Defects in GPI biosynthesis perturb Cripto signaling during forebrain development in two new mouse models of holoprosencephaly

David M. McKean and Lee Niswander*

HHMI, Department of Pediatrics, Cell Biology, Stem Cells and Development Graduate Program, and Children’s Hospital Colorado, University of Colorado Anschutz Medical Campus Aurora, CO 80045, USA

*Author for correspondence (Lee.Niswander@ucdenver.edu)

Biology Open 1, 874–883
doi: 10.1242/bio.20121982
Received 16th May 2012
Accepted 6th June 2012

Summary

Holoprosencephaly is the most common forebrain defect in humans. We describe two novel mouse mutants that display a holoprosencephaly-like phenotype. Both mutations disrupt genes in the glycerophosphatidyl inositol (GPI) biosynthesis pathway: gonzo disrupts Pigm and beaker disrupts Pgap1. GPI anchors normally target and anchor a diverse group of proteins to lipid raft domains. Mechanistically we show that GPI anchored proteins are mislocalized in GPI biosynthesis mutants. Disruption of the GPI-anchored protein Cripto (mouse) and TDGF1 (human ortholog) have been shown to result in holoprosencephaly, leading to our hypothesis that Cripto is the key GPI anchored protein whose altered function results in an HPE-like phenotype. Cripto is an obligate Nodal co-factor involved in TGFβ signaling, and we show that TGFβ signaling is reduced both in vitro and in vivo. This work demonstrates the importance of the GPI anchor in normal forebrain development and suggests that GPI biosynthesis genes should be screened for association with human holoprosencephaly.

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Key words: Holoprosencephaly (HPE), forebrain, glycerophosphatidyl inositol, GPI, Pigm, Pgap1, TGFβ, Cripto, Nodal

Introduction

Holoprosencephaly (HPE) is estimated to occur in 1 in 250 pregnancies but due to prenatal lethality, only 1 in 16,000 babies are born with HPE (Muenke and Beachy, 2000). HPE occurs when the forebrain fails to separte into the two frontal lobes. The spectrum of septal defects that result in HPE are classified as lobar (fully septated, mild), semi-lobar (incompletely septated, moderate), alobar (not septated, severe) and syntelencephaly (posterior frontal lobe and parietal lobe fail to septate). Infants born with HPE, generally representing the mildest spectra, may present with craniofacial defects, hydrocephaly, feeding issues and early mortality.

Environmental and genetic factors contribute to HPE. Environmental causes include maternal diabetes and exposure to alcohol, retinoic acid and cholesterol reducing agents. Large chromosomal defects, such as trisomy 13 account for the majority of HPE cases, but single gene disruptions are also linked to HPE. These single gene mutations segregate into the SHH pathway (SHH, PTCH, GLI2, ZIC2 and DHCR7) and TGFβ pathway (TGFβ, FAST1 and TDGF1), but only account for 15–20% of human HPE (Ming and Muenke, 2002). SHH pathway mutations are directly associated with midline defects whereas TGFβ pathway mutations are thought to act upstream of SHH, affecting SHH expression and/or activity in the prechordal plate (Rohr et al., 2001). In mouse, additional TGFβ pathway genes (Nodal, Gdf1, Smad2 and ActRIIA) have been linked to HPE (Nomura and Li, 1998; Song et al., 1999; Hoodless et al., 2001; Lowe et al., 2001; Rohr et al., 2001; Yamamoto et al., 2001; Andersson et al., 2006) and disruption of both copies of the TGFβ genes often leads to forebrain truncations, rather than classic HPE, due to the requirement for TGFβ signaling in early forebrain initiation steps.

Nodal, a TGFβ ligand, and Cripto, Nodal’s obligate co-factor (Gritsman et al., 1999) are both required for specification and localization of the distal visceral endoderm (DVE) and anterior visceral endoderm (AVE) (Varlet et al., 1997; Ding et al., 1998; Mesnard et al., 2006; D’Andrea et al., 2008; Liguori et al., 2008; Takaoka et al., 2011). The AVE is a transient organizing center that initiates forebrain specification in the underlying neuroectoderm. Although DVE progenitors initially require Nodal signaling (Varlet et al., 1997; Mesnard et al., 2006), proper migration of the DVE and AVE is only achieved by antagonism of Nodal and Wnt signaling (Yamamoto et al., 2004; Kimura-Yoshida et al., 2005). Furthermore, the AVE and future forebrain organizing centers induce and then maintain forebrain specification by antagonizing TGFβ and Wnt signaling (Perea-Gomez et al., 2002).

Cripto (the HPE gene TDGF1 in humans) protein is post-translationally modified with a GPI anchor (Minchiotti et al., 2002). This sugar-lipid anchor targets Cripto to the plasma membrane where it binds Nodal to signal in a cell autonomous function (Yan et al., 2002). Cripto’s GPI anchor may also be cleaved, releasing Cripto into the extracellular space where it may bind Nodal and signal non-cell autonomously (Yan et al., 2002; Chu et al., 2005; Watanabe et al., 2007).
Here, we describe two novel recessive mutations in mice, which result in HPE or an anterior truncation phenotype, similar to phenotypes associated with homozygous mutation of TGFβ genes. These mutations disrupt two different enzymes within the Gpi biosynthesis pathway. We hypothesize that Cripto is a key GPl-anchored protein, whose lack of a functional GPI anchor results in an HPE-like phenotype. We show that Nodal/Cripto signaling is downregulated both in vitro and in vivo in the GPl biosynthesis mutants.

Materials and Methods

Mouse strains and genotyping

The gonzo (gnz) line was derived from a forward genetic screen performed in collaboration with the laboratories of Kathryn Anderson and Elizabeth Lacy and the beaker (bkr) line was generated from mutagenized males provided by Monica Justice and the screen performed with the lab of Trevor Williams. Both lines were generated on a C57BL/6J genetic background and initially out-crossed to either C3H/HeJ (gnz) or 129Sv/SvJm (bkr) strains. Further analysis of gnn and bkr was performed in 129Sv/SvJm and C57BL/6J backgrounds, respectively. Additional strains used were Nodal^Δcd2 (Collignon et al., 1996), GPl-GFP (Rhee et al., 2006) and Hex-GFP (Rodriguez et al., 2001).

Gonzl was initially mapped between SSLP markers DM1T136 and DM1T94. Additional high-resolution markers were generated from NCBI Mouse SNP database (http://www.ncbi.nlm.nih.gov/SNP/MouseSNP.cgi) and Mouse Genome SSR search website (http://dando.mgh.harvard.edu/mouseMarkers/musssr.html). Ultimately, gnn was genotyped using ARMS primers: TGCTTTCTGTGATCCTC-CCAGCTCACAGG (Pgn outer forward), ATGACATCCGGAGGCTTTTTC-CTTAGAAA (Pgn inner A forward), CGAGATCTTTAACAATCCAGAGCA- AAGGA (Pgn inner T reverse) and GCACCTGCATCTCAATTTTTG (Pgn outer reverse) (Ye et al., 2001). Beaker was initially mapped between DM1T123 and DM1T303 and ultimately genotyped using SNP analysis with primers: CGTACAGGCTTATGCACTCCAG (Pgap1 snpF), GCAAAGGGCCTTT-CCAA (Pgap1 snpR), Pgap1 (Pgap1 snp probe). Pgap1^R (renamed Pgap1^Mnisw) have been deposited and are available from Jackson Laboratories. GFP strains were genotyped using primers oIMR0872 and oIMR1416 and LacZ identified using oIMR0039 and oIMR0040 (Jackson Labs).

Mouse embryo fibroblasts (MEFs), cell culture and immunodetection

MEFs were prepared from E13.5 embryos. Embryos were dissected in DMEM (Gibco), eviscerated, decapitated and minced with a sterile blade, followed by trypsinization for up to 30 min. MEFs were maintained in 10% FBS (Gibco) in DMEM with Penicillin/Streptomycin (Gibco).

For immunostaining, 12,000 MEFs (Pgn^+/+ or Pgn^+/gps) were plated on coverslips in 24-well plates (Falcon). MEFs were grown in 10% FBS in DMEM, fixed in 4% paraformaldehyde then analyzed by immunodetection for pGPOP (Genetex, Inc).

For in vitro Nodal/Cripto signaling assays, 100,000 MEFs (Pgn^+/+, Pgap1^+ gps, Pgap1^+/+ or Pgap1^+/gps) were plated in 6-well plates in 10% FBS in DMEM. Medium was changed to 2 ml DMEM (no serum) overnight. MEFs were treated with 250 ng/ml recombinant Nodal protein (R&D Systems) or vehicle for 4 hours, then medium changed to DMEM (no serum) overnight. MEFs were transfected with pCDNA3-HA-Cripto (Yan et al., 2002) or pCDNA3.1 (GPI-GFP strains were genotyped using primers oIMR0872 and oIMR1416 and LacZ identified using oIMR0039 and oIMR0040 (Jackson Labs).

Real time polymerase chain reaction

Total RNA was extracted from E7.5 embryos using an RNasy Micro Kit (Qiagen). Six wildtype littermates and six Pgn^−/gps and Pgap1^−/gps embryos were used for real time PCR with the Mouse TGFβ BMP Signaling Pathway R^2 Profiler PCR Array (SABiosciences) using 200 ng of reverse transcribed RNA as template.

Results

Characterization of the ENU-derived Gonzo mutant mouse line

To identify novel genes important for normal forebrain development, we employed an ENU mutagenesis screen in mice. Briefly, mutagenized C57BL/6J (C57) males were out-crossed to C3H/HeJ (C3H) females to generate founder males. Founder males were further out-crossed, and then mated to their daughters with forebrain truncations or an HPE-like phenotype. Due to the presence of a large proboscis that dominated the craniofacial region we named this line gonzo (gnz).

In the C3H background, gnn mutant embryos show three different phenotypes: dysmorphic eyes (n=14/39 embryos) (supplementary material Fig. S1A,B), gastrulation defects (9/39) (supplementary material Fig. S1J), and an HPE-like phenotype in which mutant embryos either display midline defects (n=2/39) (supplementary material Fig. S1K,L) or anterior truncations (n=14/39) (supplementary material Fig. S1E,F, Fig. 1A,D). When out-crossed into 129S1/SvJm (129S1) background, gnn mutant embryos largely present with anterior truncations. While the C3H strain was used for mutation mapping, the 129S1 strain was used for the majority of results and figures presented here.

A typical gnn mutant at embryonic day 18.5 (E18.5) shows midbrain/forebrain truncation and a large proboscis (Fig. 1D). To identify the rostral-caudal position of the truncation, bone and cartilage staining was performed (Fig. 1B,C,E,F). In gnn mutant embryos, bone and cartilage structures anterior to the interparietal (ip) element remain symmetrical yet are grossly misshapen. The ip, while recognizable, is tiny and misshapen. The tectum synoticum (tso) is largely intact, but narrower at the anterior midline. Occipital elements at the base of the skull are also dysmorphic. The supraoccipital (so) and exoccipital (eo) elements are fused and the supraoccipital is present as two lateral bony elements instead of the normal rod-like shape. The basioccipital (bo) element appears normal in gnn mutants.

In addition to the anterior truncation, gnn mutant embryos are smaller than wildtype littermates (supplementary material Fig. S2A,B). Comparison of the lengths of femurs and humeri from five mutant and wildtype E18.5 embryos (supplementary material Fig. S2C,D,I) reveal a statistically significant shortening of the femur (by 11%, P value=0.04; unpaired, 2-tailed Student’s t-test) but not the humerus (P value=0.24) in gnn mutant embryos.

Gnn mutation disrupts Pgin, encoding a glycerophosphatidyl inositol biosynthesis enzyme

We mapped the gnn mutation to a 5 Mb region on mouse chromosome 1 using Simple Sequence Length Polymorphisms (SSLPs) and Single Nucleotide Polymorphisms (SNPs) that differ between the mutagenized C57 DNA and the out-crossed C3H DNA (Fig. 1G) (between rs3654716 and rs6307336). This gene sparse region contains seven known or predicted genes. Sequencing of these seven genes revealed a single base substitution (T to A) in the splice donor region of intron 23 of the Phosphatidylinositol-glycan
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Panels R and S were immunostained for the Golgi marker observed out of number of mutant embryos analyzed. Scale bars (A–F) panels N,O and subsequent figures indicate number of similar phenotypes.

 complementary DNA (cDNA) was prepared by RT-PCR. RNA was analyzed by semi-quantitative PCR and whole mount in situ hybridization of E14.5 embryos. There were no significant changes in expression level (data not shown) or localization (Fig. 1M,N) of Pign between gonz mutants and wildtype littermates.

**Gonz** mutant embryos show mislocalized GPI anchored proteins. Pign mutants have been described in yeast and mouse cell lines (Gaynor et al., 1999; Hong et al., 1999). These mutated cells show mislocalization of GPI-anchored proteins (GPI-APs). To determine whether GPI-APs are mislocalized in gonz mutants, the gonz line was crossed into a transgenic mouse line in which GFP is targeted to the membrane by a GPI linkage (Rhee et al., 2006). While GPI-GFP is highly expressed in wildtype E12.5 embryos, gonz mutant embryos show a dramatic reduction in GPI-GFP fluorescence (Fig. 1O). The subcellular distribution of GPI-GFP in mouse embryonic fibroblasts (MEFs) from E12.5 wildtype embryos show GFP localization in the plasma membrane, whereas gonz mutant MEFs show only internal localization of GFP in numerous apparently membrane-bound structures surrounding the nucleus (Fig. 1P,Q). These structures are consistent with those seen in budding yeast with a mutation in the Pign ortholog Mcd4 (Gaynor et al., 1999). Immunostaining with bCOP, a marker of the Golgi compartment, reveals these structures express Golgi markers (Fig. 1R,S).

**Beaker** mutants exhibit anterior truncations.

A second ENU-derived mouse line that displays an HPE-like phenotype was isolated from an independent screen. Dependence on the background strain, homozygous mutant embryos from the beaker mouse line also display a range of phenotypes – normal appearance in 129S1, anterior truncations in C57 (Fig. 2B,D), and holoprosencephaly (Fig. 2A,C) and/or eye defects (supplementary material Fig. S1C,D) in mixed 129S1/C57 strains. Further analysis of the bkr line was performed in the C57 background, which gave the fully penetrant phenotype shown in Fig. 2D.

Bone and cartilage staining of wildtype and bkr mutant embryos at E18.5 (Fig. 2E–H) reveal the interparietal element is dysmorphic and not ossified and the tectum synoticum is present as two lateral elements that do not traverse the midline. All elements rostral to these are largely absent. The supraoccipital and exoccipital elements are correctly positioned, but not ossified, whereas the basioccipital element is absent. Bkr
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Between wildtype and bkr mutation, transport to the Golgi (Tanaka et al., 2004). Deacylation of GPI anchored proteins allows efficient loading into CopII coated vesicles and (supplementary material Fig. S3). Deacylation of GPI anchored glycerophosphatidyl inositol biosynthesis enzyme (Proteins factor 1 biosynthesis genes and found that Post-GPI Attachment to mutation, we cross-referenced these genes with known GPI anchored proteins. Consistent with normal trunk development, the trunk mesoderm marker Brachyury is expressed normally in E6.5 and E7.5 gnz and bkr mutants (Fig. 3A–D).

Forebrain patterning defect in Pign and Pgap1 mutant mouse embryos

Apart from anterior truncations, gnz (in 129S1 background) and bkr mutant embryos display relatively normal development, which is remarkable as there are more than one hundred GPI anchored proteins. Consistent with normal trunk development, the trunk mesoderm marker Brachyury is expressed normally in E6.5 and E7.5 gnz and bkr mutants (Fig. 3A–D).

Bkr mutation disrupts Pgap1, encoding a second glycerophosphatidyl inositol biosynthesis enzyme

We mapped the bkr mutation to a 24 Mb region, containing 145 predicted genes, on mouse chromosome 1 (Fig. 2I). Based on similarity to the gnz phenotype and discovery of the Pign mutation, we cross-referenced these genes with known GPI biosynthesis genes and found that Post-GPI Attachment to Proteins factor 1 (Pgap1) lies within this region. Pgap1 is an ER localized decacylase that removes an acyl group from the GPI anchor after covalent attachment to its target proteins (supplementary material Fig. S3). Deacylation of GPI anchored proteins allows efficient loading into CopII coated vesicles and transport to the Golgi (Tanaka et al., 2004).

PCR amplification of Pgap1 revealed a size difference between wildtype and bkr mutant cDNAs in PCR products that span exon 19 (Fig. 2J). Sequencing of genomic DNA at the exon/intron borders of exon 19 revealed a base substitution (T to C) in the splice donor of intron 19 (Fig. 2K) and sequencing of cDNA showed skipping of exon 19 in bkr mRNA (Fig. 2L). Exon 19 is 39 nucleotides, and its exclusion is predicted to result in an in-frame deletion of 13 amino acids. While these 13 amino acids do not correspond to a domain of known function, these residues are well conserved between human, mouse, chick and zebrafish, though not in S. cerevisiae (Fig. 2M,N). The Pign<sup>gnz<sub>11</sub></sup> (renamed Pgap1<sup>m1Nisw1</sup>) and Pgap<sup>bkr</sup> (renamed Pgap1<sup>m1Nisw2</sup>) mouse lines are available from Jackson Laboratories.

Fig. 3. Forebrain patterning markers are misexpressed in both Gnz and Bkr mutants. Gnz mutant embryos (B,F,J,N) and wildtype littermates (A,E,I,M) and bkr mutant embryos (D,H,L,P) and their wildtype littermates (C,G,K,O) were analyzed by whole mount RNA <i>in situ</i> hybridization for Brachyury (A–D), Six3 and Krox20 (E–H), Fgf8 (I–L) and Shh (M–P). Embryos were at E6.5 (A,B), E7.5 (C,D), E8.5 (E–H,M,N) and E9.5 (I–L,O,P). Black and white asterisks in E denote Krox20 and Six3 expression, respectively. Abbreviations: is=isthmus; ba=branchial arches; tb=tailbud. Arrows and arrowheads denote presence or absence, respectively of Fgf8 expression in the ANR (I–L). Black bars mark prechordal plate (M,O).
embryos, the anterior neural ridge (ANR) acts as a forebrain “organizing center” by secreting factors necessary to maintain forebrain specification. Fgf8 is expressed in the ANR, isthmus (midbrain-hindbrain boundary), pharyngeal arches and tail bud. Fgf8 expression in gnz and bkr mutants is normal, except in the ANR, where it is not expressed (Fig. 3I–L); lack of Fgf8 expression likely reflects that the ANR fails to develop and implicates an earlier requirement of GPI biosynthesis in forebrain specification.

Six3 is an earlier anterior forebrain marker. The Six3 expression domain was both reduced in size and level of expression in E8.5 gnz mutants, and severely reduced or absent in bkr mutants (Fig. 3E–H). Six3 function is required for forebrain development (Lagutin et al., 2003) and mutation of human SIX3 causes HPE; hence decreased Six3 expression is consistent with forebrain defects. The relative decrease in Six3 expression in bkr versus gnz mutant embryos also correlates with the extent of anterior truncation.

Shh is expressed in the prechordal plate underlying the anterior neural tube and is required for seption of the future forebrain into left and right hemispheres. Mutations in human SHH and its neural tube and is required for septation of the future forebrain defects. The relative decrease in Six3 expression in bkr versus gnz mutant embryos also correlates with the extent of anterior truncation.

Early forebrain organizing centers are disrupted in gnz and bkr mutant embryos

Otx2, which is expressed in the anterior definitive endoderm (ADE) and underlying anterior neuroectoderm in gastrulation stage (E7.5) embryos, is required for forebrain specification. The node-derived ADE migrates anteriorly and acts as an organizing center to maintain forebrain development in the neuroectoderm. Otx2 expressing cells are mislocalized at the distal tip of E7.5 gnz mutant embryos whereas E7.75 bkr mutant embryos display proper localization of Otx2 expression albeit at a reduced level (Fig. 4A–D). Alteration in Otx2 localization (gnz) and expression (bkr) suggests that ADE cellular migration or specification might be disrupted in GPI biosynthesis mutants.

To examine ADE migration and specification, we crossed gnz and bkr lines into Hex-GFP transgenic mouse line (Rodriguez et al., 2001; Stuckey et al., 2011) to visualize Hex expression and ADE localization. Both gnz and bkr E7.5 embryos showed a modest displacement of the ADE towards the distal tip compared to wildtype embryos (Fig. 4E–H). While defects in ADE migration can cause anterior truncation phenotypes, examination of Hex-GFP at an earlier stage allows visualization of both the DVE and AVE, two molecularly distinct, migratory groups of cells that contribute to forebrain specification. The DVE migrates towards the presumptive anterior pole at ~E5.5, ‘paving the road’ for subsequent AVE migration (Takaoka et al., 2011). At the anterior pole, the AVE initiates forebrain specification then is displaced and Hex expression is downregulated (Rodriguez et al., 2001). Hex-GFP fluorescence (which does not distinguish the DVE from the AVE) in gnz and bkr E6.5 embryos reveals aberrant DVE/AVE localization (Fig. 4I–L). Gnz mutants show DVE/AVE displacement at the distal tip. However, bkr mutant embryos have promiscuous GFP expression throughout the visceral endoderm, reminiscent of cultured embryos in which the extraembryonic ectoderm has been removed (Rodriguez et al., 2005). Together this indicates that GPI biosynthesis proteins are required to properly initiate forebrain development. Moreover, this allowed us to make some predictions as to the identity of the GPI-AP(s) that may be altered resulting in the HPE-like phenotype based on involvement in AVE specification and migration, and perhaps communication with the extraembryonic ectoderm.

The Nodal/Cripto signaling pathway is defective in Pign and Pgap1 deficient cell lines

Given the migration and specification defects of the AVE in E6.5 gnz and bkr mutant embryos, we hypothesized that Cripto is the GPI anchored protein whose GPI deficiency leads to the HPE-like phenotype. We analyzed Nodal/Cripto signaling in wildtype and mutant MEFs by phosphorylation of Smad2 (Watanabe et al., 2007). In the absence of exogenous Nodal stimulation, wildtype, Pgn\textsuperscript{gmc} and Pgap1\textsuperscript{bkr} MEFs, which express endogenous Cripto, have negligible phospho-Smad2 and this is not significantly increased by Cripto overexpression (transfection of HA-tagged Cripto expression vector compared to empty vector, mock stimulated cells) (Fig. 5A–C). Upon Nodal stimulation in wildtype cells, there was a 16-fold increase of phospho-Smad2 in stimulated cells) (Fig. 5A–C). Upon Nodal stimulation in wildtype cells, there was a 16-fold increase of phospho-Smad2 in stimulated cells (P value=0.003) and 35-fold increase with Cripto overexpression (Fig. 5A–C) (P value=0.007). In contrast, Nodal stimulation of gnz or bkr MEFs, in the presence or absence of Cripto overexpression, showed a significantly lower response (Fig. 5A–C) (approximately 3-fold lower for both gnz [P value=0.03 with or without Cripto] and bkr [P value=0.04 without Cripto, and 0.02 with Cripto]). Thus, GPI biosynthetic activity is required for efficient Nodal/Cripto signaling.

In Pgn\textsuperscript{gmc} and Pgap1\textsuperscript{bkr} mutant MEFs, there was some response to Nodal stimulation, suggesting that endogenous or transfected Cripto retained some signaling activity. Cripto can function non-cell autonomously via enzymatic or genetic removal of its GPI anchor (Zhang et al., 1998; Yan et al., 2002; Watanabe et al., 2007), demonstrating that secreted Cripto retains biological activity. Furthermore, secretion of GPI

Fig. 4. Forebrain organizing centers are mislocalized in both Gnz and Bkr mutants. E7.5 wildtype (A) and gnz mutant (B) littersmates and E7.75 wildtype (C) and bkr mutant (D) littersmates were analyzed by whole mount RNA in situ hybridization for Otx2. Gnz mutant embryos (F,J) and wildtype littermates (E,I) and bkr mutant (H,L) and wildtype littermates (G,K), all containing Hex-GFP transgene were analyzed by GFP fluorescence. Hex-GFP fluorescence marked the ADE in E7.5 embryos (E–H) and DVE/AVE in E6.5 embryos (I–L).
anchored proteins was detected in Mcd4 (Pign ortholog) mutant budding yeast (Gaynor et al., 1999). Cripto overexpression, while resulting in increased Smad2 phosphorylation, has no effect on intracellular Cripto protein levels (Fig. 5A) suggesting that some Cripto protein could be secreted and act in a non-autonomous manner. To test this we immunoprecipitated endogenous secreted Cripto from conditioned medium from wildtype and mutant MEFs followed by western blot detection (Fig. 5D). Although not quantitative, this showed Cripto protein in the medium, consistent with reduced but not absent Smad2 phosphorylation in both gnz and bkr MEFs.

**TGFβ responsive genes are downregulated in gnz and bkr mutant embryos**

As another measure of Cripto/Nodal activity we determined the expression of TGFβ target genes by real time PCR using TGFβ/BMP pathway arrays and E7.5 RNA from mutants compared to wildtype littermates, as the two lines are in different background strains. Seven of 22 TGFβ responsive genes were significantly (P value<0.05) downregulated in bkr mutants and 3 of 22 TGFβ responsive genes were significantly downregulated in gnz mutants as compared to wildtype littermates (Table 1). Collagen, type I alpha-2 (Coll1a2) and Interleukin6 (Il6) were downregulated in both gnz and bkr mutants. BMP signaling is not dependent on Cripto activity, so BMP responsive genes can serve as a negative control since these genes are predicted to be unchanged in GPI biosynthesis mutants. Correspondingly, only the BMP responsive gene Stat1 showed significantly altered expression, and only in bkr mutant embryos. The array also includes other genes in the TGFβ/BMP signaling pathway, many of which display significantly altered expression levels in the GPI biosynthesis mutants.

All TGFβ responsive genes that are significantly altered in gnz and bkr mutant embryos are downregulated. In contrast, 41% of the other genes in the TGFβ/BMP signaling pathway that are significantly altered are upregulated (9 out of 22). Together these data indicate that TGFβ signaling is defective in E7.5 gnz and bkr mutant embryos and corroborates the in vitro data showing that Nodal/Cripto signaling is defective in GPI deficient cell lines.

**Discussion**

**GPI biosynthesis mutants and HPE**

Herein we describe novel mutations in two GPI biosynthesis genes that result in an HPE-like phenotype in mice. In humans, mutations in the GPI biosynthesis pathway have not been implicated in HPE, although mutations are associated with other diseases, including Paroxysmal Nocturnal Hemoglobinuria (PIGA somatic mutation) and Inherited GPI Deficiency (hypomorphic mutation in the PIGM promoter). A human PIGN mutation (R709Q) has been linked to an autosomal recessive syndrome presenting with developmental delay, dysmorphic facies, seizures and severe neurological impairment (Maydan et al., 2011). It is interesting to speculate that different PIGN alleles may yield a range of phenotypes from neuroological defects to structural, HPE-like defects. Our identification of mutations in two GPI biosynthesis enzymes, which result in HPE-like phenotypes in mice, greatly expands an understanding of the genetic causes of HPE and suggests that this entire enzymatic pathway of 27 genes represent novel candidate genes for analysis in human HPE or related disorders.

Complete loss of GPI anchors is embryonic lethal in mice, as demonstrated by knockout of Piga, a critical and early GPI biosynthesis enzyme (Kawagoe et al., 1996; Tremml et al., 1999). Conditional knockout of Piga in chondrocytes leads to mice with shortened bones, similar to that in Pign<sup>−/−</sup> and Pgap1<sup>bkr/bkr</sup> mutants. Previous mutant alleles of Pgap1 show a similar forebrain truncation phenotype as Pign<sup>−/−</sup> and Pgap1<sup>bkr/bkr</sup> mutant embryos, though the authors labeled it as otocephaly, as well as growth retardation, mislocalization of GPI-APs and a sperm activation defect. (Juriloff et al., 1985; Zoltewicz et al., 1999; Ueda et al., 2007; Zoltewicz et al., 2009). Both Pign<sup>−/−</sup> and Pgap1<sup>bkr/bkr</sup> embryos are growth retarded and GPI-GFP is mislocalized in Pign<sup>−/−</sup> and Pgap1<sup>bkr/bkr</sup> mutant embryos. We also observed a genetic interaction between Pign and Nodal suggestive of a sperm defect. In a genetic cross between mice harboring a single Nodal null allele (Nodal−/−) and a single Pign<sup>−/−</sup> allele (i.e. Nodal−/−× Pign<sup>−/−</sup> or Nodal−/−× Pign<sup>−/−</sup> × wildtype) we observed a less than expected proportion of Nodal-LacZ/+ trans-heterozygotes at E7.5 (18.1%, n=115; P value<0.05 using χ² test). Moreover, skewing of the expected ratio only occurred when the father was a trans-heterozygote mated to a wildtype female (14.8%, n=216, P value=0.05). In all other combinatorial matings, the number of trans-heterozygotes was as expected (23.1%, n=199). The Pign and Nodal genetic interaction was statistically significant, although it addressed viability or perhaps sperm fitness, not forebrain development. The Pign and Nodal genetic interaction does, however, indicate a genetic link between GPI biosynthesis and TGFβ signaling.
GPI anchored proteins and HPE

Here we sought to identify the key GPI-AP(s) that Pign and Pgap1 act through to regulate normal forebrain development. In a previous report of a Pgap1 mutant allele, Zoltewicz et al. demonstrated that Wnts are modified with a GPI-like anchor and that midbrain Wnt activation is slightly premature (E8.0 vs. E8.2) (Zoltewicz et al., 2009). The importance of Wnt signaling in forebrain specification has been shown in mice with mutation of Apc (Chazaud and Rossant, 2006) or Dkkopf1 (Mukhopadhyay et al., 2001). Zoltewicz et al. surmised that premature Wnt activation was responsible for otocephaly (Zoltewicz et al., 2001). Zoltewicz et al. speculated whether another GPI-AP, Glypican 4, is in part responsible for gastrulation defects in some Pign mutants. Glypican 4 mediates non-canonical Wnt signaling, and the zebrafish mutant Knypek fails to gastrulate due to aberrant cell polarity and disrupted convergent extension (Topczewski et al., 2001). Cripto nulls also fail to undergo gastrulation (Ding et al., 1998), so it may be difficult to isolate the contribution of these two genes in gastrulation.

The GPI-anchored Cripto-related protein Cryptic may also contribute to the HPE-like phenotype in GPI biosynthesis mutants. Cryptic expression overlaps Nodal in the DVE, and its expression is downregulated in the AVE (Mukhopadhyay et al., 2001). Zoltewicz et al. surmised that premature Wnt signaling targeting the Shh pathway and the TGFβ pathways in HPE etiology. It is also interesting to speculate whether another GPI-AP, Glypican 4, is in part responsible for gastrulation defects in some Pign and Pgap1 mutants. Glypican 4 mediates non-canonical Wnt signaling, and the zebrafish mutant Knypek fails to gastrulate due to aberrant cell polarity and disrupted convergent extension (Topczewski et al., 2001). Cripto nulls also fail to undergo gastrulation (Ding et al., 1998), so it may be difficult to isolate the contribution of these two genes in gastrulation.

The following evidence supports our hypothesis that Cripto is at least one of the key GPI anchored proteins that juxtaposes GPI biosynthesis and forebrain specification: 1. Pign and Pgap1 mutations result in anterior truncations or HPE-like phenotype; Cripto hypomorphic mutations in mouse (Chu et al., 2005). Pgap1 mutants might be candidates for altered Grepl1/Cripto signaling, but it is not clear whether other proteins that functionally interact with Grepl1 are affected.

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Table 1. Real-time PCR results for TGFβ signaling genes indicate defective TGFβ responses in Pign<sup>bkr</sup> and Pgap1<sup>bkr</sup> embryos. Shown are statistically significant changes in expression levels of genes on the Mouse TGFβ BMP Signaling Pathway RT<sup>2</sup> Profiler PCR Array. Relative expression (Relative Expr.) ± standard error is shown from comparison between E7.5 Pign<sup>gnz</sup> and Pgap1<sup>bkr</sup> homozygous mutants and their respective wildtype littermates (C57BL/6J for Pign<sup>gnz</sup> and C3H/HeJ for Pgap1<sup>bkr</sup>). Data were generated using six embryos of each genotype and discarding the single largest outlier for all genes.

| Gene Symbol | Relative Expr. | p-value | Gene Symbol | Relative Expr. | p-value |
|-------------|----------------|---------|-------------|----------------|---------|
| Col1a2      | 0.57±0.15      | 0.015   | Col1a2      | 0.26±0.25      | 0.022   |
| Igf1        | 0.36±0.22      | 0.016   | Igf1        | 0.10±0.10      | 0.007   |
| Igfbp3      | 0.48±0.18      | 0.006   | Igfbp3      | 0.48±0.12      | 0.003   |
| Il6         | 0.69±0.21      | 0.037   | Il6         | 0.48±0.12      | 0.003   |
| Junb        | 0.76±0.09      | 0.001   | Junb        | 0.08±0.03      | 0.48    |
| Pdgfb       | 0.42±0.24      | 0.013   | Pdgfb       | 0.22±0.22      | 0.028   |
| Serpine1    | 0.51±0.28      | 0.049   | Serpine1    | 0.12±0.07      | 0.014   |
| Tgbi        | 0.15±0.02      | 0.015   | Tgbi        | 0.35±0.35      | 0.043   |
| BMP Responsive Genes |       |         | BMP Responsive Genes |       |         |
| Stat1       | 0.60±0.19      | 0.008   | Stat1       | 0.60±0.19      | 0.008   |
| Other TGFβ Superfamily Genes |       |         | Other TGFβ Superfamily Genes |       |         |
| Acrv1       | 1.36±0.35      | 0.043   | Acrv1       | 0.57±0.16      | 0.012   |
| Amh         | 0.56±0.21      | 0.015   | Amh         | 0.54±0.15      | 0.007   |
| Amhr2       | 0.59±0.20      | 0.009   | Amhr2       | 0.54±0.15      | 0.007   |
| Bmp1        | 1.23±0.20      | 0.037   | Bmp1        | 1.37±0.31      | 0.038   |
| Bmp7        | 0.64±0.20      | 0.020   | Bmp7        | 0.64±0.20      | 0.020   |
| Chrd        | 0.35±0.14      | 0.001   | Chrd        | 0.35±0.14      | 0.001   |
| Fst         | 0.65±0.20      | 0.017   | Fst         | 0.65±0.20      | 0.017   |
| Gdf2        | 0.35±0.19      | 0.026   | Gdf2        | 0.35±0.19      | 0.026   |
| Lefty1      | 0.65±0.21      | 0.037   | Lefty1      | 0.65±0.21      | 0.037   |
| Lhbp4       | 0.35±0.14      | 0.001   | Lhbp4       | 0.35±0.14      | 0.001   |
| Nfat1       | 0.83±0.09      | 0.001   | Nfat1       | 0.83±0.09      | 0.001   |
| Nog         | 0.83±0.09      | 0.001   | Nog         | 0.83±0.09      | 0.001   |
| Nrtf1       | 0.83±0.09      | 0.001   | Nrtf1       | 0.83±0.09      | 0.001   |
| Runx1       | 0.65±0.21      | 0.037   | Runx1       | 0.65±0.21      | 0.037   |
| Smad2       | 0.22±0.22      | 0.028   | Smad2       | 0.22±0.22      | 0.028   |
| Tgfb1       | 1.39±0.37      | 0.048   | Tgfb1       | 1.39±0.37      | 0.048   |
internalization) of GPI-anchored proteins; Cripto is a GPI anchored protein (Minichiotti et al., 2000), and its GPI anchor is critical for its function (Watanabe et al., 2007). 3. Pign and Pgap1 mutants show defective AVE migration; Cripto mutations have AVE migration defects (D’Andrea et al., 2008). 4. Otx2 expression is altered in Pign and Pgap1 mutants; Cripto genetically interacts with Otx2 to specify the AVE (Kimura et al., 2001). 5. A percentage of gnz mutant embryos display gastrulation defects; homozygous Cripto null mutant embryos display gastrulation defects (Ding et al., 1998). 6. Cripto/Nodal signaling is reduced in Pign and Pgap1 mutant cells and TGFβ responsive genes are downregulated in Pign and Pgap1 mutant embryos. These pieces of evidence support the hypothesis that Cripto is a key GPI-AP whose dysfunction leads to an HPE-like phenotype.

GPI biosynthesis mutant phenotypes are variable

Pign<sup>gnz</sup> and Pgap1<sup>bkr</sup> mutant embryos share similar phenotypes, but are remarkably different in some aspects. First, gnz mutant embryos display forebrain truncations while bkr mutants show no phenotype in the 129S1 background. Second, Hex-GFP shows differential patterns of expression with gnz embryos expressing GFP at approximately normal levels in the normal number of cells, albeit those cells are not in their correct position, whereas bkr mutants have an expanded population of Hex-GFP cells and those cells overexpress Hex-GFP. Third, TGFβ responsive gene expression is reduced in Pign<sup>gnz</sup> and Pgap1<sup>bkr</sup> mutant embryos but the specific genes that are downregulated show little overlap.

In part these differences may be related to genetic background differences as the penetrance and severity of the developmental defects depends on the genetic background. These differences may also relate to the allele generated by the point mutations. The Pgap1<sup>bkr</sup> allele causes an in-frame deletion of 13 amino acids. Pgap1<sup>bkr</sup> mutant embryos in 129S1 background are morphologically normal, which suggests that Pgap1<sup>bkr</sup> retains partial activity. In contrast, Pign<sup>gnz</sup> is predicted to encode a truncated protein that both lacks catalytic activity and is not responsive gene expression is reduced in Pign<sup>gnz</sup> and Pgap1<sup>bkr</sup> mutant embryos but the specific genes that are downregulated show little overlap.

The phenotypic differences may also relate to the different steps in GPI biosynthesis that Pign and Pgap1 catalyze. Pign catalyzes an early step in GPI biosynthesis, and disruption of Pign in cell lines leads to formation of several divergent GPI anchors, some of which localize to the plasma membrane, while others do not (Hong et al., 1999). Pgap1 catalyzes inositol deacetylation after the GPI anchor is covalently bound to target proteins (Tanaka et al., 2004). Acyl-group removal is necessary for efficient ER export via CopII coated vesicles, and Pgap1 mutant cells accumulate GPI-APs within the ER (Tanaka et al., 2004). As Pign<sup>gnz</sup> likely represents a null allele and Pign catalyzes a relatively early biosynthetic step in GPI anchor production, Pign<sup>gnz</sup> mutants likely represent a more severe class of GPI biosynthesis mutants than Pgap1<sup>bkr</sup> mutants. Alternatively, although the GPI biosynthesis pathway is largely thought to follow a linear progression, our in vivo results might suggest divergent requirements for this pathway in regulating various GPI-APs.

Cell autonomous vs. non-cell autonomous Cripto signaling

The contribution of cell autonomous versus non-cell autonomous Cripto/Nodal signaling is poorly understood. Lack of both cell autonomous and non-autonomous Cripto signaling results in a “head-without-trunk” phenotype (Ding et al., 1998). Gnz and bkr mutants, which present as a “trunk-without-head”, likely retain some non-cell autonomous Cripto signaling while lacking cell autonomous Cripto signaling. Further, overexpression of a soluble form of Cripto can rescue zebrafish Oep mutants (Zhang et al., 1998). While cell autonomous Cripto signaling is clearly required for normal development, further studies are required to determine the role of non-cell autonomous Cripto signaling during early development.

Downstream effectors of forebrain development

Nodal/Cripto signaling is important for forebrain development, yet the downstream targets of this pathway are unknown. Analysis of genes that are altered in gnz and bkr mutants may shed light upon downstream effectors that mediate this process. Col1a2 and Il6 are significantly downregulated in gnz and bkr mutants; however, phenotypes associated with mutation of these genes in humans are unrelated to forebrain development (Dickson et al., 1984; Wirtz et al., 1987; Kishimoto, 2005). Three genes involved in iron homeostasis are either downstream of TGFβ signaling or GPI-APs. Gdf2 and Il6 converge to regulate expression of Hepcidin (Truksa et al., 2007), a key regulator of iron homeostasis (Nemeth and Ganz, 2009). The GPI-AP Hemojuvelin also regulates Hepcidin, so gnz and bkr mutants may have alterations in the function of genes that regulate iron levels. Iron levels are important for forebrain development in mice and mutation of the iron transporter Ferroportin causes anterior truncations (Mao et al., 2010). It will be interesting to test whether iron homeostasis is dysregulated in bkr and gnz mutants and, if so, this could provide a context to begin to understand the molecular relationships between different pathways that alter early forebrain development.

In humans, there is no current evidence linking mutations in GPI biosynthesis genes with HPE or forebrain truncations. Our studies raise the intriguing possibility that the ~thirty GPI biosynthesis enzymes may represent a new class of genes to test for linkage to holoprosencephaly, or other cranio-facial syndromes, such as agnathia, dysgnathia, microphthalmia and otocephaly.

Acknowledgements

We thank Kathryn Anderson, Elizabeth Lacy, Monica Justice and their labs for ENU mutagenesis; Trevor Williams, Weiguo Feng and Gantz Hanson for help with the forward genetic screen; Andy Peterson for sharing unpublished results; Kat Hadjantonakis (GPI-GFP), Tristan Rodriguez (Hex-GFP) and Michael Shen (Cripto plasmids) for mice and reagents, and David Clouthier for help interpreting skull morphologies. This project was supported by NINDS F31NS060454 and NICHD U01 HD043478. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders And Stroke or the National Institutes of Health. L.N. is an investigator of the Howard Hughes Medical Institute.

Competing Interests

The authors have no competing interests to declare.

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