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RESEARCH REVIEW

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Functions of TGIF homeodomain proteins and their roles in normal brain development and holoprosencephaly

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Holoprosencephaly (HPE) is a frequent human forebrain developmental disorder with both genetic and environmental causes. Multiple loci have been associated with HPE in humans, and potential causative genes at 14 of these loci have been identified. Although *TGIF1* (originally TGIF, for Thymine Guanine-Interacting Factor) is among the most frequently screened genes in HPE patients, an understanding of how mutations in this gene contribute to the pathogenesis of HPE has remained elusive. However, mouse models based on loss of function of *Tgif1*, and the related *Tgif2* gene, have shed some light on how human *TGIF1* variants might cause HPE. Functional analyses of TGIF proteins and of *TGIF1* single nucleotide variants from HPE patients, combined with analysis of forebrain development in mouse embryos lacking both *Tgif1* and *Tgif2*, suggest that TGIFs regulate the transforming growth factor ß/Nodal signaling pathway and sonic hedgehog (SHH) signaling independently. Although, some developmental processes that are regulated by TGIFs may be Nodal-dependent, it appears that the forebrain patterning defects and HPE in *Tgif* mutant mouse embryos is primarily due to altered signaling via the Shh pathway.

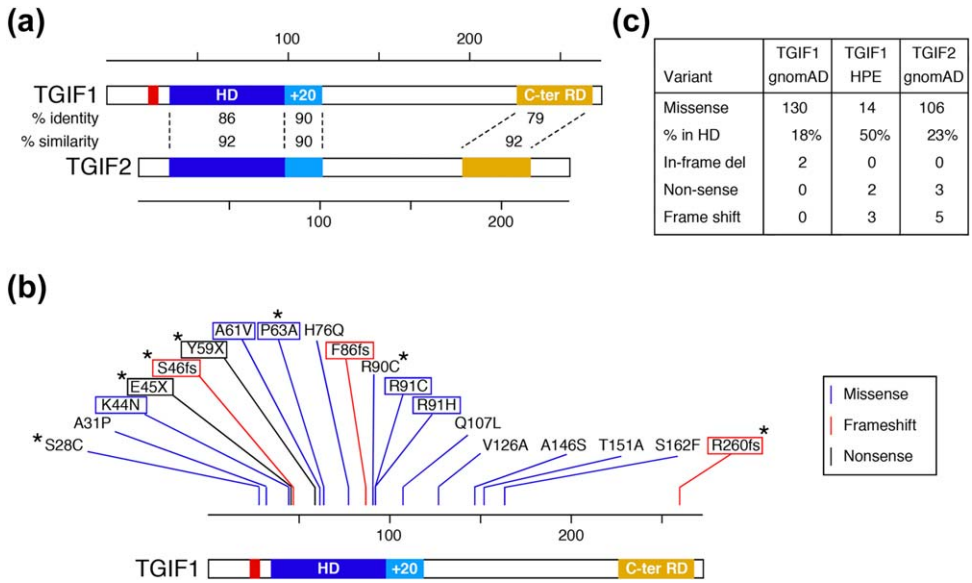
KEYWORDS
development, forebrain, holoprosencephaly, mouse, Nodal, SHH, TGIF, TGIF1, TGIF2

1 | INTRODUCTION

HPE affects ~1/8,000 live human births and up to 1/250 conceptuses, and is the most frequent human forebrain developmental disorder (Leoncini et al., 2008). The primary defect in HPE is a failure of the ventral forebrain to divide into two hemispheres, which is associated with defective midline facial structures (Golden, 1998; Rubenstein & Beachy, 1998). In addition, a number of other abnormalities can be associated with HPE, including neurological defects, such as hydrocephalus or cognitive impairment, and there is considerable variability in both the severity and the penetrance of the phenotypes (Solomon, Gropman, & Muenke, 1993). At least 14 candidate genes have been associated with nonsyndromic HPE, and the *SHH*, *ZIC2*, *SIX3*, and *TGIF1* genes are most commonly screened for mutation as part of routine genetic evaluation of HPE patients (Solomon et al., 1993). Here, we discuss the functional analysis of TGIF1 and TGIF2, their links to HPE, and analyses of mouse mutants that have been generated to interrogate the pathways regulated by Tgifs and to understand how loss of Tgif function causes HPE.

2 | TGIF HOMEODOMAIN PROTEINS

Human TGIF1 was first identified by its ability to bind to a specific DNA element from the rat CRBPII (*Rbp2*) gene promoter, and mouse Tgif1 was cloned by homology (Bertolino, Reimund, Wildt-Perinic, & Clerc, 1995; Bertolino, Wildt, Richards, & Clerc, 1996). The related *TGIF2* was also identified based on similarity to *TGIF1*, although outside the highly conserved homeodomains the proteins share limited similarity (Imoto et al., 2000; Melhuish, Gallo, & Wotton, 2001). TGIF1 and TGIF2 are members of the atypical TALE superfamily of homeodomain proteins (Bertolino et al., 1995; Burglin, 1997; Mukherjee & Burglin, 2007). The homeodomain is an ~60 amino acid DNA and protein binding domain, consisting of three helices that form a globular structure (Gehring, Affolter, & Burglin, 1994; Gehring, Qian, et al., 1994). Specific amino acid positions, primarily within the third carboxyl-terminal helix, mediate sequence-specific DNA interactions. Originally identified in the homeotic genes in *Drosophila melanogaster*, the homeodomain is found in plants, fungi, and animals, and homeodomain proteins regulate numerous important developmental functions (Burglin & Affolter, 2016). The TALE superfamily is characterized by a Three Amino acid



Variant	TGIF1 gnomAD	TGIF1 HPE	TGIF2 gnomAD
Missense	130	14	106
% in HD	18%	50%	23%
In-frame del	2	0	0
Non-sense	0	2	3
Frame shift	0	3	5

FIGURE 1 Variants in human TGIF1. (a) The human TGIF1 and TGIF2 proteins are shown schematically with the percent identity and similarity for the conserved domains shown between. Major features are shown: The homeodomain (HD), the 20 amino acid region carboxyl-terminal to it (+20), and the carboxyl-terminal repression domain (C-ter RD) are present in both. The red box amino-terminal to the HD represents the five amino acid CtBP recruitment motif that is found in TGIF1 but not TGIF2. An amino acid scale is shown above and below each. (b) Sequence variants in TGIF1 from HPE patients are shown, using the indicated color coding in the on-line version (Blue: missense, Red: frameshift, Black: nonsense). Boxed variants affect codons that show no variation in the gnomAD database. Asterisks indicate variants that have altered function in in vitro or cell-based assays, although not all variants shown here have been tested functionally. (c) Sequence variation in *TGIF1* and *TGIF2* is shown in summary form. The numbers of each type of variant affecting the coding sequence of *TGIF1* and *TGIF2* are shown for those identified in *TGIF1* from HPE patients, and for both genes from the gnomAD database. For missense variants, the percentage that are within the homeodomain is also shown

Loop Extension present between the first two helices (Burglin, 1997; Burglin & Affolter, 2016). The TALE does not affect DNA binding, but likely plays a role in interaction with other proteins, including other homeodomain proteins, as in the case of PBX–HOX interactions (Passner, Ryoo, Shen, Mann, & Aggarwal, 1999; Piper, Batchelor, Chang, Cleary, & Wolberger, 1999). Although TGIFs have a conserved TALE, it is not known if this loop in the Tgif proteins mediates specific protein–protein interactions. TGIF1 and TGIF2 have a high degree of sequence identity within the homeodomain, and a 20 amino acid region immediately carboxyl-terminal to the homeodomain that is not conserved in other members of the TALE superfamily (Hyman, Bartholin, Newfeld, & Wotton, 2003) (Figure 1a). Outside this, there is a second region of high sequence similarity toward the carboxyl-termini of the proteins, which interacts with corepressors, with the remainder of the sequence being much less similar. A conserved sequence motif (PLDLS), known to recruit the CtBP family of transcriptional corpressors (Chinnadurai, 2002, 2007; Schaeper, Subramanian, Lim, Boyd, & Chinnadurai, 1998), is present in vertebrate TGIF1 orthologs, but is absent form the TGIF2 proteins. While multiple splice variants of human *TGIF1* have been identified, the major isoform encodes the 272 amino acid protein originally identified, and all splice variants encode the homeodomain and the sequences carboxyl-terminal to it (Hamid, Patterson, & Brandt, 2008). Close TGIF1 and TGIF2 homologs are present in vertebrates. In addition to mouse and human, Tgif-related proteins from Xenopus, zebra fish, and chicken have been characterized, all having broadly similar functions as transcriptional repressors (Hyman et al., 2003; Ryan, Tejada, May, Dubaova, & Deeley, 1995; Spagnoli & Brivanlou, 2008). In contrast, in flies there are a pair of related proteins with the highly conserved homeodomain plus the 20 amino acid sequence carboxyl-terminal to it, but they share no other similarity to the vertebrate TGIFs and are transcriptional activators rather than transcriptional repressors (Hyman et al., 2003). Despite sequence differences outside the homeodomain and conserved carboxyl-terminal repression domain (Figure 1a), the vertebrate TGIF1 and TGIF2 paralogs are both transcriptional repressors and appear to have largely overlapping functions in early development (discussed below).

3 | TGIF1 VARIATION IN HPE

Of the 14 candidate genes that have been associated with nonsyndromic HPE, the *SHH*, *ZIC2*, *SIX3*, and *TGIF1* genes are the most commonly screened for mutations in HPE patients (Solomon et al., 1993). Among individuals with a family history of HPE, up to 30%–40% have variants in the *SHH* gene, *ZIC2* variants are found in only around 5%, and *SIX3* and *TGIF1* variants are each in the 1%–2% range. Thus, *SHH* appears to be the major HPE gene in humans, and this is the pathway that is best characterized as being responsible for HPE when disrupted, either genetically or by environmental teratogens (Roessler & Muenke, 2010). *TGIF1* was identified as the gene present in the minimal critical region at the HPE4 locus at 18p11.3 (Gripp et al., 2000; Overhauser et al., 1995). As with other HPE mutations, loss of *TGIF1* appears to be

inherited in an autosomal dominant manner. *TGIF1* variants found in HPE patients are associated with the full range of clinical phenotypes, but complete deletions of the HPE4 locus may cause additional craniofacial and neural defects compared to patients with intragenic *TGIF1* variants (Keaton et al., 2010). This perhaps suggests that deletion of additional genes at this locus together with *TGIF1* can contribute to a broader range of phenotypes. There has been some speculation that the incomplete penetrance of HPE-associated mutations suggests a two-hit model, where variants at two commonly affected loci are needed for the phenotype. Although, this possibility has not been fully excluded, it also appears likely that a predisposing variant combined with environmental factors and other more subtle genetic differences results in the appearance of HPE (Roessler, Velez, Zhou, & Muenke, 2012).

Since Tgif1 and Tgif2 in mice appear to have largely overlapping functions during embryonic development (Melhuish, Taniguchi, & Wotton, 2016; Powers et al., 2010; Taniguchi, Anderson, Sutherland, & Wotton, 2012), *TGIF2* represents a reasonable candidate gene that, when mutated, might cooperate with a *TGIF1* mutation in driving the HPE phenotype. However, there is as yet no evidence for HPE-associated mutations in the human *TGIF2* gene. Screening of a cohort of almost 500 HPE patients revealed no potentially pathogenic variants in the *TGIF2* gene (El-Jaick et al., 2007). Although, this suggests that *TGIF2* is unlikely to have a major role in HPE in humans, it remains possible that rare variants might have been missed in this analysis, particularly given the relatively low frequency of *TGIF1* variants in HPE. In addition to the larger deletions in human *TGIF1* that have been found in HPE patients, 19 single amino acid changes have been identified (Figure 1b) (El-Jaick et al., 2007; Keaton et al., 2010; Mercier et al., 2011). Of these 19, 10 affect amino acids at which variants are not present in the gnomAD browser (Genome Aggregation Database; http://gnomad.broadinstitute.org) (Lek et al., 2016). The remaining nine variants are also found in the gnomAD database, including two that have been shown to have functional consequences (discussed below). Thus, a significant proportion of the *TGIF1* variants found in HPE patients are present in unaffected individuals. This is consistent with the low penetrance of *TGIF1* mutations with respect to HPE, although some may be nondeleterious variants present in the wider population. The missense variants from gnomAD are relatively evenly distributed over the *TGIF1* coding sequence, with 18% of them in the homeodomain, which represents 23% of the coding sequence (Figure 1c). The proportions in *TGIF2* are similar, with 23% of missense variants in the homeodomain (27% of the coding sequence). In contrast, half of the missense variants from HPE patients are in the homeodomain (Figure 1c). While there are no truncating variants (frameshift and nonsense) in the gnomAD data for the major 272 amino acid encoding isoform of *TGIF1*, four of five truncating variants from HPE patients are within the homeodomain. The remaining variant results in a frameshift close to the carboxyl-terminus, and has been shown to affect TGIF1 function (discussed below). Although, the analysis of where missense and truncating variants lie within the coding sequence is based on a relatively limited number from HPE patients, it perhaps suggests that disruption of the homeodomain may be important for HPE pathogenesis.

4 | TRANSCRIPTIONAL REGULATION BY TGIFs

The majority of functional analysis of TGIFs has been performed with human TGIF1, with some analysis of TGIF2. Given that human and mouse Tgifs share almost identical amino acid sequences, it is likely that conclusions from these analyses apply to the mouse homologs. The initial identification of TGIF1 was by its ability to bind to a retinoid response element, and it was suggested that TGIF1 could limit transcriptional activity driven by this element by competing for binding with retinoid X receptor (RXR) nuclear receptors (NRs) (Bertolino et al., 1995). Subsequent work has shown that TGIF1 interacts with multiple general transcriptional corepreressors including mSin3, histone deacetylases, and CtBP1 (Melhuish & Wotton, 2000; Sharma & Sun, 2001; Wotton, Knoepfler, Laherty, Eisenman, & Massague, 2001; Wotton, Lo, Lee, & Massague, 1999; Wotton, Lo, Swaby, & Massague, 1999). TGIF2 also interacts with histone deacetylases and mSin3, but lacks the motif that is known to recruit CtBP corepressors (Melhuish et al., 2001; Melhuish & Wotton, 2006). Thus it is likely that in addition to competing with activators, TGIFs recruit active transcriptional repressors to limit gene expression. In support of this, when TGIFs are artificially targeted to DNA by fusion to a heterologous DNA-binding domain they drive transcriptional repression of a linked reporter gene (Melhuish et al., 2001; Wotton, Lo, Swaby, et al., 1999). Recent genome-wide analysis by ChIP-seq for Tgif1 in mouse embryonic stem (ES) cells suggests that the major way in which Tgif1 is recruited to DNA is via binding to its cognate response element, independent of other recruiting proteins (Lee et al., 2015) (Figure 2a). This appears to be consistent with transcriptome analyses in cells or tissues with reduced TGIF levels, in which the majority of genes that change in expression levels are independent of the TGFß/Nodal and NR signaling pathways (Anderson et al., 2017; Lee et al., 2015; Zerlanko, Bartholin, Melhuish, & Wotton, 2012). In addition, TGIF1 has been suggested to compete for direct binding to DNA with the Meis family of TALE homeodomain proteins, which bind the same sequence but are transcriptional activators (Willer et al., 2015; Yang et al., 2000).

This competition for binding to DNA is consistent with the original suggestion that TGIF1 competed with RXR for binding to the retinoid response element of the *Rbp2* gene (Bertolino et al., 1995). However, more recent analyses have suggested that TGIF1 can interact with RXR, such that TGIF1 could be recruited indirectly to DNA at more canonical NR binding elements without the need for a TGIF consensus site (Bartholin et al., 2006; Melhuish, Chung, Bjerke, & Wotton, 2010). The NRs comprise a large family of transcriptional regulators, which dimerize and bind to DNA in response to ligand binding, to control many complex gene expression programs (Mangelsdorf et al., 1995). Since RXR is a common partner for multiple other NRs in addition to retinoic acid receptors (Evans & Mangelsdorf, 2014), recruitment through interaction with the common RXR partner raises the possibility that TGIFs might regulate additional NR-regulated responses without the need for a TGIF consensus site (Figure 1b). Given the teratogenic effects of retinoic acids and their link to HPE-like phenotypes (Lanoue et al., 1997; Sulik, Dehart, Rogers, & Chernoff, 1995), the possibility

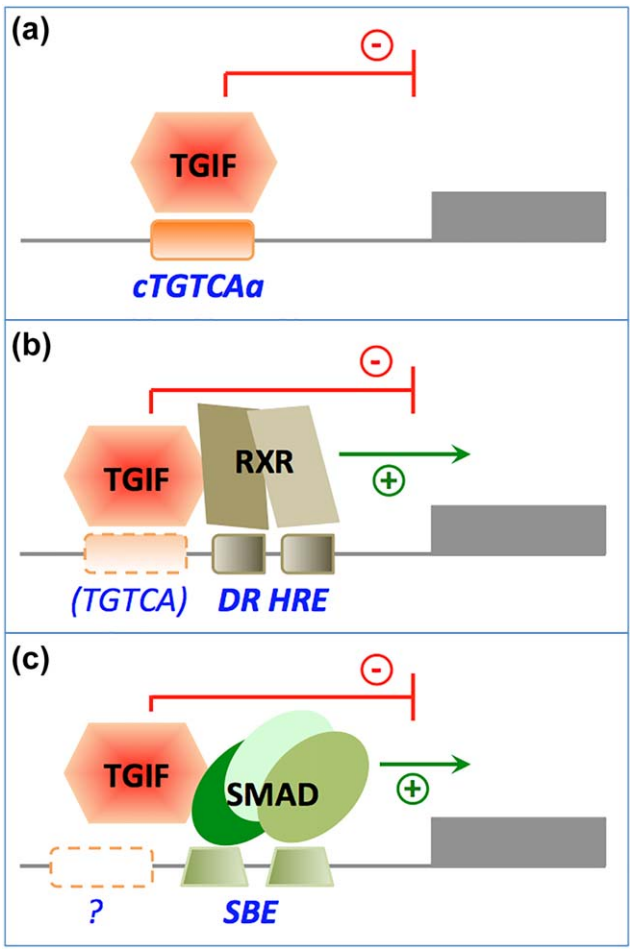


FIGURE 2 Models of transcriptional regulation by TGIFs. (a) TGIFs can bind directly to DNA via a well-defined consensus site (cTGTCAa, with the central five bases being more important). This results in repression (negative sign) of a linked target gene. (b) TGIF1 can be recruited to direct repeat (DR) hormone response elements (HRE) bound by nuclear receptors. The recruitment of TGIF1 can be by binding to the TGIF consensus site, or by indirect recruitment via interaction with the RXR, which activates gene expression (plus sign) when in a ligand bound NR complex. (c) At TGFß-responsive genes, SMAD proteins bind to SMAD-binding elements (SBE) to activate gene expression (plus sign). TGIFs can be recruited indirectly via SMAD interaction, limiting activation (minus sign). Although, there is no evidence for binding to a TGIF consensus site in this case, a requirement for DNA binding by TGIFs has not been conclusively ruled out

that reduced TGIF levels result in increased retinoid responsive gene expression has been of some interest. There is some evidence for increased sensitivity of *Tgif1* null mouse embryos to in utero exposure to retinoic acid, but not for increased HPE-like phenotypes specifically (Bartholin et al., 2006; Melhuish et al., 2016).

Much of the interest in TGIF function has centered on the roles of TGIFs in the TGFß/Nodal signaling pathway. In response to TGFß family signals, the SMAD transcription factors are phosphorylated, and accumulate in the nucleus, where they bind to DNA and regulate gene expression (Hill, 2016; Massague, Seoane, & Wotton, 2005). Signaling from TGFßs, Nodal, and Activins is primarily mediated by SMAD2 and SMAD3, in conjunction with the shared partner, SMAD4. Once bound to a SMAD-binding element, the SMAD complex primarily functions to recruit transcriptional coactivators and activate gene expression (Hill, 2016; Massague et al., 2005). TGIF1 was found to interact with SMAD2 and SMAD3, and this appears to be independent of direct DNA binding by TGIF1 to its consensus site (Wotton, Lo, Lee, et al., 1999). Interaction of TGIF1 or TGIF2 with SMADs results in competition with SMAD coactivators, recruitment of TGIF-bound transcriptional corepressors, and reduced TGFß responsive gene expression (Melhuish et al., 2001; Wotton, Lo, Lee, et al., 1999). Unlike other inhibitors of the TGFß signaling pathway, such as SMAD7 or SKIL (SnoN) (Nakao et al., 1997; Stroschein, Wang, Zhou, Zhou, & Luo, 1999), there has been little evidence that TGFß signaling regulates TGIFs directly. This has led to the model that TGIF levels limit the degree to which cells respond transcriptionally to TGFß signaling, but do not play a major role in inducible or feedback mechanisms of repression.

During embryogenesis, it appears that at least a subset of the phenotypes caused by loss of TGIF function in mice can be linked genetically to Nodal signaling, consistent with a role for TGIFs in this pathway (Powers et al., 2010; Taniguchi et al., 2012). In primary mouse embryo fibroblasts lacking Tgif1, there was some enrichment for activation of genes that are TGFß responsive in these cells (Zerlanko et al., 2012), and comparison of Tgif1 genome-wide binding in mouse ES cells with regions bound by Smad2 or Smad3 suggested that the majority of genes that were bound by Smads were also bound by Tgif1 (Lee et al., 2015). Although this is consistent with Tgifs as regulators of the TGFß signaling pathway, it is likely that regulation of TGFß-responsive gene expression represents a fraction of overall TGIF function, and it is possible that only a subset of TGFß responses are subject to regulation by TGIFs. Regulation of SMAD target genes is not thought to require DNA binding by TGIFs, suggesting an indirect recruitment model via interaction with SMADs (Figure 1c). However, it should be noted that a role for TGIF-DNA binding has not been definitively ruled out, and this might provide a mechanism for additional specificity of TGIF function in the TGFß pathway.

In addition to regulation of gene expression via recruitment to target genes, a number of other potential functions for TGIF1 have been examined. These include more indirect mechanisms for interfering with TGFß responses, such as promoting SMAD2 ubiquitylation and degradation, or preventing SMAD2 phosphorylation in response to TGFß (Seo et al., 2004, 2006). An additional, nontranscriptional function for TGIF1 has recently been proposed, whereby TGIF1 indirectly activates WNT signaling by sequestering AXIN1 and AXIN2 (Zhang et al., 2015). While TGIF1 may regulate gene expression programs by multiple mechanisms, in this review we focus on the more direct transcriptional effects. These are the most extensively characterized TGIF functions, and at least one variant in *TGIF1* from an HPE patient specifically disrupts interaction with CtBP corepressors (described below), consistent with HPE being due to effects on transcriptional regulation (Melhuish & Wotton, 2000). Thus, TGIFs are best characterized as repressors of gene expression that recruit general transcriptional corepressors to

DNA, via a combination of direct DNA binding and interaction with other transcription factors (Figure 2).

5 | FUNCTIONAL ANALYSIS OF TGIF1 VARIANTS

Although the majority of HPE-associated changes in *TGIF1* are deletions, the functional consequences of intragenic variants are of significant interest as they may give some clue as to the pathways that, when disrupted, are responsible for HPE. Of the 19 intragenic *TGIF1* variants found in HPE patients (El-Jaick et al., 2007; Keaton et al., 2010; Mercier et al., 2011), four result in truncation of the protein within the homeodomain (Figure 1). Not surprisingly, the three of these truncation mutants that have been tested functionally are inactive (El-Jaick et al., 2007; Mar & Hoodless, 2006). The fourth is likely to have a similar effect since it would remove at least part of the DNA-binding third alpha-helix (Figure 1c). A frameshift mutation close to the carboxyl-terminus of the protein, at amino acid 260, was shown to result in very low levels of expression, possibly due to decreased stability (El-Jaick et al., 2007). Of the 14 missense variants from HPE patients, half have been tested in functional assays, but only three have been shown to have functional consequences. The S28C variant is present within the conserved motif (PLDLS) that recruits CtBP corepressors, and this variant has been shown both to disrupt interaction with CtBP1 and to reduce transcriptional repression by TGIF1 (El-Jaick et al., 2007; Melhuish & Wotton, 2000). Two missense variants within the homeodomain have been shown to reduce TGIF1 function. The R90C variant, which is present in the DNA-binding helix, abolishes binding to the consensus TGIF site, reduces interaction with SMAD3 and RXR, and also reduces repression of reporter gene expression (El-Jaick et al., 2007). Although variants at the R91 position have not yet been tested, it is likely that they will affect DNA binding, since this is a direct DNA contact residue that, when altered to methionine prevents consensus site binding (Tejada, Jia, May, & Deeley, 1999). The P63A variant also decreases DNA binding and SMAD3 interaction, and reduces transcriptional repression (El-Jaick et al., 2007). A P63R alteration in TGIF1, has also been shown to have functional consequences, resulting in protein aggregation that was suggested to sequester wild type TGIF1 (El-Jaick et al., 2007; Ferrand, Demange, Prunier, Seo, & Atfi, 2007). Although this change is not found in HPE patients, it appears to have a similar effect on protein function to the HPE-associated P63A variant (El-Jaick et al., 2007). The P63R mutant and R90C variant were also unable to complement a proliferation defect in primary mouse embryo fibroblasts lacking endogenous Tgif1, whereas the S28C and Q107L variants were able to restore normal proliferation in *Tgif1* null fibroblasts (Mar & Hoodless, 2006).

For variants that did not result in apparent defects in TGIF1 function in cell-based assays, it remains possible that this is due either to the presence of endogenous wild type TGIF1, or that the appropriate assay was not used. However, it is also possible that some of these variants represent silent changes present in the population. Indeed, some of the variants that have been shown to affect function are also present in the gnomAD database, consistent with the incomplete penetrance of *TGIF1* mutations. Of the missense variants shown to have functional differences from the wild type, the S28C variant is perhaps the most informative, since this has a very specific effect on protein function, namely reducing corepressor recruitment (Melhuish & Wotton, 2000). This clearly suggests it is the loss of transcriptional repression by TGIF1 that contributes to HPE, rather than other non-transcriptional functions that have been proposed. However, none of the reduced function variants distinguish between repression of transcription via SMAD or RXR interaction, or direct DNA binding independent of these pathways, so they do not provide any clues to the signaling pathways affected.

6 | LOSS-OF-FUNCTION MOUSE MODELS

Given the low penetrance and relatively low frequency of *TGIF1* variants in HPE it was important to test if *TGIF1* is indeed an HPE-causal gene in mouse models. Several lines of mice with *Tgif1* deletions were created, in which the majority of the coding sequence of the major isoform was removed, and none revealed any HPE-like phenotypes (Bartholin et al., 2006; Jin, Gu, McKinney, & Ding, 2006; Mar & Hoodless, 2006; Shen & Walsh, 2005). A number of defects were observed in these mouse models, but they were generally of variable penetrance and often appeared to be background strain-specific. In a relatively pure C57BL6 strain background, reduced viability, growth delay, and placental defects have been observed in *Tgif1* null mice (Bartholin et al., 2008; Tateossian et al., 2013). Additionally, Tgif1 mutants in this background were shown to have otitis media with effusion, resulting in deafness, potentially linked to alterations in TGFß signaling (Tateossian et al., 2013). In a mixed 129Sv/CD1 background laterality defects and some growth delay were observed in the *Tgif1* null mice, although these phenotypes did not appear in a pure C57BL6 background (Mar & Hoodless, 2006). The identification of laterality defects in this model is supported by the later demonstration that embryos lacking both Tgif1 and Tgif2 showed loss of left–right asymmetry, associated with altered Nodal signaling (Powers et al., 2010). Primary fibroblasts from *Tgif1* null embryos have gene expression changes and proliferation defects in vitro, that were partly dependent on altered TGFß signaling (Mar & Hoodless, 2006; Zerlanko et al., 2012). Increased sensitivity of *Tgif1* mutant embryos to retinoic acid-induced teratogenecity was also observed, resulting in a higher frequency of exencephaly in exposed *Tgif1* null embryos and more severe defects in the axial skeleton (Bartholin et al., 2006; Melhuish et al., 2016). Overall, these studies suggest that Tgif1 has multiple effects in mouse development, some of which may be attributable to the TGFß or retinoic acid pathways, but do not reveal any strong link to HPE. Evidence from Xenopus supports a role for Tgif1 in regulating Nodal signaling, although, in this case by controlling expression of the *Nodal* gene (Kerr, Cuykendall, Luettjohann, & Houston, 2008). In zebrafish, reducing *Tgif1* expression was shown to alter retinoic acid synthesis and responses, and to affect forebrain patterning (Gongal & Waskiewicz, 2008). Thus, evidence from other model organisms is broadly supportive of a role for Tgifs in regulating Nodal and retinoid signaling. Combining *Tgif1* and *Shh* mutations

in mice did not reveal any evidence for cooperative effects on the development of HPE due to mutation of these genes, consistent with the scarcity of evidence for mutations in both genes together in human HPE patients (El-Jaick et al., 2007; Shen & Walsh, 2005). An additional mouse mutant, in which a single exon of *Tgif1* was deleted developed anterior defects, although not primarily HPE (Kuang et al., 2006). Again, only in the C57BL6 background, the *Tgif1* null embryos had an incompletely penetrant hypoplastic head or exencephalic phenotype, with much less frequent HPE-like defects. However, this did provide evidence for *Tgif1* as a causal HPE gene in mouse models.

The structural and functional similarities between Tgif1 and Tgif2 clearly raise the possibility of redundant phenotypes. During early embryogenesis, *Tgif2* appears to be broadly expressed throughout the mouse embryo from embryonic day (E) 6–8.5, and has a neuroepithelial expression pattern similar to that of *Tgif1* later in development (Powers et al., 2010; Shen & Walsh, 2005). Similarly, *Tgif1* expression is seen throughout the prestreak embryo (E6.0), and then at higher levels in the anterior and in the tail bud at the neural plate and head-fold stages (E7-7.5) (Jin et al., 2006). By E9.5, *Tgif1* expression is high in the telencephalon, branchial arches, and somites. Thus expression patterns of *Tgif1* and *Tgif2* are largely overlapping in the early embryo at stages when loss of their function might be expected to contribute to anterior developmental defects such as HPE. As with *Tgif1*, mutation of *Tgif2* in mice did not cause severe developmental defects, and the mice were largely normal and viable (Powers et al., 2010), although *Tgif2* null mice were later shown to have defects in bone resorption (Krzeszinski et al., 2014). The combination of mutations in both *Tgif1* and *Tgif2* resulted in gastrulation failure in embryos that were homozygous null for both genes, whereas the majority of embryos with mutations at three of the four alleles ($Tgif1^{+/-}$;$Tgif2^{-/-}$ or $Tgif1^{-/-}$;$Tgif2^{+/-}$) were normal (Powers et al., 2010). Thus Tgif1 and Tgif2 perform redundant, essential functions early in embryogenesis, but double mutant embryos do not survive to a stage where even early precursor forms of the HPE phenotype would be apparent. The gastrulation defects seen in embryos lacking both Tgifs can be bypassed by combining a *Tgif2* null allele with a conditional mutation in *Tgif1* (Powers et al., 2010). In this model, deletion of *Tgif1* is driven by a Sox2-Cre transgene that results in conditional deletion of loxP flanked sequences from all cells in the epiblast by E6.5 (Hayashi, Lewis, Pevny, & McMahon, 2002). In a *Tgif2* null background with Sox2-Cre mediated homozygous *Tgif1* deletion, the double null embryos survived to ~E10.5–11 (Powers et al., 2010; Taniguchi et al., 2012). These embryos had left–right asymmetry defects and HPE-like defects with essentially 100% penetrance, consistent with overall Tgif function being required for normal forebrain development.

7 | EARLY MOUSE FOREBRAIN PATTERNING

As the forebrain structures begin to form at around E7.75, Shh signaling is responsible for initiating dorsoventral patterning. Shh expression is first seen in the prechordal plate (PrCP) underlying the ventral forebrain precursor tissue starting at E7.75. Shh expression in the PrCP is essential for activating Shh expression in the overlying ventral diencephalon tissue by E9.0, where Shh specifies ventral identity (Geng & Oliver, 2009; Shimamura & Rubenstein, 1997). Homozygous *Shh* null embryos have a forebrain ventricle that lacks ventral identity and fails to divide into two hemispheres (Chiang et al., 1996). Gli3, a zinc-finger transcription factor that primarily acts as a repressor of Shh signaling, has been shown to play a crucial role in forebrain dorsoventral patterning (Fuccillo, Joyner, & Fishell, 2006). In the developing neural tissue, Gli3 is expressed in a dorsoventral gradient with higher Gli3 expression dorsally. *Gli3* homozygous null embryos have a forebrain with dorsally expanded ventral tissue, that lacks dorsal identity. This is consistent with an expansion of the ventral Shh signal, and suggests a requirement for the proper balance between dorsalizing Gli3 and ventralizing Shh (Aoto, Nishimura, Eto, & Motoyama, 2002; Rallu et al., 2002; Tole, Ragsdale, & Grove, 2000). The lack of ventral identity seen in *Shh* null embryos can be partially rescued when the dose of *Gli3* is reduced genetically, suggesting that the mutual antagonism of these two factors is critical for forebrain dorsoventral patterning (Aoto et al., 2009; Rallu et al., 2002). However, since the forebrain develops relatively normally in the absence of both Shh and Gli3, there must be additional pathways that specify telencephalon development. These additional pathways that can specify forebrain development likely depend on Foxg1 and FGF signaling, and there is evidence linking mutations that affect FGF signaling to HPE in humans (Dubourg et al., 2016). In mice, disrupting FGF signaling in the anterior by deletion of the Fgfr1 and Fgfr2 genes results in defective ventral telencephalon development, without disruption of the Shh signaling pathway (Gutin et al., 2006). Thus, although the Shh pathway is a major driver of forebrain patterning, other signaling pathways clearly contribute.

8 | FOREBRAIN DEFECTS IN MOUSE EMBRYOS LACKING BOTH *TGIF1* AND *TGIF2*

Although, there is no evidence for HPE-associated variants in human *TGIF2*, in mice Tgif1 and Tgif2 share overlapping functions during development. Mouse embryos lacking both Tgif genes fail gastrulation have defects in the Nodal signaling pathway, and do not survive beyond around E8.0 (Powers et al., 2010). Epiblast-specific deletion of a conditional *Tgif1* allele in a *Tgif2* null background allows for bypass of the gastrulation defects, and these conditional double null embryos survive to approximately E10.5–11. While overall forebrain size and morphology are largely normal at E8.25-9.25, embryos lacking both Tgifs show precursor forms of HPE consistent with impaired ventral specification, including failure to separate the ventral lips of the cephalic folds at E8.25, as seen in *Shh* null embryos (Figure 3) (Taniguchi et al., 2012). In addition, the midbrain neural tube fails to close in embryos lacking both Tgifs, even by E9.25. By E10.0 embryos lacking Tgif function have a significantly smaller forebrain vesicle, as well as abnormal ventral forebrain morphology with a failure to bisect the ventral head mesenchyme at the midline (Figure 4a). This reduction in forebrain size is partly due to a Nodal-dependent decrease in proliferation in the neuroepithelium (Taniguchi et al., 2012, 2017). Furthermore, marker analysis

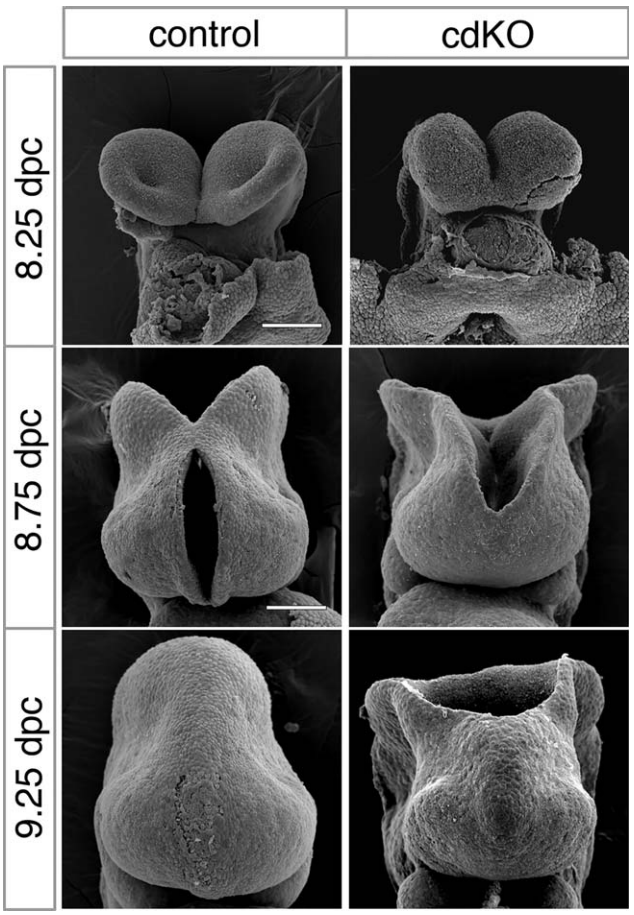


FIGURE 3 Defective neural tube development in mouse embryos lacking Tgif1 and Tgif2. Scanning electron microscopy (SEM) images of control or mutant mouse embryos (cdKO, for epiblast-specific conditional double knock-out of *Tgif1* and *Tgif2*) are shown at the indicated days post coitum (dpc). Views are from the anterior of the embryo. Scale bars: 100 μm. Images from Taniguchi et al. (2012)

demonstrates that the nasal (*Pax7*) and eye (*Pax2*) fields fail to undergo complete separation (Figure 4b) (Taniguchi et al., 2012). This is consistent with the lack of duplication of midline facial features associated with HPE, and in rare older embryos lacking both Tgifs (with a *Nodal* mutation, see below) more canonical HPE phenotypes were observed (Taniguchi et al., 2017). Expression of *Shh* is reduced in the ventral forebrain of mouse embryos lacking both Tgifs, while dorsal *Gli3* expression is increased, suggesting disruption of this major HPE-associated pathway (Taniguchi et al., 2012). Molecular studies have shown that Tgifs regulate the output of Shh signaling through direct transcriptional repression of *Gli3*, which is normally important for restricting Shh signaling (Taniguchi et al., 2017). However, it is likely the reduced *Shh* expression is not entirely due to higher dorsal Gli3 expression in *Tgif1;Tgif2* null embryos, as the increase in *Gli3* expression is relatively modest, and there are other defects, such as reduced proliferation in the ventral neuroepithelium. Reducing *Gli3* levels, by deletion of one allele, in conditional double null embryos results in some restoration of normal forebrain development, including better separation of the facial fields, consistent with the HPE-like phenotype being due in part to disruption of the Shh-Gli3 balance (Taniguchi et al., 2012). However, the combination of mutations in both *Nodal* and *Gli3* appears to further improve the phenotype, suggesting independent effects of these two pathways regulated by Tgifs (Taniguchi et al., 2017). Perhaps surprisingly, a fraction of embryos lacking both Tgifs that also have a heterozygous *Nodal* mutation survive to late gestation

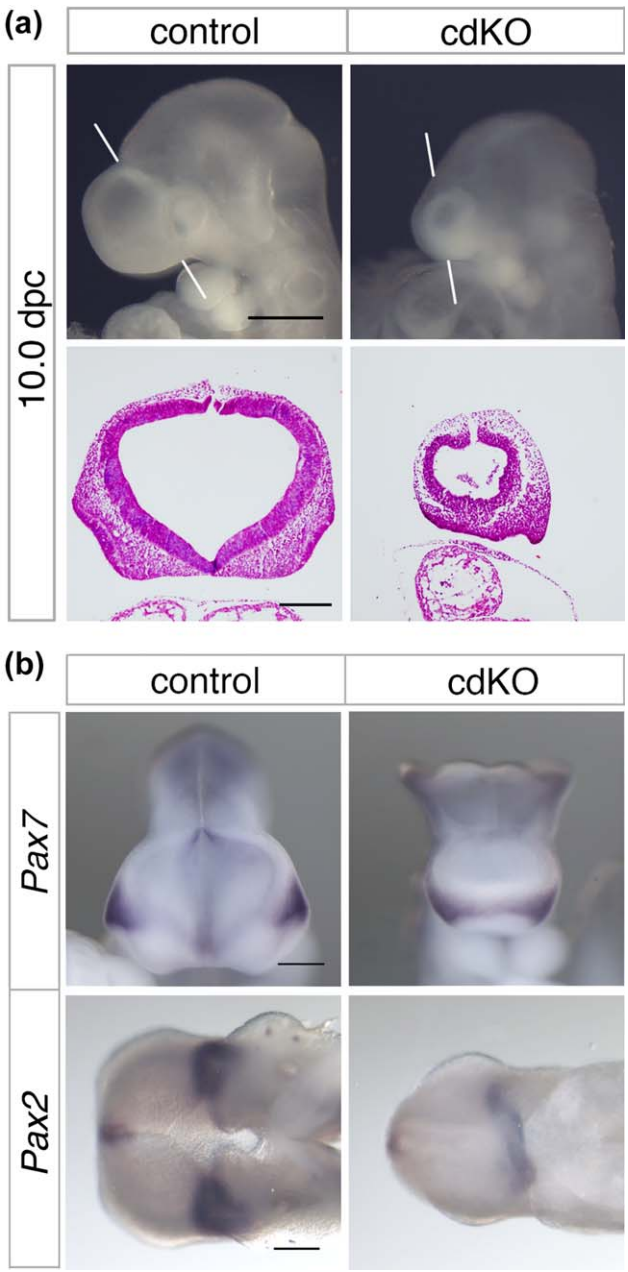


FIGURE 4 Midline separation defects in the absence of Tgifs. (a) Whole-mount images and hematoxylin and eosin (H&E) stained coronal sections of control and cdKO embryos at 10.0 dpc. The white lines indicate the plane of the coronal sections through the forebrain vesicle. Scale bars: 500 μm for whole mount and 200 μm for sections. (b) Whole mount in situ hybridization images of stage-matched (~10.0 dpc for control) embryos, showing expression of Pax7 (above, frontal view) and Pax2 (below, ventral view). Scale bars: 250 μm for *Pax7* and 200 μm for *Pax2*. Images from Taniguchi et al. (2012)

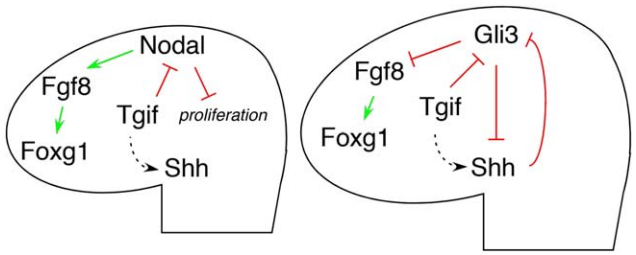


FIGURE 5 Tgif regulation of signaling in the developing forebrain. A side view of the early mouse head is shown schematically (anterior to the left), together with the major signaling interactions discussed here (Tgif represents both Tgif1 plus Tgif2 function). The left hand panel represents ~E8.0 and the right hand ~E9.0. Arrows (green) indicate positive regulation, red lines with bars indicate inhibitory effects. The dashed arrow to Shh from Tgif indicates a possible indirect permissive effect of Tgifs on Shh signaling. In addition to effects on the indicated genes/proteins, Nodal inhibition of proliferation is shown in the earlier schematic. Reduced proliferation may continue later, but by ~E9.0, Nodal is no longer expressed. The temporal separation of Tgif effects on Nodal and Gli3 is primarily for simplicity of the schematic

but have a classic HPE phenotype (Taniguchi et al., 2017). One possible explanation for this is that reducing the excess Nodal response in these embryos partially restores the impaired neuroepithelial proliferation and possibly other defects, but does not affect dorsoventral patterning of the forebrain, since a *Nodal* mutation does not reduce the excess *Gli3* expression in this context.

In addition to effects on the Shh pathway, loss of both Tgifs affects forebrain *Fgf8* expression (Taniguchi et al., 2012). Forebrain *Fgf8* expression is initially activated by Nodal signaling and, in embryos lacking Tgifs, this expression increases by E9.0 (Silvestri et al., 2008; Taniguchi et al., 2012). Telencephalic Fgf8 signaling is critical for maintaining proliferation of rostral forebrain neuroepithelial cells. Soon after the initial activation by Nodal, Gli3 expression is required to restrict *Fgf8* expression to telencephalon progenitors, where Fgf8 activates *Foxg1* expression (Aoto et al., 2002; Storm et al., 2006). At E9.5, embryos lacking both Tgifs have reduced telencephalic *Fgf8* and *Foxg1* expression, due to the ectopic Gli3 expression (Taniguchi et al., 2012). Thus, Tgifs appear to exert complex effects on telencephalic *Fgf8* expression, first limiting Nodal dependent induction, then reducing Gli3-dependent repression. However, it is not known if these effects of Tgifs on Fgf signaling contribute to the phenotypes in conditional double null embryos, and the more persistent effects on the Shh pathway may argue that this is the pathway that causes HPE when disrupted by loss of Tgifs.

Taken together, molecular and embryological analyses suggest that Tgif function impinges on multiple signaling pathways associated with early forebrain development (Figure 5). Phenotypes in the forebrain of embryos lacking all Tgif function can be ameliorated by reducing either Gli3 or Nodal, and disruption of both the Shh and Fgf signaling pathways is observed. It appears that Tgifs are required to dampen the response to Nodal signaling, which initiates telencephalic Fgf8 expression, and limits neuroepithelial proliferation (Figure 5, left). As the Shh-Gli3 balance is established to regulate dorsoventral patterning of the developing forebrain, Tgifs directly repress *Gli3* expression, preventing excess dorsalization, and also limiting Fgf8 expression (Figure 5, right). One remaining question is whether Tgifs regulate *Shh* expression in the PrCP and the ventral forebrain, other than indirectly via Gli3. While the PrCP is largely intact in Tgif double mutant embryos, *Shh* expression is reduced in the PrCP, and this may subsequently contribute to the reduced *Shh* expression in the ventral forebrain. A Gli3-independent role for Tgifs in allowing normal *Shh* expression is suggested by the fact that removing one copy of *Gli3* does not restore *Shh* expression in embryos lacking Tgifs. In addition, conditional double null embryos that were heterozygous for both *Gli3* and *Nodal* had partially restored *Shh* expression in ventral telencephalon at E9.5 (Taniguchi et al., 2017). This partial rescue of *Shh* expression may be due to better dorsoventral patterning together with a Nodal dependent improvement in forebrain proliferation, suggesting an indirect mechanism by which Tgifs are permissive for normal forebrain *Shh* expression. However, whether Tgifs regulate *Shh* expression in the PrCP and diencephalon more directly remains to be determined. Nonetheless, these studies suggest that Tgifs independently regulate the Nodal and Shh signaling pathways, and that the HPE phenotypes seen in patients with *TGIF1* variants may be primarily due to excess Gli3 expression, rather than to excess Nodal signaling as previously suggested.

9 | NODAL SIGNALING, TGIF FUNCTION AND HPE

Evidence from human studies suggests that reduced Nodal signaling can contribute to HPE. Variants in the genes encoding the NODAL ligand, the TDGF1 (Cripto) coreceptor and FOXH1, which mediates part of the Nodal transcriptional response, have been found in HPE patients (De La Cruz et al., 2002; Roessler et al., 2008). Genetic variants at these loci likely lead to reduced output of the Nodal signaling pathway, which seems difficult to reconcile with the HPE observed both in patients with heterozygous *TGIF1* mutations and in mouse embryos lacking Tgifs. Heterozygous mutations in both the *Nodal* and *Smad2* genes in mice can result in HPE, again suggesting that a reduction in Nodal signaling is important in HPE pathogenesis (Nomura & Li, 1998; Taniguchi et al., 2012). However, the defects in these embryos are primarily anterior truncations, whereas HPE-like phenotypes are less common. This is consistent with studies in mice indicating that lower Nodal signaling is associated with impaired formation of anterior visceral endoderm and the PrCP, both of which are critical for early forebrain induction (Anderson, Lawrence, Stottmann, Bachiller, & Klingensmith, 2002; Mukhopadhyay et al., 2001; Shawlot et al., 1999). While truncations in *Nodal;Smad2* mutant mouse embryos can appear similar to the proboscis-like phenotype seen with HPE (Nomura & Li, 1998), the tissue is clearly distinct with most of the presumptive forebrain tissue being absent, rather than exhibiting a clear HPE phenotype as seen in *Shh* null embryos. Human variants in *NODAL*, *FOXH1*, and *TDGF1* are relatively rare in HPE patients, and are more often associated with other congenital abnormalities, perhaps suggesting that this is a relatively rare mechanism by which HPE develops. Given that loss of FGF8

signaling has been implicated in HPE and that Nodal activates *Fgf8* expression in the telencephalon, it is tempting to speculate that loss-of-function mutations in the NODAL pathway that give rise to HPE might do so by affecting FGF8 (Dubourg et al., 2016; Silvestri et al., 2008). Based on the rescue of proliferation defects and survival of mouse embryos lacking both Tgifs by a heterozygous *Nodal* mutation, it is also be possible that reduced Nodal signaling might allow a more severely affected embryo to survive, thereby uncovering patterning defects and HPE due to additional genetic or environmental insults (Taniguchi et al., 2017). However, since variants in activators of NODAL signaling result in HPE, it is most likely that the HPE seen in the absence of TGIF function is at least partly independent of this pathway, with patterning defects primarily due to altered Shh signaling.

Despite the multiple causes of HPE in humans and mice, it is interesting to note the apparent convergence on the Shh signaling pathway. As discussed above, the Shh-Gli3 balance is disturbed in embryos lacking Tgifs, and Tgif1 binds directly to the *Gli3* promoter to repress expression (Taniguchi et al., 2017; Taniguchi et al., 2012). Recent evidence also suggests that Tgifs regulate the formation of primary cilia, which are required for the majority of Shh signaling (Anderson et al., 2017; Goetz & Anderson, 2010). Although cilia defects have not been demonstrated in the developing forebrain of Tgif mutant mouse embryos, it remains possible that effects of loss of Tgif function on cilia could reduce the Shh response in the forebrain, as seen in cultured cells (Anderson et al., 2017). Interestingly, other frequently mutated HPE genes in humans, *SIX3* and *ZIC2*, may also link functionally to either Shh signaling or to *TGIF1*. SIX3 was shown to directly activate *SHH* expression in the mouse ventral forebrain, and *SHH* and *SIX3* each positively regulate expression of the other (Geng et al., 2008; Jeong et al., 2008). Recent evidence suggests a similar direct transcriptional activation of *TGIF1* expression by ZIC2 (Ishiguro, Hatayama, Otsuka, & Aruga, 2018). However, it should be noted that ZIC2 has also been linked to both Shh and Nodal signaling via interactions with Gli and SMAD transcription factors (Houtmeyers et al., 2016; Koyabu, Nakata, Mizugishi, Aruga, & Mikoshiba, 2001). Despite the obvious complexities of potential genetic and functional interactions between the known HPE-causal genes, it is clear that the Shh signaling pathway is a major target for disruption, and recent work suggests that loss of Tgif function may also feed into this pathway to contribute to HPE pathogenesis.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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