Zebrafish model of holoprosencephaly demonstrates a key role for TGIF in regulating retinoic acid metabolism

Patricia A. Gongal and Andrew J. Waskiewicz

Department of Biological Sciences, University of Alberta, Edmonton, Canada

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Holoprosencephaly (HPE) is the most common human congenital forebrain defect, affecting specification of forebrain tissue and subsequent division of the cerebral hemispheres. The causes of HPE are multivariate and heterogeneous, and include exposure to teratogens, such as retinoic acid (RA), and mutations in forebrain patterning genes. Many of the defects in HPE patients resemble animal models with aberrant RA levels, which also show severe forebrain abnormalities. RA plays an important role in early neural patterning of the vertebrate embryo: expression of RA-synthesizing enzymes initiates high RA levels in the trunk, which are required for proper anterior–posterior patterning of the hindbrain and spinal cord. In the forebrain and midbrain, RA-degrading enzymes are expressed, protecting these regions from the effects of RA. However, the mechanisms that regulate RA-synthesizing and RA-degrading enzymes are poorly understood. Mutations in the gene TGIF are associated with incidence of HPE. We demonstrate in zebrafish that Tgif plays a key role in regulating RA signaling, and is essential to properly pattern the forebrain. Tgif is necessary for normal initiation of genes that control RA synthesis and degradation, resulting in defects in RA-dependent central nervous system patterning in Tgif-depleted embryos. The loss of the forebrain-specific RA-degrading enzyme cyp26a1 causes a forebrain phenotype that mimics tgif morphants. We propose a model in which Tgif controls forebrain patterning by regulating RA degradation. The consequences of abnormal RA levels for forebrain patterning are profound, and imply that in human patients with TGIF deficiencies, increased forebrain RA levels contribute to the development of HPE.

INTRODUCTION

Holoprosencephaly (HPE) is the most common congenital forebrain defect in humans (1–3), and results from the failure to properly specify forebrain tissue, and subsequently divide the forebrain into two distinct hemispheres during development (reviewed in 4,5). The causes of HPE are highly heterogeneous. Approximately one-quarter of cases are linked to single-gene mutations (4), but the incidence of HPE has also been linked to embryonic exposure to teratogens, including retinoid compounds (reviewed in 5). A complex interplay between environmental and genetic factors is thought to explain the high variability in the severity and incidence of HPE among individuals with genetic abnormalities.

Nonsense, missense and deletion mutations of TGIF (5’-TG-3’-interacting factor) have been associated with HPE incidence (6–11), but the precise molecular targets of TGIF in patterning the central nervous system (CNS) remain unknown. TGIF is a TALE (three amino acid loop extension)-class homeobox transcription factor, and was originally discovered in a screen for retinoid X response element binding proteins (12). Overexpression of TGIF represses transcription of a retinoid-responsive reporter in human glioma U87 and COS1 cells (12). Recently, TGIF has been shown to bind retinoid X receptors (RXRs) and less robustly, retinoic acid (RA) receptors (RARs) in COS1 cells, and repress RA-responsive reporter transcription in HepG2 cells (13). Overall, these studies suggest that TGIF can function as a repressor to regulate RA-responsive genes.
Retinoic acid signaling plays a critical role in patterning the CNS. Embryonic exposure to RA causes severe craniofacial defects, malformation of the eyes and ectopic expression of posterior CNS markers in the forebrain (14–16). Conversely, RA depletion, by maternal vitamin A-free diet, results in a single telencephalic vesicle, mispattening of the diencephalon, failure to specify the caudal hindbrain and eye abnormalities (17–19). Many of these defects strongly resemble the phenotype of HPE patients.

Levels and activity of RA are kept under multiple levels of tight regulation (reviewed in 20). The transformation of retinoid precursors into RA is accomplished by retinaldehyde dehydrogenases (raldh or aldh1a family) (reviewed in 21) and by cyp1b1 (cytochrome p450 subfamily 1B1) (22). Distinct spatial and temporal expression patterns of RA-synthesizing genes indicate complex tissue-specific regulation of RA production throughout development (reviewed in 23). Preventing the activity of aldh1a2 by mutation or chemical inhibitors results in the mispattening or elimination of hindbrain fates posterior to rhombomere (r) 4 (reviewed in 20), indicating a key role for RA in hindbrain anterior–posterior patterning. Mice lacking Aldh1a2 have profound hypoplasia in the forebrain and eyes, and display defects in forebrain Fgf (fibroblast growth factor) and Shh (sonic hedgehog) signaling (24). Thus, RA signaling is essential for early CNS patterning of both the forebrain and hindbrain, and is required for the normal function of other critical signaling pathways.

In addition to controlling RA synthesizing enzymes, precise RA levels are maintained by regulating the enzymes that catalyze its degradation. The cyp26 (cytochrome P450, subfamily XXVII) family of genes metabolizes RA, and tissues expressing these are thought to act as RA sinks (reviewed in 25). Three cyp26 genes have been reported in zebrafish: cyp26a1, cyp26b1 and cyp26c1 (26–29). cyp26a1 is expressed in the forebrain and midbrain during gastrulation, whereas cyp26b1 and cyp26c1 genes are expressed in a dynamic, rhombomere-specific pattern in the hindbrain. Zebrafish cyp26a1 mutants have a rostral expansion of the spinal cord, suggesting a slight increase in RA levels in this region (30). Embryos lacking all cyp26 genes display a severe posteriorization of the hindbrain (28), consistent with a large increase in RA levels. Embryos with reduced cyp26a1 function have eye and forebrain abnormalities, suggesting anterior regions of the head are sensitive to RA activity, although patterning of these structures in cyp26a1 mutants has yet to be characterized in detail (28,30). Together, RA synthesizing and degrading genes act to set up a complex and dynamic pattern of RA levels in the developing CNS.

Retinoic acid activity is also modulated by the presence and activity of RARs. RARs form heterodimers with RXRs, which together bind RA-responsive elements, and affect transcription of downstream targets (reviewed in 31,32). In the absence of RA ligand, the RAR–RXR complex is thought to repress transcription, but when RA is present, the complex activates target gene transcription. The majority of rar and rrx genes are expressed in distinct temporal and tissue-specific patterns (33–37), suggesting that specific pairings of rars and rrxs in specific tissues are developmentally important. The multiple levels of RA regulation that exist, together with profound loss- and gain-of-function phenotypes, demonstrate that spatial and temporal precision in RA activity is essential for normal CNS development.

The loss of TGIF, as a RA signaling repressor, would be expected to mimic exposure to higher levels of RA. While three independently generated lines of TGIF-null mice have no obvious CNS patterning defects (13,38,39), the Tgif−/− and Tgif+/− mice generated by Bartholin et al. (13) appear to have an increased susceptibility to RA-induced exencephaly. This suggests that RA regulation is altered in Tgif mutants. In addition to Tgif, mammals have three other TGIF-related genes, Tgif2, Tgif-like-on-the-X and Tgif-like-on-the-Y. These genes may be partially redundant and may compensate for the loss of Tgif in mice. Recently, Kuang et al. (40) have generated mice lacking Tgif exon 3, which they propose results in a dominant negative variant of Tgif. These mice display severe hypoplasia of the forebrain and defects in ventral forebrain patterning. Although RA signaling has not yet been directly examined in these mice, the expression patterns of two genes regulated by RA, Shh and Nkx2.2a, are disrupted. Zebrafish have only one additional TGIF-related gene, tgif2, compared with the four mammalian TGIF-like genes. Therefore, zebrafish may have reduced tgif redundancy, and thus may be more sensitive to perturbations of Tgif function. Vertebrate TGIFs are highly conserved, and zebrafish and human TGIF have been shown to have functional similarities (41). Furthermore, as zebrafish develop externally, they are an excellent model in which very early patterning events can be examined, namely RA signaling activity during early neural patterning.

In this work, we demonstrate that a reduction of Tgif in zebrafish strongly reduces the initiation of both cyp26a1 and aldh1a2 during gastrulation, suggesting Tgif activates both RA synthesis and degradation. Tgif-depleted embryos show reduced expression of RA-dependent genes in the hindbrain, suggesting RA levels are lower at this axial level. Tgif is also necessary to properly specify forebrain tissues. The forebrain phenotype of tgif morphants resembles that of embryos lacking cyp26a1, suggesting higher anterior levels of RA may be responsible for forebrain defects. Additionally, Tgif overexpression is sufficient to initiate ectopic cyp26a1 expression. We propose a model in which Tgif is a high-level regulator of RA levels, and controls forebrain patterning by regulating RA degradation. The complex effect of Tgif activity on RA levels and its effects on forebrain patterning in zebrafish provides important insights into the development of HPE in humans with TGIF deficiencies.

RESULTS

Tgif regulates the expression of cyp26a1 and aldh1a2

The human HPE gene TGIF was originally discovered through its ability to bind an RA response element (12). TGF also binds RARs and RXRs, and represses RA-dependent transcription in HepG2, U87 and COS1 cells (12,13). However, TGIF’s effects on the RA signaling pathway have not been examined in the developing CNS of an animal model. Vertebrate TGIFs are highly conserved, and are expressed broadly during early development (Supplementary Material, Fig. S1, 38,39). To model Tgif loss-of-function in zebrafish, we used
We then examined the expression of two essential components of the RA pathway: *cyp26a1*, which metabolizes RA, and *aldh1a2*, which synthesizes this critical morphogen, at a variety of stages in *tgif* morphants.

During gastrulation (9 h post-fertilization; hpf), *cyp26a1* expression is initiated in the anterior neurectoderm, and is thought to protect this tissue from the RA produced in the posterior. In 85% of *tgif* morphants, the size of the expression domain, as well as the expression level of *cyp26a1* is strongly reduced (n = 33; Fig. 1B, compared with wild type in 1A). *cyp26a1* is also expressed in the germ ring during gastrulation, the migration of which was used to precisely stage embryos. This suggests that when Tgif is depleted, RA degradation by Cyp26a1 is reduced in the anterior neurectoderm. At 14 hpf, *cyp26a1* is expressed at low levels in the branchial arches, which flank the forebrain, and is RA inducible (28,30,42,43). Expression in this region is higher in *tgif* morphants (Fig. 1D, compared with wild type in 1C), suggesting that RA activity is higher than normal at the level of the forebrain.

At 9 hpf, *aldh1a2* is initiated in the presumptive paraxial mesoderm. Levels of expression and the size of the domain are reduced in 90% of Tgif-depleted embryos (n = 20; Fig. 1F, compared with wild type in 1E). *aldh1a2* levels remain reduced at 10.5 hpf; the posterior portion of this gene’s expression domain is most strongly affected (Fig. 1H, compared with wild type in 1G). By 14 hpf, *aldh1a2* is normally present in the somites and the lateral mesoderm adjacent to the hindbrain. In *tgif* morphants, expression in the somites remains slightly reduced, but expression in the lateral mesoderm at the level of the hindbrain is expanded (Fig. 1J, compared with wild type in 1I). *aldh1a2* expression is directly repressed by RA (42,44). Therefore, the expansion seen in *tgif* morphants suggests that lower than normal RA activity occurs at the level of the hindbrain.

We examined *cyp26a1* and *aldh1a2* expression together to determine relative changes in expression domains at 9 hpf, when early neural patterning takes place. In addition to lower levels of expression of both genes, the distance between the two expression domains is increased in *tgif* morphant embryos (Fig. 1L, compared with wild type in 1K). While the mean size of the gap in wild-type embryos is 97.1 ± 21.0 μm, *tgif* morphants have a 21% larger gap (117.5 ± 25.2 μm), a statistically significant change (t = 1.69, df = 30, P = 0.01). Overall, our results define a new role for Tgif in the initiation of RA signaling.
and regulation of the expression of RA-synthesizing and RA-degrading genes throughout development.

Although tgif morphants have no obvious morphological defects at the MO doses used for analysis (Supplementary Material, Fig. S3), MO knockdown is known to cause non-specific activation of p53-dependent apoptotic pathways (45). We determined that MO-induced cell death was not responsible for the early reduction in aldh1a2 and cyp26a1 expression. To suppress p53-dependent apoptosis, we co-injected tgif MO with p53 MO (45). We saw no rescue of expression of either aldh1a2 or cyp26a1 expression by injecting p53 MO in tgif morphants, indicating that non-specific p53-dependent apoptosis caused by MO injection was not the basis of the deficiencies in aldh1a2 and cyp26a1 expression (data not shown).

Tgif is required for initiation of hindbrain RA target genes

Changes in the expression patterns of cyp26a1 and aldh1a2 imply that complex changes in RA levels occur in Tgif-depleted embryos. We sought to clarify the effects of Tgif-depletion by examining the expression of genes strongly dependent on RA. Overall, our findings suggest that the loss of Tgif results in examining the expression of genes strongly dependent on RA.

Embryos. We sought to clarify the effects of Tgif-depletion by examining the expression of genes strongly dependent on RA. We compared the changes in RA-responsive genes in cyp26a1 or by pharmacological treatment, and strongly upregulated when RA levels are increased either by the loss of Cyp26a1 or by pharmacological treatment, and strongly reduced when RA is inhibited (43,46). tgif morphants show strongly decreased levels of meis3 and vhnf1 expression, and the expression domain of vhnf1 is strongly reduced (100%, n = 23; Fig. 2B and F, compared with wild type in 2A and E).

We compared the changes in RA-responsive genes in tgif morphants to those found in embryos lacking cyp26a1, and embryos treated with diethylaminobenzaldehyde (DEAB), which specifically inhibits retinaldehyde dehydrogenases (47–49). While tgif morphants have reduced vhnf1 and meis3 expression, cyp26a1 morphants have an increased domain of meis3, whereas vhnf1 is unchanged (Fig. 2C and G). DEAB treatment of embryos eliminates expression of meis3 and vhnf1 (Fig. 2D and H). Thus, the expression patterns of RA-responsive genes in the hindbrain of tgif morphants resemble (albeit are less severe than) the loss of retinaldehyde dehydrogenase activity.

To determine whether defects in RA-dependent patterning persist throughout development in Tgif-depleted embryos, we examined the expression of an additional RA-dependent gene at a slightly later stage. We observe mild hindbrain defects at 14 hpf, suggesting that RA signaling activity is slightly less than normal in the hindbrain at this stage, compared with strongly reduced hindbrain gene expression during gastrulation. At 14 hpf, the level of hoxd4 expression, known to be directly activated by RA (50–52), is ostensibly unchanged in tgif morphants. However, the distance between r5, marked by egr2b (early growth regulator 2b; also known as krox20), and hoxd4 expression is substantially decreased (Fig. 3B, compared with wild type in 3A). This result could be interpreted as either an anterior spread of hoxd4, or the loss of r6. Given that vhnf1 is required for r6 identity (53), and that vhnf1 expression is strongly reduced in tgif morphants, we favor the latter interpretation. The mild hindbrain phenotype observed at this stage is consistent with the more normal aldh1a2 expression present at this time.

Tgif synergizes with RA signaling to pattern the hindbrain

To corroborate that a reduction in Tgif results in a decrease in RA levels in the hindbrain, we tested whether Tgif synergistically interacts with RA signaling to pattern the hindbrain. We injected embryos with a dose of tgif MO that produces no discernable hindbrain phenotype (2 ng), and treated them either with 1 μM DEAB until 10 hpf, a dose that alone causes no hindbrain phenotype at 14 hpf, or an equivalent amount of solvent (DMSO). Then, we examined the hindbrain markers egr2b, expressed in r3 and r5, hoxd4, expressed in r7 and spinal cord, and vhnf1, expressed at the r5–r6 boundary and posterior. When embryos are treated with DMSO, tgif MO, or DEAB alone (Fig. 3C–E and G–I), no defects in egr2b, hoxd4, or vhnf1 expression occur. However, in tgif morphants treated with DEAB, the r5 stripe of egr2b is eliminated (Fig. 3F and J). Additionally, hoxd4 (Fig. 3F) and vhnf1 (Fig. 3J) expression is reduced. Next, we examined egr2b expression relative to the first somite, marked by myoD (myogenic differentiation), to examine the overall size of the hindbrain. Compared with embryos treated with DMSO, tgif MO, or DEAB alone (Fig. 3K–M), DEAB-treated tgif morphants have a strongly reduced distance between r3 and the first somite, suggesting a failure to specify caudal rhombomeres (Fig. 3N). Overall, the phenotype of embryos treated with low doses of DEAB and tgif MO resembles that observed in embryos treated with a higher dose of DEAB alone (47 and Gongal and Waskiewicz, unpublished data), implying that Tgif is required for normal RA signaling, in order to properly pattern the caudal hindbrain.

RA acts downstream of tgif

To determine whether RA acts downstream of Tgif, we performed epistasis experiments. tgif morphants show reduced expression of the RA-dependent hindbrain gene meis3 (Fig. 2J, compared with wild type in 2I). Treating embryos with RA strongly induces meis3 expression throughout the anterior neuroderm (Fig. 2K). RA still induces ectopic meis3 expression in 100% of tgif morphants (n = 24; Fig. 2L), indicating RA activity does not require, and is therefore genetically downstream, of Tgif. However, the magnitude of meis3 induction is slightly lower in tgif morphants. This result implies that Tgif also has a role in regulating the downstream response to RA, namely RA-mediated meis3 induction, in addition to influencing RA metabolism. Overall, our results indicate that there may be multiple points of genetic interaction between Tgif and the RA signaling pathway.

Loss of tgif causes forebrain defects

Mutations in TGF1 have been associated with human HPE (reviewed in 11), and zebrafish tgif expression is slightly enriched in the presumptive forebrain at the end of gastrulation (Supplementary Material, Fig. S1). Given this association
between TGIF and forebrain patterning, we analyzed the expression of genes required for early forebrain development in tgif morphants. We find that Tgif-depleted embryos have substantial defects in diencephalic patterning at its earliest stages and that patterning defects persist through later development.

At 10 hpf, when early neural patterning is occurring, we examined the presumptive diencephalon markers barhl2 (BarH-like 2) and arx (aristaless-related homeobox), the presumptive telencephalic marker tlc (secreted frizzled-related protein), emx3 (empty spiracles homeobox 3), which marks both telencephalon and diencephalon, and the eye field markers rx1 and rx2 (retinal homeobox 1 and 2). Expression of the two diencephalic markers barhl2 and arx is reduced in tgif morphants (Fig. 4B and D, compared with wild type in 4A and C). No change is apparent in the telencephalic domain of emx3 (Fig. 4F, compared with wild type in 4E). The diencephalic domain of emx3 is reduced, whereas no changes are visible in the telencephalic domain (Fig. 4H, compared with wild type in 4G). No changes are apparent in the size of the eye field or the expression levels of the eye field markers rx1 and rx2 at 10 hpf (data not shown). Further, the migration of the prechordal plate, an essential ventral signaling center for the developing forebrain, appears normal in tgif morphants, as marked by ctsl1b (cathepsin L1b, formerly known as hgg1) expression (data not shown).

At 14 hpf, as the neural tube becomes more finely patterned, we examined the expression of additional CNS markers. pax6a (paired box gene 6a) is normally expressed in the diencephalon, hindbrain and spinal cord at this stage. Tgif morphants fail to upregulate the expression in the diencephalic domain, whereas no changes are visible in the telencephalic domain (Fig. 4J, compared with wild type in 4I). Expression levels of the telencephalic and eye marker six3b (sine oculis homeobox homolog 3b) are unchanged in tgif morphants (data not shown).

Because aberrations in other HPE genes specifically affect ventral forebrain structures, we examined the expression of genes required for ventral forebrain specification, namely the hypothalamic markers tif1a and tif1b (thyroid transcription...
Figure 3. Tgif is required for caudal hindbrain patterning and interacts with RA signaling. tgif morphants (B, compared with wild type in A) have a reduced distance between r5, marked by egr2b (red) and hoxd4 (blue), which is normally expressed in hindbrain r7 and the spinal cord, such that the anterior hoxd4 boundary nearly contacts r5. Line indicates r6. While low doses of tgif MO (D, H and L), DEAB (E, I and M) or DMSO control (C, G and K) have a negligible effect on caudal hindbrain patterning, DEAB combined with tgif MO results in the elimination of the r5-specific expression of egr2b and a reduction in hoxd4 (F) and vhnf1 (J) expression. DEAB combined with tgif MO causes the deletion of presumptive hindbrain tissue between r3 and the first somite, marked by myoD expression (N). Brackets on panels K–N indicate the distance between r3 and the first somite.
Figure 4. Tgif is required for proper forebrain patterning. *tgif* morphants show reduced expression of the presumptive diencephalic markers *barhl2* (B, compared with wild type in A) and *arx* (D, compared with wild type in C), at 10 hpf. Presumptive telencephalic territory expressing *tlc* appears unchanged (F, compared with wild type in E). The diencephalic domain of *emx3* appears reduced (H, compared with wild type in G), whereas expression in the telencephalon is unchanged. At 14 hpf, *tgif* morphants have reduced expression of *pax6* in the diencephalon, and increased expression of *pax6* in the anterior hindbrain (J, compared with wild type in I). Arrows in (A–I) indicate the diencephalon. *tgif* morphants show reduced domains of the ventral hypothalamic-specific genes *titf1a* (L, compared with wild type in K) and *titf1b* (N, compared with wild type in M), and a subtle reduction in anterior *shh* expression (P, compared with wild type in O). Arrows in (O–P) indicate the telencephalon. Compared with wild-type embryos (Q), expression of *ptc1* is unchanged in *tgif* morphants (R).
factor 1a and 1b, also known as nk2.1a and nk2.1b) at 18 hpf. In tgif morphants, both of these transcription factors display truncated expression domains (Fig. 4L and N compared with wild type in 4K and M). To quantify this phenotype, we measured the length of the expression domain of titf1a. We note a 15% reduction in the size of the titf1a expression domain in tgif morphants (t = 2.67, P = 0.014, n = 10). These data demonstrate that tgif morphants have a reduced territory specified as hypothalamus. shh is known to regulate titf1a and titf1b expression (54). We therefore hypothesized that tgif-dependent changes in shh expression might underlie the alteration in the hypothalamus of tgif-depleted embryos. At 18 hpf, tgif morphants show a subtle decrease in shh’s anterior-most domain (Fig. 4P, compared with wild type in 4O). No changes in shh expression are apparent in more posterior domains, or at earlier stages (data not shown).

To assess the consequences of the mild reduction in shh expression, we examined the expression of the well-characterized Shh target, patched1 (ptc1). We observed no detectable differences in tgif morphants (Fig. 4R), compared with wild-type embryos (Fig. 4Q), suggesting that there is little change in Shh activity. Therefore, deficiencies in the Shh pathway are likely not responsible for the forebrain defects of tgif morphants.

Regulation of cyp26a1 explains Tgif-dependent forebrain defects

We wondered whether the changes in the anterior neuroectoderm expression of cyp26a1 in tgif morphants were responsible for the defects in forebrain patterning. The loss of cyp26a1 has been hypothesized to result in higher than normal embryonic RA levels (28,30). We reasoned that the reduction in cyp26a1 in tgif morphants may result in higher than normal RA levels in the presumptive forebrain, even in the presence of lower levels of aldh1a2 in the posterior. If this were the case, the forebrain of tgif morphants would resemble that of cyp26a1 mutants. We find that indeed, the forebrain defects of tgif morphants bear striking similarity to those of cyp26a1−/− mutants. In embryos lacking cyp26a1, the diencephalic markers barhl2 and arx are nearly eliminated (Fig. 5B and D, compared with wild type in 5A and C). This is similar to, although more severe than, tgif morphants (Fig. 4B and D). We also examined the hypothalamic markers titf1a and titf1b. cyp26a1−/− embryos showed a shortening of the expression domains of these genes (Fig. 5F and H, compared with wild type in 5E and G) again mimicking the tgif morphant phenotype (Fig. 4L and N). The length of the expression domain of titf1a in cyp26a1−/− embryos was significantly reduced by 19% (t = 14.5, P < 0.05, n = 5) compared with wild type controls. This change is comparable with the 15% reduction in forebrain territory observed in tgif morphants. The strong similarities between cyp26a1− and tgif-depleted embryos suggest that reduced levels of cyp26a1 are responsible for the tgif morphant forebrain phenotype.

cyp26a1 acts downstream of Tgif

To confirm cyp26a1 acts downstream of Tgif, we performed an epistasis experiment. otx2 (orthodenticle homologue 2) is expressed in the presumptive forebrain and midbrain, and is repressed by RA (43). The loss of Tgif results in a subtle reduction in otx2 expression and posterior expansion of the expression domain (Fig. 5J, compared with wild type in 5I), consistent with a reduction in RA signaling activity. We synthesized cyp26a1 mRNA and injected it into one-cell embryos, alone, and in combination with tgif MO. Overexpressed cyp26a1 alone caused a slight increase in expression of otx2 (Fig. 5K). Treating tgif morphants with cyp26a1 mRNA resulted in a rescue of otx2 levels to approximately wild-type levels (Fig. 5L), indicating Cyp26a1 functions genetically downstream of Tgif.

Overexpression of zebrafish or human TGIF induces cyp26a1

Our loss-of-function work indicates that Tgif is required for the normal expression of cyp26a1, which is required for normal forebrain patterning. To confirm the genetic interaction between these two genes, we examined the effects of Tgif overexpression. We find that when Tgif is overexpressed, cyp26a1 expression is strongly induced in cells posterior to cyp26a1’s normal expression domain in 62% of injected embryos (n = 60; Fig. 5N and P, compared with wild type in 5M and O). Injection of a control RNA (gfp) has no effect on cyp26a1 expression (data not shown). To quantify the induction of cyp26a1 by Tgif, we measured the distance between the posterior limit of cyp26a1 expression and the germ ring. The mean length of the cyp26a1-free domain in wild-type embryos is 165.3 ± 20.8 μm, while this domain in Tgif overexpressing embryos is decreased to a mean of 106.8 ± 28.7 μm, a significant reduction of 35% (t = 7.12, P < 0.0001, df = 42). Further, tgif RNA rescues the expression of cyp26a1 in tgif morphants: 48% of morphants show wild type or higher cyp26a1 expression levels when co-injected with tgif RNA (n = 27, Supplementary Material, Fig. S1). Coinjecting control RNA (gfp) with tgif MO results in a comparable proportion of embryos with reduced cyp26a1 expression as tgif MO alone (78%, n = 36). This result provides additional evidence for the specificity of the tgif MO used in this study.

To examine the functional relationship between zebrafish and human TGIF, we overexpressed the human version of TGIF in zebrafish embryos. Similar to its counterpart in zebrafish, overexpression of human TGIF results in ectopic cyp26a1 expression posterior to its normal domain (53% of injected embryos, n = 64; Fig. 5R and T compared with wild type in 5Q and S). We also measured the distance between the posterior boundary of cyp26a1 expression and the margin in human TGIF overexpressing embryos. We found that this distance is reduced to a mean of 86.9 ± 43.7 μm, a 47% reduction compared with wild type embryos (t = 6.66, P < 0.0001, df = 28). Human TGIF can also rescue cyp26a1 expression to wild type or higher levels in 77% of tgif morphants (n = 30; Supplementary Material, Fig. S2). This finding reiterates that the tgif MO used in this study is specific. Overall, our Tgif overexpression studies indicate that human and zebrafish Tgif have significant functional homology, and confirm that Tgif regulates (directly or indirectly) the initiation of cyp26a1 expression.
Figure 5. Forebrain defects in cyp26a1−/− embryos resemble those of tgif morphants, and Tgif overexpression induces cyp26a1 expression. At 10 hpf, cyp26a1−/− mutant embryos show nearly eliminated expression of the diencephalic markers barhl2 and arx (B, D), compared with wild type embryos (A, C), similar to, but more severe than tgif morphants. Arrows in (A–D) indicate the diencephalon. cyp26a1−/− mutants also show a shortened hypothalamic domain at 18 hpf, marked by titf1a (F, compared with wild type in E) and titf1b (H compared with wild type in G). Compared with wild type embryos (I), tgif morphants embryos show a slight reduction in otx2 levels (J). Overexpression of cyp26a1 RNA causes a subtle upregulation of otx2 levels (K), and rescues the phenotype of tgif morphants (L), indicating cyp26a1 acts downstream of Tgif. Overexpression of zebrafish tgif results in a strong posterior expansion of cyp26a1 expression (N), compared with wild-type embryos (M). (O–P) depicts the region of the embryo indicated by the brackets on (M–N), deyolked and flat mounted. Overexpression of human TGIF causes a similar induction of cyp26a1 posterior to its normal expression domain (R), compared with wild type (Q). (S–T) depicts the region of the embryo indicated by the brackets on (Q–R), deyolked and flat mounted. Brackets in (M–N) and (Q–R) indicate the distance between the posterior boundary of cyp26a1 expression and the margin. Arrows in (O–P) and (S–T) indicate the germ ring.
DISCUSSION

Tgif regulates RA signaling during CNS development

Our study is the first to examine the effect of the HPE gene TGIF on RA signaling components and RA-dependent transcription during animal development. We propose that Tgif functions at the top of the hierarchy of genes regulating RA signaling. Tgif is required for the transcriptional activation of both aldh1a2 and cyp26a1, genes that are responsible for establishing differential levels of RA in the developing embryo. As such, a reduction in Tgif strongly influences CNS patterning at multiple axial levels, and has important implications for the etiology of human HPE.

When Tgif is depleted, RA activity is reduced in the hindbrain, assessed by changes in RA-dependent gene expression and synergistic activity with DEAB, an inhibitor of RA synthesis. In tgif morphants, posterior hindbrain genes that are known RA targets, such as vhnf1 and meis3, are strongly decreased, whereas the gene otx2, repressed by RA, is expanded. Further, aldh1a2 expression is repressed by RA; in 14 hpf tgif morphants, mesodermal expression of aldh1a2 at the level of the hindbrain is increased. Lastly, tgif MO synergistically interacts with DEAB, in the patterning of the caudal hindbrain. Together, these findings strongly argue that RA activity is reduced in the hindbrain, and can most easily be explained if Tgif functions to regulate RA production via control of aldh1a2.

tgif and forebrain patterning

tgif expression is slightly enriched in the forebrain, and Tgif-depleted embryos display tissue-specific defects in diencephalic and hypothalamic specification. Although RA levels appear reduced in the hindbrain, our work suggests increased levels of RA in the forebrain, caused by a reduction in cyp26a1 levels, may be the basis of the tgif loss-of-function forebrain phenotype. At 14 hpf, the expression of cyp26a1 is upregulated in regions flanking the forebrain. Expression of cyp26a1 is highly RA-inducible (28,30,43), which suggests that RA levels are increased in anterior tissues of tgif morphants. Indeed, when cyp26a1 itself is mutated, and anterior degradation of RA is thought to be attenuated, cyp26a1 is induced in somitogenesis-stage embryos in a pattern strikingly similar to that in tgif morphants (30). Further, in both tgif morphants and cyp26a1 mutants, expression of several diencephalic markers is diminished, and hypothalamic territory is reduced. Together, these data suggest that diminished levels of cyp26a1, and therefore a reduced ability to process RA in the anterior neuroectoderm, is responsible for tgif morphant forebrain mispatterning.

High levels of RA in the forebrain cause a wide range of morphological defects (14–16). Recent studies have begun to clarify the mechanism by which excess RA causes forebrain mispatterning. Shh signaling plays an essential role in patterning the CNS by activating ventral-specific gene expression, and loss-of-function has been linked to HPE (reviewed in 4,11,55). RA has an early role in post-translationally regulating SHH activity, and has the additional later role of regulating Shh expression (24). Mice mutant for Aldh1a2 have reduced expression of Shh in ventral diencephalic cells, but increased Shh in the infundibulum (24). These results suggest that RA is required to both activate and repress shh expression within different forebrain regions. Overall, the effect of RA on shh signaling is highly dependent on developmental timing and the properties of target cells. In tgif morphants, there is no change in shh expression during gastrulation, and expression of shh at 18 h is only slightly reduced. Although the expression domains of two Shh-target genes, titf1a and titf1b, are slightly reduced, the expression of ptc1, a more direct reporter of Shh signaling, is unaffected. Therefore, it seems unlikely that changes in Shh signaling are responsible for the forebrain defects of tgif morphants.

The Nodal pathway is also critical for proper early forebrain patterning, and has been linked to HPE (reviewed in 4). Notably, TGIF has been reported to bind SMAD2 and repress TGF-β-dependent transcription in L17 mink lung epithelial cells (56). However, if Tgif acts during early development to repress Nodal signaling, Tgif loss-of-function ought to result in shh overexpression, rather than a reduction in expression, as reported here. As others note (55), it is difficult to understand how a derepression of Nodal signaling could result in the loss of ventral forebrain tissue that is a hallmark of HPE. Indeed, we are unable to detect a genetic interaction between Tgif and components of the Nodal signaling pathway in the developing zebrafish (Gongal and Waskiewicz, unpublished data). We therefore support Hayhurst and McConnell’s suggestion (55) that while TGIF’s regulation of the RA signaling pathway is critical for early development, its role as a TGF-β corepressor may be more important at later stages of development, or in specific cell types not examined in this study.

Our work suggests the loss of tgif results in higher than normal RA levels in the developing zebrafish forebrain, concomitant with a reduction in diencephalic gene expression, including that of pax6. However, overexpression of Tgif in the chick represses Pax6 expression in the spinal cord (57). Although these results appear contradictory, RA has been previously shown to both repress and activate Pax6, depending on the tissue. For example, studies in the vitamin-A-deficient quail model indicate that RA represses pax6 in the retinal pigmented epithelium (58). However, expression of anRAR dominant negative construct in chick spinal cord indicates that RA signaling is in fact required for pax6 expression in this region of the CNS (59). These studies again highlight the tissue-specific effects of RA, and that this molecule can have a markedly different role at one level of the CNS compared with another. Therefore, the contrasting role of Tgif in repressing pax6 expression in the spinal cord, while activating it in the forebrain, likely reflects the different effects of RA in these tissues.

Initiation of aldh1a2 and cyp26a1

Other than Tgif in the present study, the regulators required for the initiation of cyp26a1 and aldh1a2 transcription are entirely unknown. The expression of cyp26a1 is RA-inducible: RA treatment strongly upregulates cyp26a1 expression during gastrulation (28,43), and in cyp26a1−/− mutant embryos, which are thought to have higher than normal RA levels, cyp26a1 expression is higher than normal during somitogenesis (30).
However, the initiation of cyp26a1 in the anterior neuroectoderm is unaffected by DEAB treatment, maternal vitamin A deprivation, or loss of cyp26a1 itself (28,42,60,61), suggesting that cyp26a1 initiation is likely RA-independent. aldh1a2 expression during somitogenesis is directly repressed by RA (42,44), but similarly to cyp26a1, early activators, other than Tgif in this work, have not yet been described. TGF has been shown to bind the co-repressors CtBP and mSin3 (13,56,62), and may activate cyp26a1 and aldh1a2 expression by repressing intermediate transcription factors, the identity of which are clearly of interest. Alternatively, Tgif may have an activating function in certain cellular contexts not yet identified. Notably, other TALE-class homeodomain transcription factors can act as both activators and repressors, depending on the presence or absence of cofactors (63). Tgif has been shown to bind RXRs and, less robustly, RARs (13). Whether Tgif’s RA receptor binding abilities are required for normal initiation of aldh1a2 and cyp26a1, and whether it is a direct or indirect interaction between Tgif and these two transcriptional targets, remains to be determined. Our experiments suggest that RA is less effective at inducing target gene expression in the absence of Tgif, suggesting a role for Tgif in transducing the RA signal. This finding seems inconsistent with the model proposed by Bartholin et al. (13), whereby TGF directly competes with RA to represses RA signaling. If this was the case, upon RA treatment, tgif morphants would be expected to show a stronger induction of RA target genes than wild-type embryos, rather than the weaker response reported here. However, RA activity is known to be highly tissue specific, and Tgif may similarly have different activities in different cell types. Pinpointing the mechanism of Tgif’s activity during neural patterning will be necessary to clarify the potential multiple interactions between Tgif and the RA signaling pathway, and is the subject of ongoing studies.

A novel animal model for Tgif function

The phenotype of HPE patients is highly variable, and no convincing phenotype—genotype correlation exists for the known TGIF mutations. In fact, TGIF mutations detected in patients can sometimes be found in a phenotypically normal parent (64). Because of this variability, it has been widely hypothesized that environmental effects play a major role in the etiology of HPE. Our results support this idea, demonstrating that the loss of TGIF causes aberrant RA metabolism. In particular, the misregulation of cyp26a1, and diminished ability to process RA, may cause TGIF-deficient individuals to be highly sensitive to the teratogenic effects of RA on the forebrain. Although no obvious forebrain abnormalities are present in the three independently generated Tgif knockout mouse lines (13,39,65), one line does indeed show increased susceptibility to RA-induced teratogenesis (13), consistent with our model of TGF function.

Recently, Kuang et al. (40) have reported a mouse line with Tgif lacking exon 3 that they propose functions as a dominant negative, potentially overcoming compensatory effects of the three other mammalian Tgif-like genes. The Tgif<sup>exon3</sup> mouse has several features recapitulated in the zebrafish tgif morphant model. Both have defects in shh and pax6, as well as in members of the nk family of genes involved in forebrain patterning. Zebrafish tgif morphants have defects in titf1a and titf1b (also known as nk2.1a and nk2.1b), whereas the Tgif<sup>exon3</sup> mouse has defects in Nkx2.2 expression. A zebrafish model has the advantage of having only one potentially redundant tgif-like gene, and the ability to examine Tgif’s role in the earliest stages in neural patterning. Yet, the mouse model may reveal a role for Tgif in generating mammalian-specific forebrain morphology and patterning. Both of these animal models will undoubtedly further our understanding of Tgif function and HPE in future work.

We demonstrate that human TGIF, when overexpressed in zebrafish, has activity that is clearly relevant to early CNS development. Therefore, this study lays the groundwork for an in-depth analysis of mutant versions of TGIF that are linked to HPE. The ease of overexpression is a key advantage of the zebrafish as a model, and the activity of TGIF sequence variants in a developing animal will provide important insights into the structure and function of Tgif protein, and will help elucidate the nature of TGIF-related forebrain mispatterning.

CONCLUSIONS

Changes in RA levels cause severe CNS patterning defects that resemble HPE. Reduced function of Tgif, an HPE candidate gene, results in changes in RA signaling and significant CNS patterning abnormalities. Our work suggests Tgif is involved in establishing early RA levels in the CNS, which has profound implications for early patterning events. HPE can be caused by a wide variety of environmental factors and genetic abnormalities. The variable penetrance of mutations in HPE genes suggests that embryonic environmental insults and genetic deficiencies interact to cause this syndrome (4). Diminished Tgif function and subsequent misregulation of RA metabolism may lead to an individual being highly sensitive to variations in exposure to retinoids, resulting in severe developmental patterning defects, such as HPE.

MATERIALS AND METHODS

Embryos and <i>in situ</i> hybridization

Embryos were grown at 25.5–33°C and staged according to Kimmel et al. (66). cyp26a1<sup>−/−</sup> mutant embryos were unmounted after photography and genotyped as described (30). In all cases, experiments were performed in their entirety on a minimum of three independent occasions. The n values we report typically represent data collected from one to three representative experiments.

<i>In situ</i> hybridization with digoxigenin-labeled RNA probes was performed essentially as described previously (67) with the following modifications: probes were not hydrolyzed; protease K treatment (10 μg/ml in PBS-T) time was 1 min for 8–10S embryos and 3:20 min for 18S embryos, and Iodo-Nitrotetrazolium Violet was used as the alkaline phosphatase substrate for red coloration reactions. Coloration reactions were terminated when one reaction was saturated.

Probes for <i>in situ</i> hybridization were generated using digoxigenin-UTP (Roche) or fluorescein-UTP with T3, T7 or
SP6 polymerase (Roche) and were purified using SigmaSpin columns (Sigma). Plasmid containing cDNA for ptc1 was the kind gift of S. Scholpp and A. Lumsden. Plasmids containing cDNA for gsc, shh, twhh and cyc were the kind gift of M. Halpern. Plasmids containing cDNA for six3b were the kind gift of H.-C. Seo and A. Fjose. Plasmids containing cDNA for titf1a, tinf1b and nkd2a were the kind gift of S. Wilson. Plasmids containing cDNA for cyp26a1, vhnf1, and hoxd4 were the kind gift of R. Hernandez and A. Lumsden. Plasmids containing cDNA for axr, emx3, emx2, barhl2 and tlc were the kind gift of M. Mione and C. Houart.

Cloning of zebrafish and human TGIF

To clone zebrafish tgif, we performed PCR on a 24 hpf zebrafish cDNA library, with primers based on the reported zebrafish sequence of tgif, accession numberAY391467 (forward: 5'-CACAGGATCAGATGAAAGCCAAAGGGTGTGTTTGCCCG-3' and reverse: 5'-CACAGGATCCTGCTTATAATTCCTCATCCG-3'). We amplified a fragment of 877 nt, which was cloned into the pCR4-TOPO vector (Invitrogen) and confirmed by automated sequencing (DYEnamic ET, Amersham). To generate probes for in situ hybridization, pCR4-Tgif was linearized with Pmel (NEB) and digoxigenin-UTP (Roche) probe was synthesized with T7 polymerase (Roche).

To generate our human TGIF construct, we amplified TGIF from pCMV-SPORT6 vector containing the full-length cDNA of TGIF, accession number BC000814 in (Open Biosystems, MHS1010-57645), with primers based on the sequence (forward: 5'-CACAGGATCAGATGAAAGCCAAAGGGTGTGTTTGCCCG-3' and reverse: 5'-CACACTCGAGGCTTTAATTCCTCATCCG-3').

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from zebrafish embryos using the RNaseque isolation system as recommended by the manufacturer (Ambion), and purified with RNeasy columns (Qiagen). One-step RT-PCR was performed with the SuperScript III RT-PCR kit (Invitrogen) using zebrafish tgif-specific primers (as above). An equivalent amount of ExTaq (Takara) was substituted for the reverse transcriptase/Taq enzyme mixture to generate a –RT control. The housekeeping gene efi1α (elongation factor 1α) was amplified as a control (forward: 5'-GGCCACGTCGACTCCGGAAAGTCC-3' and reverse: 5'-TCAAAACGAGGCTTGGCTTAAGG-3').

RNA synthesis and microinjection

cyp26a1, human TGIF and zebrafish tgif were subcloned and ligated into pCS2+MT vector, creating a myc epitope tagged coding sequence. Prior to in vitro transcription, DNA was linearized with NorI (Promega), and was treated with 100 μg/mL proteinase K and 0.5% SDS for 30 min. Capped mRNA synthesis was performed with the mMessage mMachine SP6 kit (Ambion) on 1 μg of template DNA, as suggested by the manufacturer. mRNA was subsequently purified by two sequential microcon YM-50 columns (Amicon, Millipore). Myc-tagged mRNA was injected into embryos at the one-cell stage. Unless otherwise noted, the amount of cyp26a1 mRNA delivered to each embryo was 280 pg; the amount of human TGIF mRNA delivered to each embryo was 180 pg; the amount of zebrafish tgif mRNA delivered to each embryo was 250 pg.

MOs and MO specificity controls

Morpholinos (GeneTools) were diluted in Danieau solution, heated to 65°C for 10 min and allowed to cool to room temperature before microinjection into 1- to 2-cell embryos. Embryos used for the analysis were given a dose of 6 ng of MO1 (5'-ACACCCCTTTGCTTTACGCGT-3') unless otherwise noted. To ensure the specificity of tgif MOs, we designed a second, non-overlapping translation-blocking MO (tgif MO2: 5'-TTACGGTGGCCACCAAGGAAGTCTGCA-3'). tgif MO2 injection showed a phenotype identical to that of tgif MO1 (Supplementary Material, Fig. S2 and data not shown). To demonstrate the binding specificity of MOs, we synthesized mRNA from a fusion of the tgif MO target sequence and eGFP. We amplified eGFP using the following tgif MO1 primer (5'-CACAGGATCGCGACATGAAG ACCAAACATATGTGACAGGCGAGGAGCTG-3') and tgif MO2 primer (5'-CACAGGATCTCGACCTCTCTGTTGCCACGTGAACATGGTGAGCAAGGGCGAGGAGCTG-3'), and a common reverse primer (5'-CACACTCGAGGCTTTAATTCCTCATCCG-3'). These were subcloned into pCS2+ and used for in vitro transcription and mRNA injection. Three nanograms of tgif MO (tgif MO1 or tgif MO2) was injected in conjunction with mRNA. When MO1::eGFP or MO2::eGFP mRNA is injected alone, we observed an efficient translation of eGFP. When MO1::eGFP mRNA injection is followed by injection of MO1, eGFP translation is eliminated. When MO1::eGFP injection is followed by injection of MO2, however, no knockdown occurs. Likewise, when MO2::eGFP injection is followed by MO2 injection, translation of the mRNA is eliminated; injection of MO1 has no effect (Supplementary Material, Fig. S2). These data demonstrate that both tgif MOs specifically bind their tgif target sequence and block the translation of bound mRNA. We confirmed that Tigf protein levels were reduced in tgif morphants by generating whole embryo lysates for western blotting (1/50 dilution, αTGIF H-172, Santa Cruz Biotechnology; Supplementary Material, Fig. S2). Finally, injecting either the zebrafish or human version of TGIF rescues the effect of tgif MO on cyp26a1 expression in gastrulating embryos, while injecting control RNA (GFP) fails to do so (Supplementary Material, Fig. S2 and data not shown).

cyp26a1 MO was of identical sequence to that used by Kudoh et al. (43) (5'-CGCAACGATCGCCAAAGGCAAGAGG-3'). An amount of 2.5 ng of cyp26a1 MO was injected into the yolk of one- to two-cell embryos. p53 MO was used to suppress apoptosis (5'-GGCCCATTTGCGCCAGAGG-3'); 45).

Pharmacological treatments

Diethylaminobenzaldehyde has previously been shown to specifically inhibit retinaldehyde dehydrogenases and reduce RA signaling in zebrafish (47–49). DEAB was stored as a
100 mM stock in DMSO and diluted into embryo medium to 1 μM. Embryos in their chorions were placed into DEAB-containing medium immediately after collection, and were grown at 33°C in the dark. For the suboptimal dose with which embryos were treated in Figure 3, DEAB-containing medium was poured off embryos at 10–11 hpf, and fresh medium added. Control embryos are siblings treated with an equivalent amount of DMSO. All-trans RA was stored as a 12.5 mM stock in ethanol and diluted into embryo medium to 1 nM. Embryos were treated in their chorions from 5.3 to 10 hpf. Control embryos are siblings treated with an equivalent amount of ethanol.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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