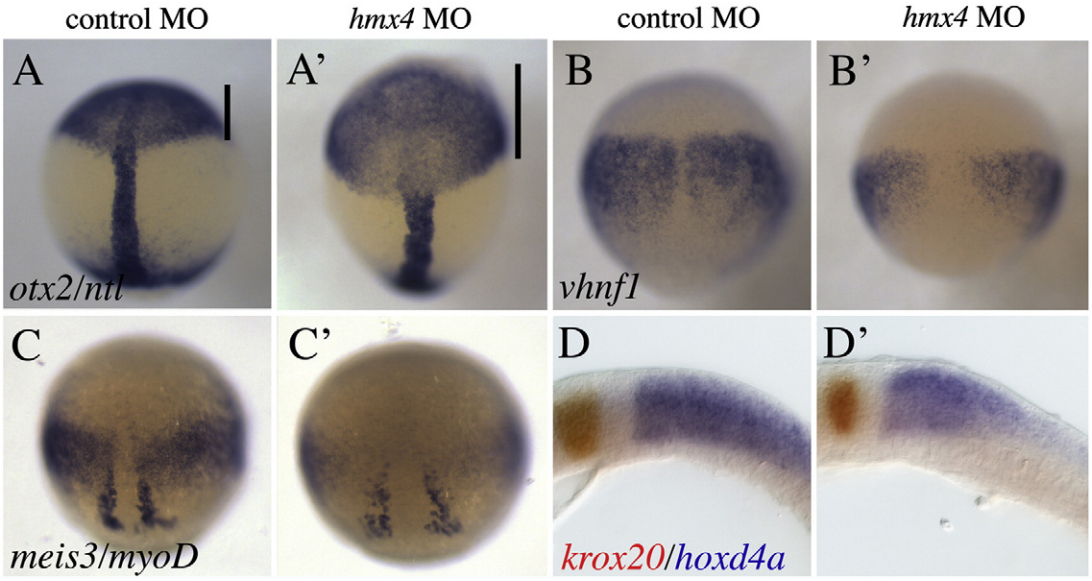


Fig. 3. *hmx4* morphants have defects in RA signaling. (A) *otx2* is expanded in *hmx4* morphants (extent of domain marked by the black line), while *vhnf1* (B) and *meis3* (C) are reduced in *hmx4* morphants at 90% epiboly. (D) *hoxd4a* (blue) in 10s *hmx4* morphants is reduced, while *krox20* (red) expression in r3 and r5 is unchanged.

degrade RA during early embryogenesis. Until mid-somitogenesis, *aldh1a2* is the only known aldehyde dehydrogenase gene expressed in zebrafish, and is present in the paraxial mesoderm during gastrulation. This expression domain is reduced in *hmx4* morphants (Fig. 4A). During somitogenesis, *aldh1a2* is robustly expressed in the somites, in mesoderm flanking the hindbrain, and in the dorsal optic vesicle. All three domains are strongly downregulated in *hmx4* morphants (Fig. 4B–C), suggesting RA synthesis during early embryogenesis is reduced when *hmx4* is depleted. To quantify the overall change in expression, we performed real-time quantitative PCR (qPCR) to detect *aldh1a2* transcripts. Compared to control embryos, *hmx4* morphants have a 50.4% decrease ($t=6.66$, $p<0.0001$, $df=12$) in *aldh1a2* 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 neurectoderm 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. 4E), 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.

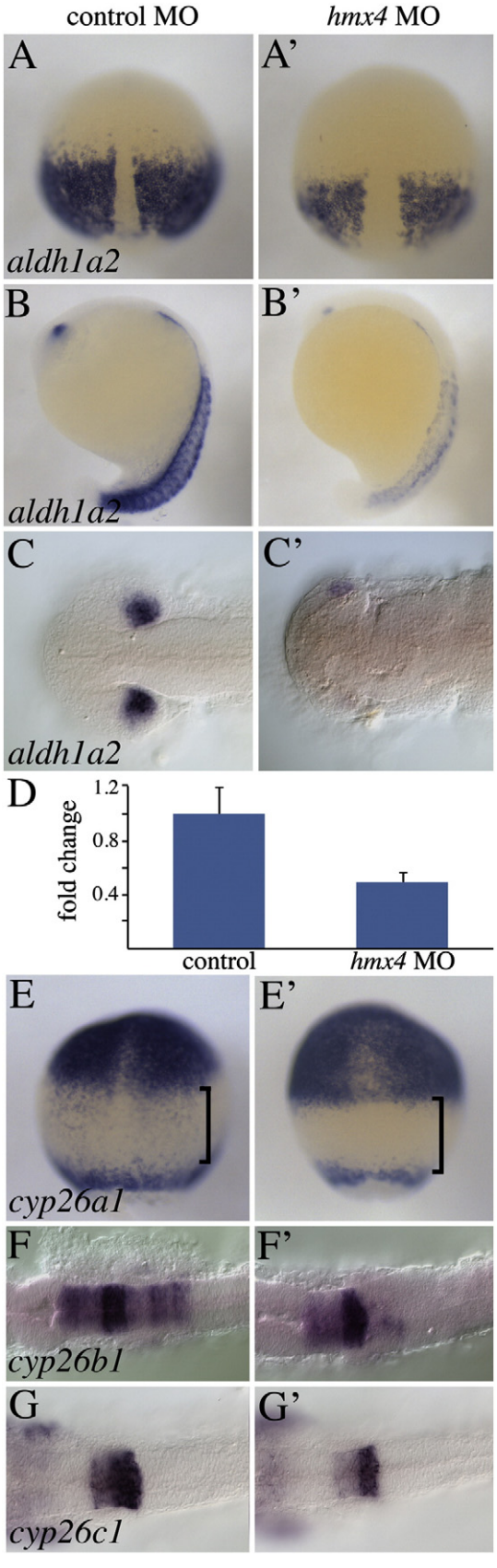
We therefore hypothesized that the phenotypes we observe in *hmx4* morphants are caused by an early loss of RA synthesis. To test this idea, we treated morphants with a low dose of exogenous RA. A 1 nM dose causes no detectable morphological phenotypes, and does not induce *hmx4* expression (Fig. S2). *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% ($\pm 17\%$, $n=97$) of *hmx4* morphants display this phenotype. When *hmx4* morphants are treated with 1 nM RA, this frequency decreases to 24% ($\pm 11\%$, $n=83$), a statistically significant rescue ($\chi^2=6.95$, $p<0.01$, $df=1$). 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% ($\pm 1\%$, $n=59$) of *hmx4* morphants display an open neural tube. When *hmx4* morphants are treated with 1 nM RA, this frequency decreases to 34% ($\pm 16\%$, $n=76$), a statistically significant rescue ($\chi^2=10.8$, $p<0.01$, $df=1$). 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% ($\pm 5\%$, $n=49$) 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% ($\pm 10\%$, $n=71$), a statistically significant rescue ($\chi^2=23.7$, $p<0.001$, $df=1$). 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 *nkx2.2a* 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 *nkx2.2a* 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. *aldh1a2* 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 *aldh1a2* 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).

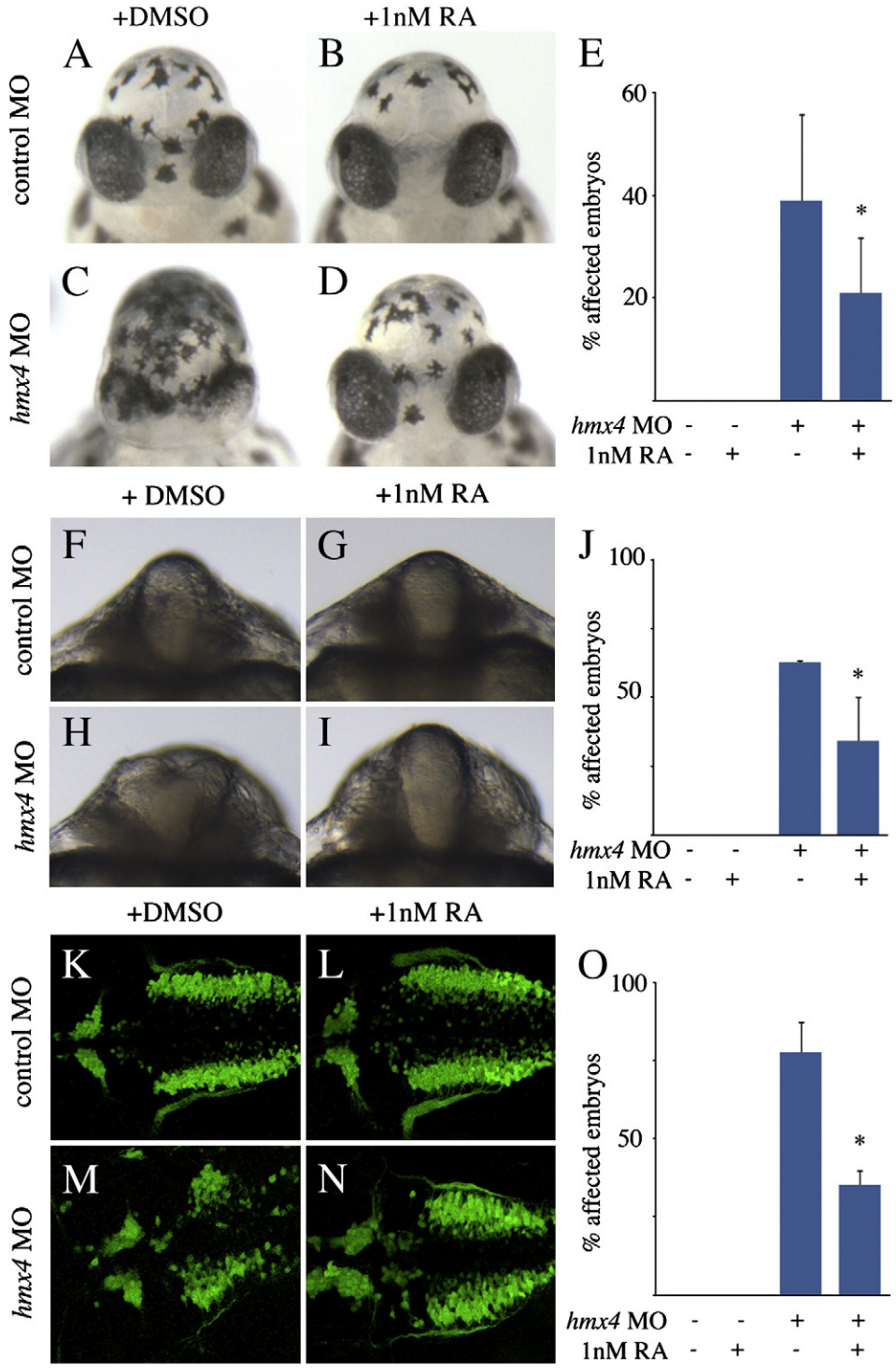


Fig. 5. RA rescues *hmx4* morphant phenotypes. Eye field narrowing (A–D), the neural tube closure defect (F–I), and loss of vagal motor neurons (K–N) in *hmx4* morphants are rescued by treatment with 1 nM RA. Frequencies of morphant phenotypes are scored for eye field narrowing (E), open neural tube (J) and loss of vagal motor neurons (O). All rescues are statistically significant (see text for statistical tests). Error bars represent standard error.

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

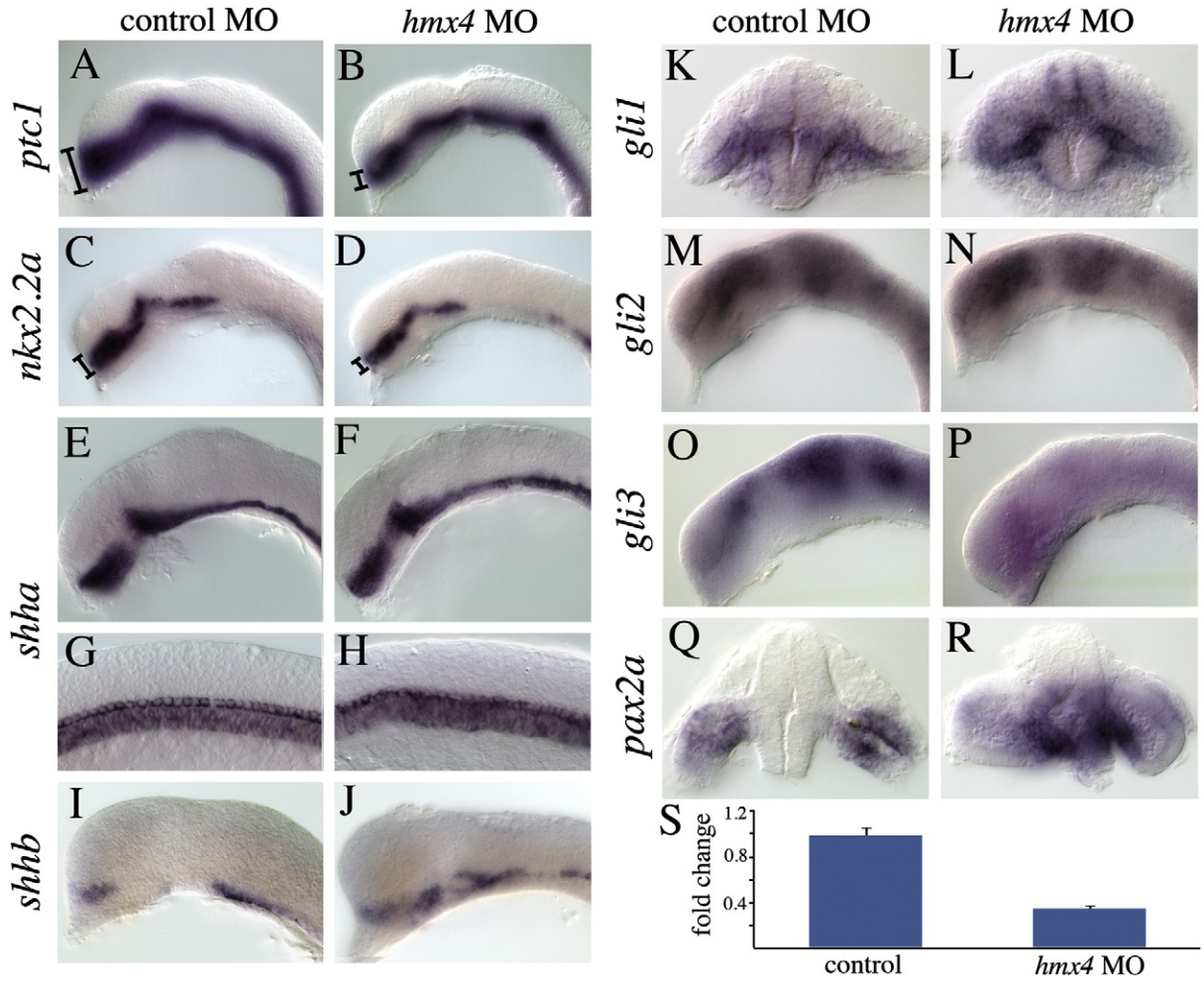


Fig. 6. *hmx4* deficiency causes defects in Shh signaling. The Shh target genes *ptc1* (A–B) and *nkx2.2a* (C–D) are reduced in 18 hpf *hmx4* morphants. Dorsal–ventral extents of expression are denoted by the bracket. *shha* expression is unaffected in the forebrain (E–F), notochord and floor plate (G–H) in *hmx4* morphant embryos. (I–J) *shhb* expression is upregulated in the ventral floor plate of *hmx4* morphants. Sections through the forebrain reveal that *hmx4* morphants have a dorsal expansion of *gli1* expression (K–L). *gli2* expression is unchanged in morphants (M–N), while *gli3* expression is strongly reduced (O–P). Sections through the forebrain reveal that *pax2a* is expanded throughout the forebrain of *hmx4* morphants (Q–R). (S) qPCR showing the significantly reduced relative levels of *gli3* transcription in *hmx4* morphants, with the levels in wild type embryos set to 1 (see text for statistical tests). Error bars indicate standard deviation.

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

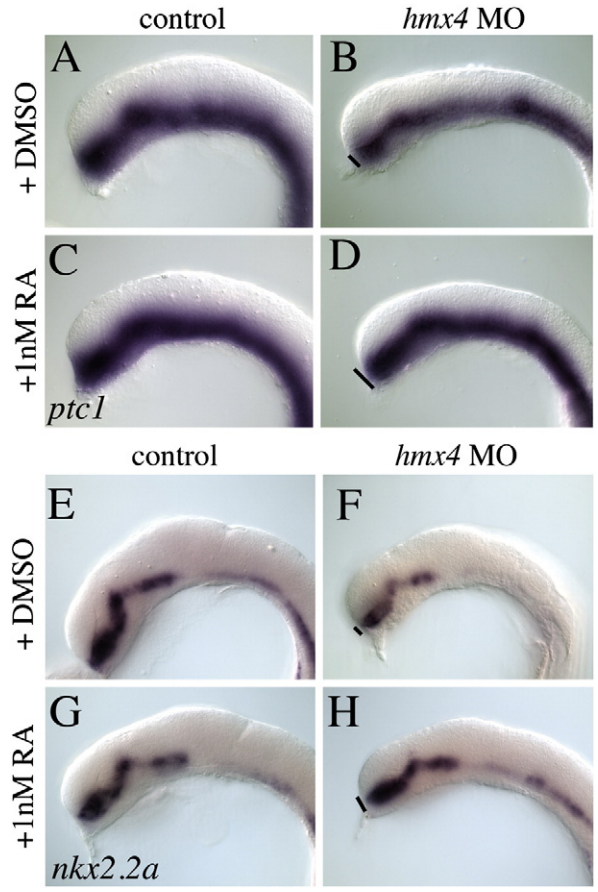


Fig. 7. Shh signaling defects in *hmx4* morphants are rescued by RA. 18 hpf *hmx4* morphants show a reduction of *ptc1* (A–D) and *nkx2.2a* (E–H) that are significantly rescued by RA treatment (see text for frequencies and statistical tests). Black lines indicate the size of gene expression domains on the dorsal–ventral axis, for comparison of control *hmx4* morphants and RA-treated *hmx4* morphants.

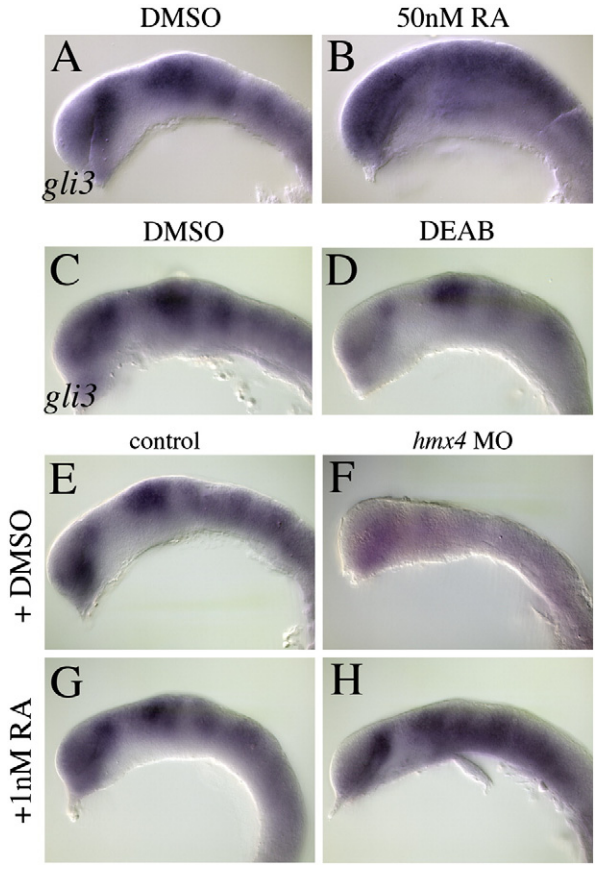


Fig. 8. Retinoic acid regulates *gli3* transcription and rescues *gli3* levels in *hmx4* morphants. Treatment with RA induces *gli3* expression throughout the forebrain (A–B) of 18 hpf embryos, while inhibiting RA synthesis with DEAB causes its reduction (C–D). (E–H) *hmx4* morphants have reduced *gli3* expression, which is significantly rescued by RA treatment (see text for frequencies and statistical tests).

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 prosencephalic 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 ShhN 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 phenotype suggests that zebrafish Hmx4 may function similarly to mammalian Hmx1.

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 eye 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 QEII graduate scholarships, and PG is currently the recipient of Fondation de Pierre-Gilles de Gennes and École 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

Abu-Abed, S., Dolle, P., Metzger, D., Beckett, B., Chambon, P., Petkovich, M., 2001. The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev. 15, 226–240.

Alique, M., Moreno, V., Kitamura, M., Xu, Q., Lucio-Cazana, F.J., 2006. Kinase-dependent, retinoic acid receptor-independent up-regulation of cyclooxygenase-2 by all-trans retinoic acid in human mesangial cells. Br. J. Pharmacol. 149, 215–225.

Aoto, J., Nam, C.I., Poon, M.M., Ting, P., Chen, L., 2008. Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. Neuron 60, 308–320.

Aza-Blanc, P., Ramirez-Weber, F.A., Laget, M.P., Schwartz, C., Kornberg, T.B., 1997. Proteolysis that is inhibited by hedgehog targets *Cubitus interruptus* protein to the nucleus and converts it to a repressor. Cell 89, 1043–1053.

Bai, C.B., Auerbach, W., Lee, J.S., Stephen, D., Joyner, A.L., 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 129, 4753–4761.

Bai, C.B., Stephen, D., Joyner, A.L., 2004. All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev. Cell 6, 103–115.

Begemann, G., Schilling, T.F., Rauch, G.J., Geisler, R., Ingham, P.W., 2001. The zebrafish neckless mutation reveals a requirement for raldh2 in mesodermal signals that pattern the hindbrain. Development 128, 3081–3094.

Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H.F., Donis-Keller, H., Helms, C., Hing, A.V., Heng, H.H., Koop, B., Martindale, D., Rommens, J.M., Tsui, L.C., Scherer, S.W., 1996. Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. Nat. Genet. 14, 353–356.

Deak, K.L., Dickerson, M.E., Linney, E., Enterline, D.S., George, T.M., Melvin, E.C., Graham, F.L., Siegel, D.G., Hammock, P., Mehltretter, L., Bassuk, A.G., Kessler, J.A., Gilbert, J.R., Speer, M.C., 2005. Analysis of ALDH1A2, CYP26A1, CYP26B1, CRABP1, and CRABP2 in human neural tube defects suggests a possible association with alleles in ALDH1A2. Birth Defects Res. A Clin. Mol. Teratol. 73, 868–875.

Dickman, E.D., Thaller, C., Smith, S.M., 1997. Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. Development 124, 3111–3121.

Emoto, Y., Wada, H., Okamoto, H., Kudo, A., Imai, Y., 2005. Retinoic acid-metabolizing enzyme Cyp26a1 is essential for determining territories of hindbrain and spinal cord in zebrafish. Dev. Biol. 278, 415–427.

Farzan, S.F., Singh, S., Schilling, N.S., Robbins, D.J., 2008. The adventures of sonic hedgehog in development and repair. III. Hedgehog processing and biological activity. Am. J. Physiol. Gastrointest. Liver Physiol. 294, G844–G849.

Franco, P.G., Paganelli, A.R., Lopez, S.L., Carrasco, A.E., 1999. Functional association of retinoic acid and hedgehog signaling in *Xenopus* primary neurogenesis. Development 126, 4257–4265.

Franz, T., 1994. Extra-toes (Xt) homozygous mutant mice demonstrate a role for the Gli-3 gene in the development of the forebrain. Acta Anat. (Basel) 150, 38–44.

Furimsky, M., Wallace, V.A., 2006. Complementary Gli activity mediates early patterning of the mouse visual system. Dev. Dyn. 235, 594–605.

Geissmann, F., Revy, P., Brousse, N., Lepelletier, Y., Folli, C., Durandy, A., Chambon, P., Dy, M., 2003. Retinoids regulate survival and antigen presentation by immature dendritic cells. J. Exp. Med. 198, 623–634.

Geng, X., Speirs, C., Lagutin, O., Inbal, A., Liu, W., Solnica-Krezel, L., Jeong, Y., Epstein, D.J., Oliver, G., 2008. Haploinsufficiency of Six3 fails to activate Sonic hedgehog expression in the ventral forebrain and causes holoprosencephaly. Dev. Cell 15, 236–247.

Gongal, P.A., Waskiewicz, A.J., 2008. Zebrafish model of holoprosencephaly demonstrates a key role for TGIF in regulating retinoic acid metabolism. Hum. Mol. Genet. 17, 525–538.

Grandel, H., Lun, K., Rauch, G.J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Kuchler, A.M., Schulte-Merker, S., Geisler, R., Holder, N., Wilson, S.W., Brand, M., 2002. Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. Development 129, 2851–2865.

Gray, P.A., Fu, H., Luo, P., Zhao, Q., Yu, J., Ferrari, A., Tenzen, T., Yuk, D.I., Tsung, E.F., Cai, Z., Alberta, J.A., Cheng, L.P., Liu, Y., Stenman, J.M., Valerius, M.T., Billings, N., Kim, H.A., Greenberg, M.E., McMahon, A.P., Rowitch, D.H., Stiles, C.D., Ma, Q., 2004. Mouse brain organization revealed through direct genome-scale TF expression analysis. Science 306, 2255–2257.

Grove, E.A., Tole, S., Limon, J., Yip, L., Ragsdale, C.W., 1998. The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. Development 125, 2315–2325.

Gu, X., Xu, F., Wang, X., Gao, X., Zhao, Q., 2005. Molecular cloning and expression of a novel CYP26 gene (cyp26d1) during zebrafish early development. Gene Expr. Patterns 5, 733–739.

Halilagic, A., Zile, M.H., Studer, M., 2003. A novel role for retinoids in patterning the avian forebrain during presomite stages. Development 130, 2039–2050.

Hernandez, R.E., Putzke, A.P., Myers, J.P., Margaretha, L., Moens, C.B., 2007. Cyp26 enzymes generate the retinoic acid response pattern necessary for hindbrain development. Development 134, 177–187.

Higashijima, S., Hotta, Y., Okamoto, H., 2000. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. J. Neurosci. 20, 206–218.

Ingham, P.W., Placzek, M., 2006. Orchestrating ontogenesis: variations on a theme by sonic hedgehog. Nat. Rev. Genet. 7, 841–850.

Jacob, J., Briscoe, J., 2003. Gli proteins and the control of spinal-cord patterning. EMBO Rep. 4, 761–765.

Johnson, D.R., 1967. Extra-toes: a new mutant gene causing multiple abnormalities in the mouse. J. Embryol. Exp. Morphol. 17, 543–581.

Kagechika, H., Shudo, K., 2005. Synthetic retinoids: recent developments concerning structure and clinical utility. J. Med. Chem. 48, 5875–5883.

Karlstrom, R.O., Tyurina, O.V., Kawakami, A., Nishioka, N., Talbot, W.S., Sasaki, H., Schier, A.F., 2003. Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. Development 130, 1549–1564.

Ke, Z., Emelyanov, A., Lim, S.E., Korzh, V., Gong, Z., 2005. Expression of a novel zebrafish zinc finger gene, gli2b, is affected in Hedgehog and Notch signaling related mutants during embryonic development. Dev. Dyn. 232, 479–486.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Kudoh, T., Wilson, S.W., Dawid, I.B., 2002. Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development 129, 4335–4346.

Maden, M., 2002. Retinoid signalling in the development of the central nervous system. Nat. Rev. Neurosci. 3, 843–853.

Maurus, D., Harris, W.A., 2009. Zic-associated holoprosencephaly: zebrafish Zic1 controls midline formation and forebrain patterning by regulating Nodal, Hedgehog, and retinoic acid signaling. Genes Dev. 23, 1461–1473.
Maves, L., Kimmel, C.B., 2005. Dynamic and sequential patterning of the zebrafish posterior hindbrain by retinoic acid. Dev. Biol. 285, 593–605.
Munroe, R.J., Prabhu, V., Acland, G.M., Johnson, K.R., Harris, B.S., O'Brien, T.P., Welsh, I.C., Noden, D.M., Schimenti, J.C., 2009. Mouse H6 Homeobox 1 (Hmx1) mutations cause cranial abnormalities and reduced body mass. BMC Dev. Biol. 9, 27.
Niederreither, K., Dolle, P., 2008. Retinoic acid in development: towards an integrated view. Nat. Rev. Genet. 9, 541–553.
Paparidis, Z., Abbasi, A.A., Malik, S., Goode, D.K., Callaway, H., Elgar, G., deGraaff, E., Lopez-Rios, J., Zeller, R., Grzeschik, K.H., 2007. Ultraconserved non-coding sequence element controls a subset of spatiotemporal GLI3 expression. Dev. Growth Differ. 49, 543–553.
Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Bose, J., Ruther, U., Ericson, J., Briscoe, J., 2002. Dorsal–ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes Dev. 16, 2865–2878.
Pillay, L.M., Forrester, A.M., Erickson, T., Berman, J.N., Waskiewicz, A.J., 2010. The Hox cofactors Meis1 and Pbx act upstream of gata1 to regulate primitive hematopoiesis. Dev. Biol. 340, 306–317.
Pittlik, S., Domingues, S., Meyer, A., Begemann, G., 2008. Expression of zebrafish aldh1a3 (raldh3) and absence of aldh1a1 in teleosts. Gene Expr. Patterns 8, 141–147.
Plack, P.A., Miller, W.S., Ward, C.M., 1964. Effect of Vitamin A deficiency on the content of three forms of vitamin a in hen's eggs. Br. J. Nutr. 18, 275–280.
Rat, E., Billaut-Laden, I., Allorge, D., Lo-Guidice, J.M., Tellier, M., Cauffiez, C., Jonckheere, N., van Seuningen, I., Lhermitte, M., Romano, A., Gueant, J.L., Broly, F., 2006. Evidence for a functional genetic polymorphism of the human retinoic acid-metabolizing enzyme CYP26A1, an enzyme that may be involved in spina bifida. Birth Defects Res. A Clin. Mol. Teratol. 76, 491–498.
Ribes, V., Wang, Z., Dolle, P., Niederreither, K., 2006. Retinaldehyde dehydrogenase 2 (RALDH2)-mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling. Development 133, 351–361.
Ribes, V., Le Roux, I., Rhinn, M., Schuhbaur, B., Dolle, P., 2009. Early mouse caudal development relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways. Development 136, 665–676.
Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., Ekker, S.C., 2007. p53 activation by knockdown technologies. PLoS Genet. 3, e78.
Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S.W., Tsui, L.C., Muenke, M., 1996. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat. Genet. 14, 357–360.
Ruiz i Altaba, A., Nguyen, V., Palma, V., 2003. The emergent design of the neural tube: prepattern, SHH morphogen and GLI code. Curr. Opin. Genet. Dev. 13, 513–521.
Schneider, R.A., Hu, D., Rubenstein, J.L., Maden, M., Helms, J.A., 2001. Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH. Development 128, 2755–2767.
Schorderet, D.F., Nichini, O., Boisset, G., Polok, B., Tiab, L., Mayeur, H., Raji, B., de la Houssaye, G., Abitbol, M.M., Munier, F.L., 2008. Mutation in the human homeobox gene NKX5-3 causes an oculo-auricular syndrome. Am. J. Hum. Genet. 82, 1178–1184.
Schug, T.T., Berry, D.C., Shaw, N.S., Travis, S.N., Noy, N., 2007. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell 129, 723–733.
Theil, T., Alvarez-Bolado, G., Walter, A., Ruther, U., 1999. Gli3 is required for Emx gene expression during dorsal telencephalon development. Development 126, 3561–3571.
Theodosiou, M., Laudet, V., Schubert, M., 2010. From carrot to clinic: an overview of the retinoic acid signaling pathway. Cell. Mol. Life Sci. 67, 1423–1445.
Tyurina, O.V., Guner, B., Popova, E., Feng, J., Schier, A.F., Kohtz, J.D., Karlstrom, R.O., 2005. Zebrafish Gli3 functions as both an activator and a repressor in Hedgehog signaling. Dev. Biol. 277, 537–556.
Wang, W., Lufkin, T., 2005. Hmx homeobox gene function in inner ear and nervous system cell-type specification and development. Exp. Cell Res. 306, 373–379.
Wang, W., Lo, P., Frasch, M., Lufkin, T., 2000. Hmx: an evolutionary conserved homeobox gene family expressed in the developing nervous system in mice and Drosophila. Mech. Dev. 99, 123–137.
Wang, W., Grimmer, J.F., Van De Water, T.R., Lufkin, T., 2004. Hmx2 and Hmx3 homeobox genes direct development of the murine inner ear and hypothalamus and can be functionally replaced by Drosophila Hmx. Dev. Cell 7, 439–453.
Yoshiura, K., Leysens, N.J., Reiter, R.S., Murray, J.C., 1998. Cloning, characterization, and mapping of the mouse homeobox gene Hmx1. Genomics 50, 61–68.