Intragenic deletion of Tgif causes defects in brain development

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TG-interacting factor (TGIF) is a homeodomain-containing protein and functions as a transcriptional repressor within the TGF-β and retinoic acid signaling pathways. Heterozygous mutations of TGIF have been found in patients with holoprosencephaly (HPE), which is the most common congenital brain malformation in humans. However, targeted null deletions of the entire Tgif gene in mice surprisingly revealed no apparent brain defects. We report here that deletion of the third exon of Tgif gene resulted in a defined spectrum of brain developmental defects including exencephaly, microcephaly, HPE, and abnormalities in embryonic brain ventricle formation and cleavage. These defects could be detected in mice both heterozygous and homozygous for the targeted Tgif deletion. Moreover, expression of dorsal–ventral patterning genes including Shh, Pax6 and Nkx2.2 was altered. The ventricular neuroepithelium exhibited focalized increase of cell proliferation rate and resultant tissue expansion. The incidence of brain abnormalities within the mutant mice was dependent on its genetic background, suggesting that additional genetic modifiers functionally interact with Tgif during embryonic brain development. The intragenic Tgif deletion mouse, therefore, would serve as a useful model that can be used to unravel the genetic complexity implicated in the pathogenesis of HPE.

INTRODUCTION

Holoprosencephaly (HPE) is the most common congenital malformation of forebrain in humans. It occurs with a prevalence of one in 250 during early embryogenesis, decreasing throughout gestation to a frequency of one in 16 000 live births (1,2). The pathogenesis of HPE comprises a failure of the brain hemispheres to separate along the mid-sagittal axis into discrete hemispheres during early development. In its most severe form, HPE is characterized by alobar brain development, cyclopia and other craniofacial abnormalities. HPE can also be associated with milder brain malformations and midfacial dysmorphisms, as well as abnormalities such as microcephaly, mental retardation and epilepsy. These anomalies are extremely heterogeneous in etiology and involve interplay of both genetic and environmental factors (1,2). Impairment of the genetic program controlling neural development is able to cause HPE. To date, mutations in seven genes have been identified to cause human HPE: Sonic hedgehog (SHH) (3,4), ZIC2 (zinc finger protein of cerebellum) (5), SIX3 (a sine oculis homeobox gene) (6), TGIF (TG-interacting factor) (7), PATCHED (a receptor for SHH) (8), TDGF1 (or CRIPO, an EGF-CFC family member) (9), and GLI2 (a transcription factor involved in SHH signaling) (10). In addition, HPE has also been noted in association with prenatal exposure to drugs such as retinoic acid and cholesterol biosynthesis inhibitors (11,12). Moreover, the phenotype of familial HPE is extremely variable, ranging from clinically normal individuals to patients with severe HPE. Therefore, genetic heterogeneity, phenotype variability, incomplete penetrance and etiological heterogeneity constitute the unique features and complexity of HPE.

TGIF is a member of the TALE (three amino acid loop extension) superfamily of atypical homeodomain

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(HD)-containing proteins (13) and functions as a transcriptional repressor within the TGF-β and retinoic acid signaling pathways (14,15). The significance of TGIF in HPE came from human genetic studies. Deletions or mutations of a single copy of the TGIF gene, which maps to the HPE4 locus at 18p11.3 region, have been found in various HPE patients (7). Eleven mutations/truncations of TGIF gene have been reported in human HPE patients so far (7,16–21). TGIF was initially identified as a factor with an ability to bind a retinoid X receptor (RXR) response element (13). It contains two repression domains (RD1 and RD2) and the HD, RD1, at N-terminus, can bind a co-repressor C-terminal binding protein (CtBP) that inhibits gene transcription (22). The HD of TGIF recognizes the consensus RXR response element and represses RXR-mediated gene expression by competing with retinoid X receptor (RXRα) and Meis2 for DNA binding (15). The C-terminal RD2 is able to interact with Smad2/3 and functions as a co-repressor for transcription of TGF-β target genes (14). The repressive activity of RD2 is mediated by interaction of this domain with histone deacetylases (HDACs) and Sin3 (14,23). Collectively, TGIF has been shown to repress transcription that is mediated by two critical signaling pathways: TGF-β and retinoic acid. It is interesting to note that missense mutations of TGIF have been found within the RD1 domain, the HD and the RD2 domain in human HPE patients, indicating the functional importance of these domains in TGIF implicated in early brain development. Three nonsense or frameshift mutations found in human HPE generates an early stop codon with the HD yet leaves RD1 intact (17,21). Human and mouse TGIF are 82 and 90% identical at the mRNA and protein levels, respectively. During embryonic development, Tgif is highly expressed in the mouse developing brain, including the ventricular neuroepithelium lining the third and fourth ventricles as well as in the walls of the midbrain and cerebellar plate (24). In adult mouse, expression level of Tgif is very low in the brain, and intermediate to high expression are detected in spleen, lung, skeletal muscles, liver, kidney and testis (24). Although mutations of TGIF have been reported to be linked to human HPE, the role of TGIF in the pathogenesis of HPE remains to be determined. Recently, several studies have been attempted to explore the function of Tgif in brain development using a mouse knockout strategy. Surprisingly, null deletion of the entire Tgif gene revealed no overt brain defects in these mouse models (15,25–27).

We report here a mouse model that has a targeted deletion of the third exon of the Tgif gene. In contrast to the null deletion of the entire Tgif gene, the intragenic deletion of Tgif leads to various forms of brain developmental defects including exencephaly, microcephaly, HPE and abnormalities in brain ventricle formation and cleavage. More importantly, our characterization of the Tgif deletion mouse suggests that the mouse model contains many features that recapitulate the pathogenesis of HPE in humans.

RESULTS

Intragenic deletion of mouse Tgif gene

Given that TGIF mutations have been found in HPE patients (7,17–20), we decided to determine if Tgif is required during normal embryonic brain development and the establishment of ventral midline structures in the mouse. Thus, we generated mice with targeted deletion of the third exon of Tgif (referred to as TgifΔexon3) (Fig. 1A) and this will leave intact the N-terminal Tgif transcriptional repression domain RD1 (28). Targeting of the Tgif allele was confirmed by Southern blotting analysis (Fig. 1B) and PCR with primers specific for exon 3 (data not shown). Tgif protein expression levels were measured by western blot analysis (Fig. 1C). As expected, Tgif protein migrates as a doublet around 37 kDa and the expression was only detected in wild-type and heterozygous embryos but not in TgifΔexon3−/− samples.

Normal mice have two Tgif splice variants, with an alternative first exon followed by two common exons. Reverse transcription-PCR (RT-PCR) analyses revealed that the two spliced mRNA transcripts between exon 1 (or exon 1′) and exon 2 could be found in the TgifΔexon3−/− embryos (Fig. 1D). However, PCR with primers that span the region from exon 2 to exon 3 had a negative result in the TgifΔexon3 null samples (Fig. 1D). The longer wild-type splice variant encodes 272aa and our deletion of exon 3 results in the loss of the last 191aa. This TgifΔexon3 mutant retains the first 81aa that contains the first repression domain and the N-terminal half of the HD of Tgif protein. Coincidently, similar truncation mutations (Y59X, E45X and a frameshift mutation at S46) that leave intact the N-terminal repression domain of TGF was also found in human HPE patients (17,21).

The brain developmental defects of TgifΔexon3 mouse is affected by genetic background

The chimeric targeted mice were crossed onto the C57BL/6 background and the offspring was genotyped to verify germ-line transmission of the mutant TgifΔexon3 allele (Fig. 2A). Heterozygous TgifΔexon3 mice were used in two strategies of breeding to identify potential brain developmental defects as indicated in Figure 2A. Initially, the F0 TgifΔexon3+/− mice were intercrossed and their 183 embryos were all normal (Fig. 2B). The F1 offspring resulting from the aforementioned intercross was then intercrossed among themselves. Of the 168 embryos generated from the F1 intercross, two TgifΔexon3 null embryos exhibited exencephaly (Fig. 2B). Incidentally, we found that the parents of these two embryos with exencephaly had dark coats, and that this is in contrast to the usual agouti color present in most other F1 mice that gave rise to phenotypically normal embryos. Given that this is probably due to genetic segregation, we hypothesized that the C57BL/6 genetic background may alter the penetrance of the defective brain phenotypes associated with the TgifΔexon3 deletion. To test this hypothesis, we carried out a second sequential backcross breeding scheme via crossing the F1 mice with wild-type C57BL/6 mice. As shown in Figures 2 and 3, we found that the incidence of brain developmental defects increased as the genetic background of the mice comprised greater proportions of the C57BL/6 genetic background. In ‘F1-C57’ mice, the C57BL/6 genetic contribution is theoretically 75%.

In this background, three TgifΔexon3 heterozygous embryos exhibited exencephaly and another 22 had a hypoplastic head phenotype (n = 149). Furthermore, 10 TgifΔexon3−/−
embryos exhibited hypoplastic head and 14 had exencephaly \((n = 67)\). In addition to the brain developmental defects, the ratio of the live \(Tgif^{\Delta e x o n 3/-}\) embryos at E10.5–12.5 was lower than what would be expected for Mendelian distribution (only 67 \(Tgif^{\Delta e x o n 3/-}\) compared to 112 wild-type embryos), indicating that homozygous deletion of \(Tgif\) exon 3 can result in embryonic lethality. In ‘F2-C57’ mice, the C57BL/6 genetic component is theoretically 87.5%. In agreement with our hypothesis, the incidence of brain developmental defects further increased within both heterozygous and homozygous \(Tgif^{\Delta e x o n 3/-}\) mutant embryos (Figs 2 and 3). Significantly, with each successive backcross on the C57BL/6 genetic background, there was a corresponding increase in the penetrance of brain anomalies and lethality (Fig. 3). It is noteworthy that the survival rate of \(Tgif^{\Delta e x o n 3/-}\) mutant embryos was substantially decreased in each generation with increasing contribution of C57BL/6 genetic background for both homozygous and heterozygous \(Tgif^{\Delta e x o n 3/-}\) mice (Fig. 3B). In ‘F3-C57’ and ‘F4-C57’ mice with over 90% of C57BL/6 background in theory, the ratios of surviving embryos with \(Tgif^{\Delta e x o n 3/-}\)
and $Tgif^{Dex3-/-}$ genotypes were significantly lower than those of the wild-type (Fig. 3B).

**Brain development is impaired in $Tgif^{Dex3-/-}$ deficient mice**

We observed various types of brain developmental defects with different degrees of severity in $Tgif^{Dex3}$ mutant embryos; including exencephaly, microcephaly, HPE and a hypoplastic head phenotype (Figs 4 and 5). Exencephaly typically reflects a defect in closure of the anterior neural tube, which normally occurs between E8.5 and E9.5 (29). The majority of exencephalic embryos displayed openings of the roofplate within either the forebrain or hindbrain regions, but the midbrain seemed largely unaffected (Fig. 4A). Peculiar midface malformations were also observed (Fig. 4A). Typical features include a protruded nose and long upper lip with deficient philtrum. In limited numbers, microcephaly (Fig. 4B) and HPE (Fig. 5B) were observed, but the vast majority of mutants exhibited a hypoplastic head phenotype (Fig. 4C–E). Scanning electron microscopy was used to further analyze the exencephaly phenotype (Fig. 4F–H). Although wild-type E11.5 littermate embryos had a closed and normally developed brain (Fig. 4F), affected $Tgif^{Dex3}$ embryos exhibited exencephaly and the neuroepithelium was exposed outward with abnormal pitting (Fig. 3G and H). This suggests that brain development was severely impaired, resulting in the failure of closure of the lateral hemispheres in the $Tgif$-deficient embryos.

The hypoplastic head phenotype was the most frequently observed defect in the mutant embryos and manifested as a reduced or underdeveloped forebrain and/or hindbrain in comparison with their wild-type littermates (Fig. 4C–E). This phenotype appears to be caused by reduced brain ventricle size, as was observed in retinoic acid-treated mice that harbor a deletion of the entire $Tgif$ gene (15). Histological analysis revealed that the wild-type neuroepithelial layer at E11.5 and E12.5 was intact and the forebrain and hindbrain ventricular chambers were well expanded (Fig. 5). However, in $Tgif^{Dex3}$ null embryos, neuroepithelial folding was abnormal and there appeared to be local thickenings that prevented...
normal ventricular expansion (Fig. 5). In the sagittal sections, it appeared that the thickening of ventricular neuroepithelium in the hypoplastic head phenotype was mainly localized in the ventral region of forebrain (Fig. 5B). In the most severely affected phenotype, rather than exhibiting the normal two telencephalic vesicles and a large diencephalic centrally located vesicle, the brain failed to generate separated telencephalic and diencephalic ventricles and only a single vesicle was evident (Fig. 5A, lower right), indicative of HPE. In most affected embryos, the ventricular cavity was significantly smaller than the wild-type controls (Fig. 5). The surface of the mutant ventricular neuroepithelium was rough with formation of crests and ridges and some of the ventricles were clearly deformed. In addition, the neuroepithelium lining the hindbrain ventricle was defective in Tgif\textsuperscript{Dexon3} nulls as it was much thicker than the wild-type controls in most regions, accompanied by multiple folds and protrusion into the hindbrain cavity (Fig. 5A). Asymmetrical expansion of the neuroepithelium was also observed in the Tgif\textsuperscript{Dexon3} null hindbrain (Fig. 5A). Collectively, those observations suggest that Tgif is involved in the folding and patterning of neural ventricular neuroepithelium throughout the forebrain and hindbrain.

Dorsal–ventral patterning in Tgif\textsuperscript{Dexon3} mouse brain

Since mutations in human TGIF gene are associated with HPE and our Tgf\textsuperscript{Dexon3} mutant embryos showed brain developmental defects, we next examined dorsal–ventral patterning of the brain. SHH signaling has been shown to be pivotal for ventral patterning and mutations of genes within SHH pathway are commonly found in human HPE patients (2,30). As shown in Figure 6A, Shh expression is normally localized to the ventral midline, but in Tgf\textsuperscript{Dexon3} null embryos with severe brain defects, Shh expression was significantly reduced and only residual signal could be detected in the ventral midline region. We next examined Pax6 expression in the brain as Pax6 is implicated in the regulation of Shh activity (31,32). In contrast to the highly localized wild-type spatiotemporal patterns of Pax6 expression within the cortical ventricular
zone of diencephalon and telencephalons, the $Tgif^{\Delta\text{exon}3}$ mutant embryos had markedly disregulated Pax6 expression (Fig. 6B). Ectopic Pax6 could be found in the dorsal region of forebrain as well as in the optic placodes in $Tgif^{\Delta\text{exon}3}$ null embryos (Fig. 6B). Nkx2.2 is another dorsal–ventral patterning marker and has been shown to expand dorsally in Pax6 mutants dependent upon Shh activity (33). Consistent with our findings with Shh and Pax6 expression, Nkx2.2 was also misexpressed in $Tgif^{\Delta\text{exon}3}$ mutant embryos with hypoplastic head phenotype (Fig. 6C). In wild-type embryos, Nkx2.2 was expressed at the ventral forebrain and hindbrain regions. Nkx2.2 expression could be found in the ventral portions of forebrain and hindbrain in the $Tgif$-deleted embryos. However, the pattern of Nkx2.2 expression was deformed or misplaced in affected embryos with hypoplastic head phenotype, consistent with the abnormality of brain ventricle formation revealed by hematoxylin and eosin (H&E) staining (Fig. 5).
Increased proliferation in cortex of neuroepithelium in Tgif mutant embryos

The major sites affected within the Tgif<sup>Δexon3</sup> mutants are ventricular neuroepithelium, including its formation, thickness and folding. In order to understand how neuroepithelium growth is perturbed, we analyzed both cell apoptosis and proliferation status during brain development. TUNEL assays revealed that the rate of apoptosis was similar between wild-type and mutants (Supplementary Material, Figure S1). There was no obvious apoptosis observed with either the wild-type or Tgif<sup>Δexon3</sup> mutant brains. We next analyzed cell proliferation rate by immunofluorescent staining with an anti-Ki-67 antibody, a selective marker for cycling cells (34). Compared to wild-types, the Tgif<sup>Δexon3</sup> mutants displayed significantly elevated index of Ki-67 positive cells in localized regions of the cortex within the mutant embryo ventricles (Fig. 7A). Such accelerated proliferation was most evident within the ventral region of forebrain and the thickened region of hindbrain. To further analyze the changes of cell proliferation status, we used another marker, PCNA. As shown in Figure 7B, staining with the PCNA antibody revealed a similar pattern as the Ki-67 staining. In Tgif<sup>Δexon3</sup> mutants, the ventricular neuroepithelium close to the midline region had an expanded proliferation. In addition, the regions of an increased proliferation within both the forebrain and the hindbrain coincided with the locations of the neuroepithelium anomalies (Fig. 5). Collectively, these data suggested that the defective folding and thickening observed in Tgif<sup>Δexon3</sup> mutants may be, at least in part, caused by an altered cell proliferation rate in localized neuroepithelial regions of the brain.

DISCUSSION

HPE is a severe brain development defect due to both genetic and environmental causes. Although heterozygous TGIF
mutations are associated with HPE in human patients (7,17–20), the underlying pathology and molecular mechanisms responsible for these defects remain unclear. Paradoxically, a number of TGIF mutation carriers do not develop HPE, highlighting the genetic complexity and variable penetrance of this disorder. Our studies using mouse gene targeting strategy indicate that an intragenic deletion of the \textit{Tgif} gene is able to cause developmental brain defects, including exencephaly, HPE and anomalies in brain ventricle formation and cleavage. Intriguingly, these abnormalities occurred in both homozygous and heterozygous animals, very similar to what is found in human HPE subjects. Moreover, expression of dorsal–ventral patterning genes including Shh, Pax6 and Nkx2.2 was altered, consistent with the structural defects observed within the brain ventricles. The ventricular neuroepithelium of the \textit{Tgif}^{\Delta e x o n 3} mutant mouse exhibited an

\textbf{Figure 6.} Analyses of dorsal–ventral patterning markers. (A) Immunofluorescent staining of Shh with transverse sections of the embryonic brain. In wild-type embryos at E11.5, Shh is expressed in the ventral midline portions of both forebrain and hindbrain. In \textit{Tgif}^{\Delta e x o n 3} mutant embryos, Shh expression was markedly reduced and only residual signals could be detected in the midline regions of the brain. Nuclear staining was performed with Hoechst 33342 to help visualize the neuroepithelium. (B) Immunofluorescent staining of Pax6. While Pax6 is mainly expressed in the ventral regions of telencephalon and diencephalon in wild-type embryo, in \textit{Tgif}^{\Delta e x o n 3} mutant brains, Pax6 could be detected in eyes and the dorsal region of forebrain that exhibits severe structural abnormalities. (C) Immunohistological staining of Nkx2.2. Note that Nkx2.2 expression marks the dorsal–ventral boundary of the forebrain and hindbrain, but in mutant embryos, the pattern of Nkx2.2 expression was perturbed and not symmetrical within the neuroepithelium.
increased rate of proliferation, in agreement with the observed abnormal folding and thickening of the neuroepithelium. Interestingly, the incidence of phenotypic mutant embryos was elevated with an increasing percentage of C57/BL6 background, suggesting that genetic modifiers may play a role in determination of the penetrance of HPE through functional interaction with the mutant Tgif. Therefore, it appears that the Tgif\textsuperscript{D\textsubscript{exon3}} mouse recapitulates the pathogenesis of human HPE in many aspects including a highly variable phenotype (ranging from exencephaly, HPE to hypoplastic head phenotype), autosomal dominant inheritance (defects found in heterozygous animals), incomplete penetrance and genetic complexity (phenotype affected by genetic background).

In contrast to previous attempts to generate Tgif knockout mice in which the entire Tgif gene is deleted (15,25-27), we only deleted exon3 of Tgf. This strategy resulted in the expression of an 81aa truncated form of Tgf. Coincidently, our deletion mimics several truncation mutations identified in human HPE patients. So far, eleven TGIF mutations have been found in human HPE patients and three of them (Y59X, E45X and a frameshift mutation at S46) result in TGIF protein truncation at the N-terminus (17,21). Interestingly, all these truncations would leave the N-terminal repression domain of TGIF intact, similar to our intragenic Tgif deletion. The N-terminal 81aa region of Tgf covers the sequences encoding intact RD1 domain that functions as a CtBP-binding domain and partial HD domain that binds consensus DNA sequence and regulates target gene expression.

One of the possible mechanisms by which Tgif deletion leads to brain developmental defect is interference of retinoic signaling. This is supported by our finding that Shh expression was suppressed in the Tgif\textsuperscript{D\textsubscript{exon3}} mutant mouse. The truncated Tgif protein may function in a dominant-negative way to suppress Shh expression, consequently resulting in brain developmental defects. The lack of Shh could be due to a reduced

![Figure 7. Analysis of cell proliferation with immunohistological staining for Ki-67(A) and PCNA (B). The nuclei were counterstained with methyl green. Note that the Tgf\textsuperscript{D\textsubscript{exon3}} mutant embryos contain significantly more Ki-67 and PCNA positive cells in the ventral region of forebrain and in the extra folds in hindbrain (indicated by arrows), suggesting accelerated proliferation of neuroepithelium in these areas.](image-url)
replicative activity of the Tgif\textsuperscript{Δexon3} allele on retinoic acid signaling, as Tgif can bind to RXR response element and inhibit RXR binding via steric hindrance (13,15). Consistently, it has been reported that teratogenic dose of retinoic acid is able to inhibit of Shh signaling and lead to HPE-like phenotypes in chick embryos (35). The dominant-negative form of the truncated Tgif may interfere with the binding of the RXR response element by the wild-type Tgif or its close homologue Tgif2 (36), leading to a decreased repression that would result in overexpression of retinoic target genes as well as inhibition of Shh signaling. All of these changes would lead to a HPE-like phenotype. This hypothesis is partly supported by the finding that deletion of the entire Tgif gene can increase the susceptibility of the mouse to retinoic acid-induced brain defects (15).

TGF\textsuperscript{β} inhibits TGF-β signaling as a transcriptional co-repressor through interaction with Smad2 and Smad3. Signaling of TGF-β family members regulates early embryonic development, and aberrance of response to TGF-β superfamly members such as alteration of Nodal signaling has been associated with severe HPE in the mouse (37). The RD2 domain of Tgif is involved in interaction with Smads (14) and this domain is deleted in Tgif\textsuperscript{Δexon3} mouse. Given the postulated dominant-negative nature of the truncated Tgif protein in Tgif\textsuperscript{Δexon3} mouse, it cannot be ruled out that such a mutant Tgif protein also affects TGF-β signaling. However, the loss of Tgif in Tgif\textsuperscript{Δexon3} mice is expected to relieve the repressive activity of Tgfα on TGF-β-mediated transcription, leading to an upregulation of gene transcription and an overall hyperactivity of TGF-β signaling pathway. As the major biological function of TGF-β is negative regulation of cell proliferation (38), the reduced repressive activity by loss of Tgif is expected to cause reduction of cell proliferation. This is supported by the finding that the growth rate of mouse embryonic fibroblasts isolated from Tgif knockout mouse is significantly reduced (27). However, such a reduced proliferation does not seem consistent with the brain defects in these animals. In fact, the abnormal embryos in our studies exhibited an increased cell proliferation rate in the cortex regions of embryonic ventricles, leading to thickening of ventricular neuroepithelium and resulting in extra ridges and crests in various regions. Therefore, it is likely that the loss of Tgif has different effects on the cell proliferation rate dependent on the cellular context.

One of the important features unveiled with the Tgif\textsuperscript{Δexon3} mouse is that the brain developmental defects caused by Tgif deletion are affected by genetic background. The incidence of brain developmental defects and embryonic lethality increased sequentially within both heterozygous and homozygous Tgif\textsuperscript{Δexon3} mutant embryos when the C57BL/6 genetic background was elevated. Our study suggest that genetic modifiers are present in C57BL/6 mouse strain and these modifiers functionally interact with the loss of Tgif and lead to increased penetrance of the brain developmental defects. Intriguingly, our findings are consistent with the multiple-hit theory of human HPE patients. For many of Mendelian conditions, genotype–phenotype correlations do not perfectly match and variability of penetrance is extremely common in human HPE, as evidenced by the finding that ~36% of obligate carriers with disease-causing mutations have no clinical abnormalities with normal intelligence (39). Moreover, currently identified HPE genes only account for a small portion of all sporadic HPE cases. Therefore, there are still unidentified susceptibility or modifier genes that are required for the full penetrance of HPE phenotype. We postulate that similar modifiers may also exist in humans and affect the penetrance of HPE. In this regard, the Tgif\textsuperscript{Δexon3} deletion mouse would serve as a useful model system that can be used to unravel the genetic complexity implicated in the pathogenesis of HPE (40).

**MATERIALS AND METHODS**

**Targeted disruption of mouse Tgif**

The knockout targeting vector was designed to replace exon 3 of mouse Tgif with a neomycin selection cassette (Fig. 1A). Briefly, a 129SVJ mouse genomic DNA library in the Lambda FIXII vector (Stratagene, La Jolla, CA, USA) was screened with a full-length mouse Tgif cDNA sequence. Two Tgif genomic clones were isolated and verified by DNA sequencing. A 4.4 kb genomic fragment that contains exon 2 was cloned into the Xhol and HindIII sites of the pKO-Neo\textsuperscript{loxP} vector (41). A 4.1 kb genomic fragment that contains the sequences downstream of exon 3 was cloned into the EcoRI and KpnI sites of the same vector. Homologous recombination between the targeting vector and the genomic DNA would give rise to deletion of exon 3 but retain exons 1 and 2 (Fig. 1A). The linearized construct was electroporated into embryonic stem (ES) cells that were subsequently grown in G418-containing medium. Correctly targeted ES cell clones were identified by Southern blotting analysis using a probe indicated in Fig. 1A. Chimeric mice were generated by blastocyst injection and used to intercross with C57BL/6 mice to obtain heterozygous animals to check for germline transmission.

**Genotyping of Tgif\textsuperscript{Δexon3} deletion mice by Southern blotting**

Genomic DNA was extracted from mouse tails using the Genomic DNA Extraction Kit (Promega, Madison, WI, USA). For Southern blotting, genomic DNA was digested with BamHI, separated on an agarose gel, and transferred to the Hybond-N membrane (GE Healthcare, Piscataway, NJ, USA). Membranes were hybridized with a 32P-labeled DNA probe, followed by autoradiographic detection. The Tgif\textsuperscript{Δexon3} null allele yields a 7.7 kb band, whereas the wild-type allele gives a 5.4 kb band (Fig. 2).

**Western blotting analyses**

Mouse embryos at E12.5 were homogenized and solubilized, and total protein was extracted. Mouse Tgif and Actin were detected by using rabbit anti-Tgif and rabbit anti-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A secondary antibody, conjugated to horseradish peroxidase was used for ECL detection.

**RNA isolation and RT-PCR**

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from E12.5 mouse whole brain tissues.
following genotyping. The RNA was treated with RNase-free DNase I (Invitrogen) to remove any residual DNA, and reverse-transcribed with oligo(dT) primer using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Oligonucleotides used for PCR reactions (Fig. 1D) to detect mRNA of mouse Tgif and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) are: 5'-AAAGTTGCACTGCGGAGTGTCAG-3' and 5'-GAATGTGGACAGGTGTCATC-3' for splicing variant of Tgif between exon 1 and exon 2 (Fig. 1D); 5'-GCTGAAAGCAGGACACCTTGTAG-3' and 5'-TCTGGACTGACCTTGGGCAGATTTG-3' for splicing variant between exon 1 and exon 2 (Fig. 1D); 5'-TTCTTGGACTGCTGTAGAACAC-3' and 5'-TACGGAGGAAATGGCCTCTC-3' for Tgif mRNA between exon 2 and exon 3; 5'-GTCTTCACCACCAGATGGAGAAG-3' and 5'-TACTCCAGGTGGTGACAAACG-3' for G3PDH.

Histology, immunohistochemistry and TUNEL analyses

Pregnant mice were sacrificed via carbon dioxide euthanasia. E10.5–12.5 embryos were dissected in cold PBS and fixed in 4% paraformaldehyde at 4°C overnight. Embryos were embedded in paraffin by using standard processing, and 5 μm serial sections stained with H&E to determine the histological features. For cryosectioning, fixed samples were dehydrated in 30% sucrose in PBS and embedded in Tissue-Tek OCT (Sakura USA, Torrance, CA, USA). For immunofluorescence, cryostat sections of 10 μm thickness were air-dried and incubated with 1× PBS followed with 1 h of blocking in 10% goat serum. Slides were incubated at 4°C with primary antibody overnight, followed by incubation with a fluorescence-conjugated secondary antibody for 2 h at room temperature. Nuclei were counter-stained with Hoechst33342 (Invitrogen). Primary antibodies used were as follows: mouse anti-Pax6 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), rabbit anti-Shh antibody (Santa Cruz Biotechnology). Indirect immunofluorescence was performed with goat anti-mouse Cy3-coupled antibody (Jackson ImmunoResearch, West Grove, PA, USA) or goat anti-rabbit FITC-coupled secondary antibody (Santa Cruz Biotechnology). Immunohistological staining was performed on 5 μm paraffin sections by using Histoplus immunostaining kit according to manufactures instructions (Invitrogen). The following primary antibodies were used: mouse anti-Ki-67 antibody (BD PharMingen, Franklin Lakes, NJ, USA), PCNA (Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-Nkx2.2 antibody (Developmental Studies Hybridoma Bank). TUNEL assays were performed on 5 μm paraffin sections by using the Apoptag kit (Chemicon, Temecula, CA, USA). Positive controls were performed via treatment with 0.1 μg/ml DNaseI (Sigma-Aldrich) for 10 min at room temperature for generation of nicked on double-strands DNA. Methyl green was used as a counter-stain.

Animal protocols

All protocols involving the use of animals were in compliance with the National Institutes of Health’s and the Indiana University Laboratory Animals Research Committee’s Guide for the Care and Use of laboratory Animals. C57BL/6 mouse spouse strain was obtained from Harlan (Indianapolis, IN, USA).

For timing of the pregnant mice and embryos, the noon of the day that the vaginal plug was observed was designated embryonic day 0.5 (E0.5).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors wish to declare no competing financial interests.

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