Deletion of *Porcn* in Mice Leads to Multiple Developmental Defects and Models Human Focal Dermal Hypoplasia (Goltz Syndrome)

Wei Liu¹, Timothy M. Shaver²,⁵, Alfred Balasa³, M. Cecilia Ljungberg⁴,⁷, Xiaoling Wang¹, Shu Wen¹, Hoang Nguyen²,⁵, Ignatia B. Van den Veyver¹,⁶,⁷*

¹Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas, United States of America, ²Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, United States of America, ³Pediatrics, USDA/ARS Children’s Nutrition Research Center, Houston, Texas, United States of America, ⁴Pediatrics-Neurology, Baylor College of Medicine, Houston, Texas, United States of America, ⁵Stem Cells and Regenerative Medicine Center, Baylor College of Medicine, Houston, Texas, United States of America, ⁶Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, United States of America, ⁷The Jan and Dan Duncan Neurological Research Institute, Texas Children’s Hospital, Houston, Texas, United States of America

Abstract

**Background:** Focal Dermal Hypoplasia (FDH) is a genetic disorder characterized by developmental defects in skin, skeleton and ectodermal appendages. FDH is caused by dominant loss-of-function mutations in X-linked *PORCN*. *PORCN* orthologues in Drosophila and mice encode endoplasmic reticulum proteins required for secretion and function of Wnt proteins. Wnt proteins play important roles in embryo development, tissue homeostasis and stem cell maintenance. Since features of FDH overlap with those seen in mouse Wnt pathway mutants, FDH likely results from defective Wnt signaling but molecular mechanisms by which inactivation of *PORCN* affects Wnt signaling and manifestations of FDH remain to be elucidated.

**Results:** We introduced intronic loxP sites and a neomycin gene in the mouse *Porcn* locus for conditional inactivation. *Porcn-ex3-7floxed* mice have no apparent developmental defects, but chimeric mice retaining the neomycin gene (*Porcn-ex3-7Neo-flox*) have limb, skin, and urogenital abnormalities. Conditional *Porcn* inactivation by *Ela*-driven or *Hprt*-driven Cre recombinase results in increased early embryonic lethality. Mesenchyme-specific *Prx-Cre*-driven inactivation of *Porcn* produces FDH-like limb defects, while ectodermal *Krt14-Cre*-driven inactivation produces thin skin, alopecia, and abnormal dentition. Furthermore, cell-based assays confirm that human *PORCN* mutations reduce Wnt3A secretion.

**Conclusions:** These data indicate that *Porcn* inactivation in the mouse produces a model for human FDH and that phenotypic features result from defective Wnt signaling in ectodermal- and mesenchymal-derived structures.

Citation: Liu W, Shaver TM, Balasa A, Ljungberg MC, Wang X, et al. (2012) Deletion of *Porcn* in Mice Leads to Multiple Developmental Defects and Models Human Focal Dermal Hypoplasia (Goltz Syndrome). PLoS ONE 7(3): e32331. doi:10.1371/journal.pone.0032331

Editor: Shree Ram Singh, National Cancer Institute, United States of America

Received December 15, 2011; Accepted January 25, 2012; Published March 6, 2012

Copyright: © 2012 Liu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding for this work was provided by The March of Dimes Birth Defects Foundation (6-FY08-268), the National Foundation for Ectodermal Dysplasias (Research Grant 27568), and the National Institutes of Health (R01-AR059122). This project was also supported by the administrative, neuropathology, and mouse embryonic stem cell cores of the Baylor College of Medicine Intellectual and Developmental Disabilities Research Center, award number P30HD024064 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Eunice Kennedy Shriver National Institute of Child Health and Human Development or the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: iveryver@bcm.edu

Introduction

Focal dermal hypoplasia (FDH), also known as Goltz syndrome or Goltz-Gorlin syndrome, is an X-linked disorder that predominantly affects females. Individuals with FDH have a pleiotropic phenotype consisting primarily of defects of skeleton, skin, and ectodermal appendages. They often have ectrodactyly, syndactyly, brachydactyly, and oligodactyly of hands and feet, and can have shortened or absent long bones combined with typical streaks of atrophic, hypo- and hyperpigmented skin with abnormal subcutaneous fat deposition in a thin dermis. Other common features consist of eye and ear abnormalities, brittle and sparse hair, dystrophic nails, hypodontia, and supernumerary nipples. More variable findings in FDH include short stature, pointed chin, cleft lip and palate, osteopathia striata of long bones, diastasis pubis, kidney abnormalities, abdominal wall defects, and papillomas of lips, gingivae, and tonsils. Strikingly, central nervous system abnormalities and intellectual disability are uncommon in FDH [1,2,3,4,5,6]. It has been suggested that the phenotype of FDH results from a developmental defect in signaling between ectoderm and mesoderm [7].

We and others first described that FDH is caused by mutations in the X-linked *PORCV* gene, which has facilitated the clinical diagnosis of FDH [8,9,10,11,12,13]. Females with FDH have heterozygous or mosaic loss-of-function mutations or large deletions of *PORCV* and the few affected males have mosaic mutations [14]. This explains the female predominance of the disorder, absence of male-to-male transmission, and presumed
male lethality of germline mutations [1,15]. PORCN is the human orthologue of the Drosophila segment polarity gene, parcunine [16]. The Porcupine protein is a multipass transmembrane endoplasmic reticulum (ER) protein and a member of the superfamily of membrane-bound O-acyl transferases (MBOAT) [17]. Drosophila and mouse Porcupine lipid-modify Wnt proteins by covalently linking palmitic acid at a conserved serine residue (S209 in mouse Wnt3a), which is required for the attachment of a second acyl group, palmitate at a conserved cysteine (C77 in Wnt3a) [18,19,20]. This is important for processing, secretion, and signaling of Wingless (Wg) in Drosophila, Wnt3a in mouse, and mouse Wnt3a (Fig. 1B–D), which is required for the attachment of a second acyl group, palmitate at a conserved cysteine (C77 in Wnt3a) [17]. Drosophila orthologue of the segment polarity gene, Drosophila Wg, is the human FDH-like developmental defects in a chimeric mouse.

Results

FDH-like developmental defects in a chimeric Porcn genetrap mutant mouse

We performed two sets of injections into blastocysts of mouse ES cells (line CSD256) that were targeted with a genetrap cassette containing a splice-acceptor sequence upstream of a β-galactosidase-neomycin fusion (βgeo) reporter gene inserted 3′ to exon 2, which contains the translation initiation site of Porcn (Fig. 1A). Consistent with the prediction that this allele would result in inactivation of Porcn, only a single liveborn male chimera (Fig. 1B–I) was found in 28 offspring. This animal had fused and hypoplastic digits on 1 to all 4 extremities (Fig. 1B and 1E), 2 midline ventral wall defects, and a midline dorsal wall defect (Fig. 1C). The skin lesions showed a thin epidermis with presence of a dermoid cyst in one of the ventral lesions (Fig. 1F). Interestingly, this mouse also had external male genitalia, but was a hermaphrodite with a single ovary and a hypoplastic testis (Fig. 1G). It developed a cystic intra-abdominal structure, suspected to be a dilated obstructed cystic intra-abdominal structure, which was also observed in their mosaic parents, but they had no other defects. We propose that skewed X-inactivation patterns in these survivors to wild type mice, but only obtained 2 heterozygous mosaic females carrying the Porcn-ex3-7del allele (not shown). The recovery of these mosaic floxed/inscribed ES cell clones from which 3 independent lines of Porcn-ex3-7del/7del mice were bred to homozygous X/Y; PORCNfl/fl ES cell clones. We obtained 5/288 (1.7%) correctly targeted clones from which 3 independent lines of chimeric mice were generated after successful blastocyst injections with different clones. Of 17 chimeric mice, there were 9 high-level male and female chimeras from 2 different clones that displayed a mild phenotype reminiscent of human FDH (Fig. 1A–G). These mice had absent, fused, and shortened digits on 1 all 4 of their extremities (Fig. 2A and B), vertebral anomalies of the tail (Fig. 2C), hydromecephrosis (Fig. 2D), small testicles in males (Fig. 2E), and uteri with rudimentary uterine horns in females (Fig. 2F). We confirmed the presence of the targeted allele in various tissues by PCR amplification with primers P1 and P2 (Fig. 2G). Therefore, intronic integration of the Neo gene and loxP sites created at least a hypomorphic Porcn allele. The phenotype observed in chimeric mice further confirmed that as in human FDH, mosaic expression of mutant Porcn is sufficient to generate developmental abnormalities. We excised the Neo gene by mating XPorcn-ex3-7del/7del; Hprt-Cre mice expressing Flp recombinase to generate XPorcn-ex3-7del/7del; X/Y mice (Fig. 1I), which had no observable phenotype and were fertile.

Zygotic inactivation of Porcn by Ella-Cre and Hprt-Cre causes early embryonic lethality

To generate Porcn-ex3-7del alleles (Fig. 1E), in the zygote and earliest stages of embryonic development, XPorcn-ex3-7del/Y mice were bred to homozygous X/Y; Ella-Cre/Ella-Cre mice, which express Cre recombinase under control of the Ella viral promoter [30]. From 13 litters, we obtained 38 female and 40 male offspring, but only 3 male and 3 female liveborn mice carried the Ella-Cre transgene together with a Porcn-ex3-7del allele (Table 1) and had low-level mosaicism for the deleted Porcn-ex3-7del allele (not shown). The recovery of these mosaic floxed/deleted survivors can be explained by mosaic expression of the Ella-Cre transgene as previously described [31]. We then bred these survivors to wild type mice, but only obtained 2 heterozygous females carrying the Porcn-ex3-7del allele out of 133 offspring total. These mice were viable and showed diminished hair growth, which was also observed in their mosaic parents, but they had no other defects. We propose that skewed X-inactivation patterns may explain the fairly mild phenotype in these heterozygous offspring. Timed matings yielded 14 female and 4 male live embryos at E10.5, but only 5 out of 14 female embryos carried the Porcn-ex3-7del allele; we did not recover any XPorcn-ex3-7del/Y embryos or liveborn mice, indicating that lethality of embryos with this genotype occurs before E10.5. This is consistent with data that became available during the course of this work that support an essential role for Porcn in gastrulation [25,27].
Because of the observed mosaic inactivation of Porcn with the Ehlo-Cre transgene [31] and to further examine the embryonic defects in heterozygous mutant females in this animal model for FDH, we next bred floxed mice to mice that express Cre recombinase under control of the endogenous X-linked Hprt promoter that drives expression of Cre in oocytes and zygotes. Ubiquitous non-mosaic deletion of floxed alleles can be obtained when conditionally targeted mice are bred with either female or male Hprt-Cre mice [32]. We reasoned that breeding floxed X\(^{Porcn-ex3-7del}/X\) females to X\(^{Hprt-Cre}/Y\) males would yield heterozygous deleted females and allow us to study the full phenotypic spectrum of Porcn mutations. It would also allow us to address whether germline heterozygous mutations in females are usually embryonic lethal, since it has been proposed that most surviving female offspring, none of which carried the X\(^{Porcn-ex3-7del}/Y\) genotype. Most of these (>80%) had open neural tubes, defects in ventral body wall closure, and axial/tail truncation (Figure 3A). And mutant embryos appeared progressively smaller compared to wild type counterparts at advancing embryonic stages. By E12.5 most X\(^{Porcn-ex3-7del}/X\) embryos were resorbed. These findings indicate that the majority of females with heterozygous inactivation of Porcn are lost prenatally, consistent with some clinical data in human FDH [9]. The axial/tail truncation and neural tube defect partially resemble Wnt3a mutants and the phenotype of conditional inactivation of β-catenin in 3 germ layers by Cre recombinase under control of a promoter fragment of the caudal-related homeobox gene (Gdx) [33,34]. The severe ventral body wall defects in heterozygous female mice are very interesting in light of recent case reports of pentalogy of Cantrell in infants or fetuses with FDH and mutations in PORCN [35,36]. The open ventral body wall was also reported in dermal β-catenin mutant mice and Dvl2/Dvl3 double mutant mice [37,38].

RNA from embryos collected at E9.5 and E10.5 was extracted to evaluate gene expression of Porcn, Wnt pathway, and Wnt target genes, as well as other developmental markers by quantitative RT-PCR. Expression of Porcn itself and the canonical Wnt signaling pathway gene Axin2 and the target gene c-myc were significantly reduced at E9.5, indicating that reduced Porcn levels affects Wnt signaling and its targets (Figure 3B). Reduced expression of Porcn and c-myc persisted in E10.5 mutant embryos. There was also decreased expression of the neuroectoderm marker Gbx2 (Figure 3C). Gbx2 plays an essential role in cerebellum development and maintenance of the mid/hindbrain organizer and it is also required to pattern the neural tube [39,40,41]. The decreased levels of Gbx2 suggest that reduced function of Porcn in X\(^{Porcn-ex3-7del}/X\) embryos affects neuroectoderm development. This could in part explain the observed open neural tube defects. It also implies that, even though central nervous system anomalies and neural tube defects are not typically seen in human FDH patients, mutations of Porcn impair the development of these structures. We speculate that in humans these defects are too severe or associated with additional developmental defects to permit survival to birth.
Conditional inactivation of Porcn in limb mesenchyme causes mesomelic limb shortening and digital abnormalities

To further investigate the mechanisms by which loss of Porcn results in the limb defects of FDH, and to initiate studies into the role of Porcn in mesenchyme-derived tissues, we bred Porcn-ex3-7flox mice with Prx-Cre mice, reported to drive Cre expression in mesenchyme at early stages of forelimb development [42]. Analysis of 6 litters (Table 1) indicated preserved Mendelian ratios in liveborn offspring. Male offspring (14/14, 100%) with X\(^{\text{Porcn-ex3-7flox}}\)/Y; Prx-Cre genotypes and predicted Porcn-ex3-7del in tissues where Prx-Cre is expressed, have significantly shorter limbs

![Image of phenotypes](image_url)

**Figure 2. Phenotype of Porcn-ex3-7-Neo-flox chimeric mice.** (A) Hypoplastic, fused, and missing digits on right (R) and left (L) fore- (F) and hindlimbs (H) in different chimeras (C1–C4) compared to wild type (WT). (B) Skeletal preparations of extremities shown in panel A. (C) Vertebral abnormalities in the tail of chimera 1 (C1). (D) Hydronephrosis of the right kidney and normal left kidney in C1. (E) Hypoplastic testicle in C1 and normal testicle in C5. (F) Uterine abnormalities: asymmetrical hypoplastic uterine horn in C6. (G) Gel picture showing amplification of the targeted allele in various tissues of C1. H, heart; K(L), left kidney; LU, lung; SK, skin; SP, spleen; T, testis; K(R), right kidney; Li, liver; TG, targeted allele; WT, wild type allele.

doi:10.1371/journal.pone.0032331.g002

| Mating                        | X\(^{\text{Porcn-ex3-7flox}}\)/X with X/Y; Ella-Cre | X\(^{\text{Porcn-ex3-7flox}}\)/X with X/Y; Prx-Cre/- | X\(^{\text{Porcn-ex3-7flox}}\)/X with X/Y; Krt14-Cre/- |
|-------------------------------|---------------------------------------------------|------------------------------------------------------|------------------------------------------------------|
| Number of litters (average litter size) | 13 (6)                                             | 6 (7.3)                                              | 7 (6.6)                                              |
| Female:male ratio              | 38:40                                              | 19:25                                                | 19:27                                                |
| X\(^{\text{Porcn-ex3-7flox}}\)/Y; Cre | 3                                                  | 10                                                   | 7                                                    |
| X\(^{\text{Porcn-ex3-7flox}}\)/X; Cre | 3                                                  | 2                                                    | 2                                                    |
| X/Y; Cre                       | 37                                                 | 5                                                    | 6                                                    |
| X/X; Cre                       | 35                                                 | 4                                                    | 5                                                    |
| X\(^{\text{Porcn-ex3-7flox}}\)/Y | n/a                                                | 5                                                    | 6                                                    |
| X\(^{\text{Porcn-ex3-7flox}}\)/X | n/a                                                | 6                                                    | 5                                                    |
| X/Y                            | n/a                                                | 5                                                    | 8                                                    |
| X/X                            | n/a                                                | 7                                                    | 7                                                    |

doi:10.1371/journal.pone.0032331.t001
as early as E18.5 (Figure 4A). They are also smaller than their littermates at postnatal day 7 (P7) (Figure 4B) and become increasingly stunted by P28 (Figure 4C). Their limbs remain very short with apparent syndactyly of digits of both fore- and hindlimbs in some animals (3/14, 21%) (Figure 4D). Skeletal preparations show that long bones of all extremities and digits are shortened, but we did not observe bony fusion of digits, indicating that the syndactyly is limited to soft tissues and does not include skeletal elements (Figure 4E–J). As expected, the Porcn-ex3-7del allele was detected by PCR in affected tissues from limbs of these mice (Figure S2A). These data indicate that Porcn deletion in mesenchymal derivatives is sufficient to cause severe defects of fore- and hindlimbs. As expected from the known expression pattern of Prx-Cre [42], defects were more severe in forelimbs. The shortened limb phenotype is similar to that of Wnt5a null except that our mice did not show loss of distal digits as Barrott et al.

Table 2. Liveborn offspring from breeding of $X^{Porcn-ex3-7flo/x}$ mice to Hprt-Cre mice.

| Mating                  | $X^{Porcn-ex3-7flo/x}$ with $X^{Hprt-Cre/Y}$ |
|-------------------------|-----------------------------------------------|
| Number of litters (average litter size) | 5 (5.2)                                      |
| Female:male ratio       | 9:17                                         |
| $X^{Porcn-ex3-7flo/x}$  | 9                                             |
| $X^{Porcn-ex3-7flo/x}$ $Hprt-Cre$ | 0                                             |
| $X/X^{Hprt-Cre}$        | 9                                             |
| $X/Y$                  | 8                                             |

doi:10.1371/journal.pone.0032331.t002

Figure 3. Phenotype at E9.5 and gene expression analysis of $X^{Porcn-ex3-7del/X}$ embryos. (A) $X^{Porcn-ex3-7del/X}$ embryo with open neural tube and abdominal wall closure defect (arrows). (B) Bar graph of quantitative real-time PCR analysis at E9.5 (top panel) and E10.5 (bottom panel) of Porcn wild type (n=4 each) and mutant (n=3 each) embryos. Bars are color-coded by gene type and indicate the log2 of the fold-deviation of gene expression levels in Porcn mutant embryos compared to wild type (set as 1). Error bars indicate standard errors of the mean. (*) indicates statistical significance at p<0.05 (Student’s t-test). doi:10.1371/journal.pone.0032331.g003
observed [25,43]. This observation supports that Porcn may be required for “noncanonical” Wnt signaling; yet, the reason we observe a milder phenotype in X\(^{\text{Porcn-ex3-7flox}}\)/Y;Prx-Cre mice remains to be discovered. The shortened limb phenotype models the features of severe cases of FDH, except for absence of bony syndactyly [44].

Conditional inactivation of Porcn by Krt14-Cre-mediated deletion causes thinning of the skin with absent hair follicle development and dental defects

To identify the origin of defects of the skin and ectodermal appendages present in FDH and to investigate the contribution of altered ectodermal \(\text{PORCN}\) function to the FDH phenotype, we bred X\(^{\text{Porcn-ex3-7del}}\)/X mice with X/Y;Krt14-Cre\(^{-}/\) mice. The Krt14 promoter drives Cre expression in the basal layers of developing epithelia, causing deletion of floxed alleles in the epidermis and other ectodermal derivatives [45]. Mendelian ratios were preserved among liveborn offspring of these matings (Table 1). Heterozygous X\(^{\text{Porcn-ex3-7del}}\)/X,Krt14-Cre\(^{-}/\) females had barely detectable hair loss, but males with Krt14-Cre-driven Porcn deletion had large areas of thin skin with alopecia (Figure 5A and B). The mosaic pattern of this phenotype likely results from variable expression of the Krt14-Cre transgene, as we have previously observed. Consistent with Krt14-driven Cre expression in multiple ectodermal derivatives, the mutant mice also had abnormally formed incisors (Figure 5C–E).

We detected the Porcn-ex3-7del allele in affected skin (Figure S2B) and confirmed reduced Porcn expression by RNA in situ hybridization with a Porcn riboprobe in the skin of E16.5 mutant embryos (Figure 5F–I). Histological analysis of skin at P9 showed that affected areas are completely devoid of hair follicles (Figure 5J and K). This phenotype is a phenocopy of that obtained when β-catenin is conditionally inactivated in skin by the same Krt14-Cre (Figure 5L and M), supporting the conclusion that it results from defective Wnt signaling. Analysis at E16.5 revealed that early budding of epidermal cells to form the hair placodes that should give rise to hair follicles did not take place in these embryos (Figure 5N and O). Absent staining in mutant skin for the early hair follicle marker P-cadherin and for Sox9, which is present in hair follicles from the placode stage, confirmed that placode formation was drastically compromised (Figure 5P–S), and indicates that hair follicle morphogenesis is blocked. This suggests that these aspects of the epidermal phenotype can result from ectodermal loss of Porcn (Figure S2B).

Therefore, although Prx drives Cre expression primarily in mesenchymal derivatives and Krt14 drives it primarily in ectodermal derivatives, mice with Porcn inactivation in either of
these Cre lines have phenotypes that are remarkably similar to those seen in human patients, implicating that disruption of both mesenchymal and ectodermal PORCN contributes to the FDH phenotype. Interestingly, we also noted that in the areas of thinned skin, the subcutaneous fat was directly adjacent to the outermost epidermal layers of the skin (Figure 6). While this will require further detailed investigation, this finding may be relevant to the observed areas of apparent herniating fat found in the skin defects of patients with FDH.

Effects of overexpressed wild type and mutant PORCN on cellular WNT3A secretion

It has been shown that Drosophila Porcupine and, in cell culture assays, its murine ortholog lipid-modify Wnt proteins (in particular Wnt3a) to promote their secretion from the ER and consequently from Wnt-producing cells, allowing them to act as signaling molecules that activate receptors on Wnt-responding cells [18,22,23]. However, a direct effect of Porcupine on WNT-protein secretion and accordingly a link between FDH-causing mutations and WNT signaling had not yet been demonstrated in human cells when we initiated our studies. We therefore performed a cell-based assay to investigate how human wild type (wt) and mutant PORCN influence secretion of WNT proteins (Figure 7). Co-expression of wt PORCN and WNT3A in human embryonic kidney (HEK293T) cells showed that, as expected, transiently overexpressed wt PORCN reduced WNT3A in the cell to virtually undetectable levels, indicating increased secretion of WNT3A. In contrast, PORCN with mutations p.M1I (c.3G>A) and p.R124X (c.370C>T), known to cause human FDH, resulted in WNT3A retention in cells compared to wt PORCN (Figure 7A). However, other PORCN mutations found in FDH, p.S136F (c.407C>T), p.G168R (c.502G>A), and p.Y359X (c.1077C>A), did not or only mildly affect WNT3A retention in cells. It remains to be investigated how these mutations affect WNT protein signaling. Interestingly, other recent studies also indicate that Porcupine proteins without lipid adducts are still secreted but...
Discussion

PORCN was first identified as the mutated gene in Goltz-Gorlin syndrome or FDH in 2007 [8,9]. Since then, at least 80 different mutations and large genomic deletions of the PORCN gene have been found in FDH patients [36]. The resulting phenotype in human patients varies widely from mild skin and distal skeletal defects to severe forms of FDH, with multiple defects that include severe limb abnormalities involving long bones, aplasia cutis, limb-body wall complex anomaly, pentalogy of Cantrell, and Van Allen-Myhre syndrome [35,36,47]. Identification and characterization of PORCN mutations as the cause of FDH was important for genetic counseling and diagnostic testing in suspected cases. However, an animal model for the condition and functional cell-based assays are needed to determine the functional in vivo consequences of Porcn mutations on Wnt signaling and to investigate new therapies.

We first attempted to generate mice from ES cells with a genetrap insertion in the Porcn locus. This produced a single chimera, but not unexpectedly, there was no germline transmission of this null allele. We then generated a conditional allele of Porcn by introducing losP sites flanking exons 5 and 7. When targeted mice are bred with early embryonic Cre-expressing mice (EIIa-Cre, Hprt-Cre, ectoderm-specific Krt14-Cre, and limb mesenchyme-specific Prx-Cre mice), a variety of interesting phenotypes are observed that recapitulate various aspects of the human condition and prove that these mice provide a reliable animal model for human FDH. Our findings are in agreement with and significantly expand on those reported by Barrott and colleagues during the course of this work in a mouse model with a deletion of exons 2 and 3 of Porcn [25]. We also made important novel observations that may be relevant for other human conditions potentially resulting from Porcn mutations or other defects in the Wnt signaling pathway.

Although lethality of Porcn mutations in hemizygous males has already been confirmed in studies with embryos resulting from aggregation of CSD256 ES cells with tetraploid blastocysts [25] and in vivo [27], our data provides the first in vivo evidence that many heterozygous females with inactivating mutations of PORCN also die in utero. We also demonstrate that, even though neural tube defects are not typically present in liveborn humans with FDH, severe cranial neural tube defects are common in the heterozygous embryos. This is consistent with expression of Porcupine and Wnt proteins in the developing central nervous system and suggests that associated defects could be responsible for the lack of surviving humans with FDH that have NTDs or other severe brain abnormalities [34]. We also demonstrated in different ways that mosaicism for a Porcn mutation is important for the phenotype: we observed limb, skin, and internal organ abnormalities in the CSD256 genetrap chimera and in chimera animals carrying the Porcn-ex3-7Neo-flox allele, as well as mosaic skin defects in mice with Porcn-ex3-7 deletion driven by Krt14-Cre and by EIIa-Cre. Having the current mouse model will also allow us to study the contribution of X-inactivation patterns for the mosaic expression of the phenotype in females.

Krt14-Cre-mediated Porcn inactivation also causes complete absence of hair follicle development and differentiation. This is in agreement with the lack of hair follicles described with ubiquitous Cre recombinase-mediated inactivation [25], but our observation that inactivating Porcn only in ectoderm using Krt14-Cre is sufficient to cause this phenotype is novel. In addition, we found that the defects in hair follicle development phenocopy those observed after Krt14-Cre-driven β-catenin inactivation, implying that they are caused by defective canonical Wnt signaling in ectodermal derivatives. The absence of P-cadherin and Sox9 indicates a very early defect in specification of the hair placode. Our findings of abnormal dentition in the animals are also novel and may result from similar disruption in ectodermal Wnt signaling in the tooth placode.

Another novel observation is that the chimeric animals have some of the rarer phenotypic features occasionally described in FDH, such as urogenital anomalies. Considering the known role of Wnt proteins in the development of the urogenital tract, the Porcn-ex3-7floxed mice will be valuable to study how Porcupine regulates Wnt signaling during the development of the urogenital tract.

Finally, the cell culture assays provide evidence that PORCN containing mutations that cause human FDH can influence the secretion and signaling of WNT proteins from the cell. Consistent
with prior data [18,25,27,46], we demonstrate that knockdown of \( \text{PORCN} \) by siRNA, as well as some of the FDH-causing mutations that we studied, cause retention of WNT3a in the cell. In contrast, similar experiments with WNT1 show a lesser and different effect on WNT protein secretion and indicate that the effects of identical mutations in \( \text{PORCN} \) cannot be generalized to all WNT proteins.

A recent study also showed that differential palmit(e)oylation of two residues of Wnt1 has both overlapping and distinct consequences, whereby one is \( \text{Porcn} \)-dependent and the other is \( \text{Porcn} \) independent. It has been proposed that this differential residue modification may play a role in determining participation in the canonical versus non-canonical Wnt-signaling pathway [48]. This data provides further evidence that Porcupine has a complex role in the regulation of secretion and signaling of Wnt ligands and that individual Wnt-proteins are differently influenced. This is consistent with data by others that indicate that effects of Porcupine on Wnt signaling can be uncoupled from Wnt ligand secretion [19].

The mouse \( \text{Porcn} \) alleles that we generated, supported by cell-based assays, provide evidence for an important role of Porcupine in ectodermal and mesodermal signaling of Wnt proteins. This and other generated mouse models [25] will be important tools for further detailed characterization of the function of \( \text{PORCN} \) in development and disease, and have provided a model for investigating potential therapies for some of the features of FDH that progress postnatally, such as skin defects and peri-orificial papillomas.

**Materials and Methods**

All animal procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. All animals are monitored daily by experienced personnel, animal staff and veterinarians. Animals were euthanized when they were unable to stand or eat, or when they displayed agonal breathing, vocalization, self-mutilation, had dehiscent wounds, hypothermia, weight loss of >20%, or seemed otherwise to be suffering. For animals that were impaired in reaching standard food supply due to skeletal abnormalities, food pellets were placed on the bottom of the cage, and we confirmed that animals were reaching these pellets and were also able to reach drinking water.

**Generation and analysis of the \( \text{Porcn} \) genetrap mouse**

Mouse embryonic stem (ES) cells (line CS256) carrying a genetrap mutation in intron 2 of \( \text{Porcn} \) [Figure 1A] previously generated as described [49] were purchased from the Mutant Mouse Regional Resource Centers (MMRRC). ES cells were injected into blastocysts that were transferred into pseudopregnant females, yielding 1 male chimera out of 28 offspring. This mouse was sacrificed at 3.5 months of age and tissues were snap frozen for genetic analysis or fixed in 10% formalin and embedded in paraffin for sectioning and hematoxylin-eosin (H&E) staining for histopathology. Skeleton reconstruction of the bony skeleton (without cranium) was performed by micro-CT using a Gamma

---

**Figure 7. Cell-based WNT3A secretion assay.**

(A) Western blot showing WNT3A levels in total cell lysates after transient transfection. (NTC, non-transfected control). (B) Quantification of WNT3A levels in cell lysates. Co-expression of WNT3A with wild type \( \text{PORCN} \) (WNT3A+PORCN) results in disappearance of WNT3A from the cell lysate compared to WNT3A alone or WNT3A+GFP. In contrast, co-expression of mutant \( \text{PORCN} \) forms p.M1I and p.R124X with WNT3A causes WNT3A retention in cells, but other mutations, p.S136F, p.G168R, and p.Y359X, do not affect WNT3A secretion. We compared WNT3A levels in cells co-transfected by WNT3A and either wild type or mutant \( \text{PORCN} \) forms to WNT3A levels in cells expressing WNT3A+GFP, because these contain similar amounts of transfected DNA. Fold changes with standard deviation are shown; all data were normalized to \( \beta \)-tubulin; (*) indicates significant difference at \( p < 0.05 \); the experiment was repeated 4 times.

doi:10.1371/journal.pone.0032331.g007
Deletion of Porcn in Mice Models Human FDH

Medica FLEX X-O.X-SPECT SPECT/CT instrument (Gamma Medica Ideas Inc.), and analyzed with Amira 3.1.1 software. Genotyping of various tissues was performed by PCR-based detection of the lacZ gene present in the βgeo cassette of the inserted transgene.

Construction of the targeting vector, embryonic stem cell (ES) culture, and gene targeting

The pFRT-LoxP plasmid used to construct the targeting vector was kindly provided by Dr. James Martin (Institute of Biosciences and Technology, Houston). The pFRT-LoxP contains 2 loxP sites, a neomycin gene flanked by two FRT sites, and a TK cassette for negative selection. The 4.7 kb 5’ targeting arm, 1.7 kb mid arm, and 5 kb 3’ targeting arm of the Porcn targeting vector and diagnostic probes for correct targeting were amplified from BAC clone bMQ-207H18 [129S7/AB2.2, Sanger Center] using Takarai LA Taq™ DNA Polymerase (Takara Bio, Inc.) and cloned into a TA-vector (TOPO® TA Kit, Invitrogen). A 360 site for future characterization was introduced into the forward PCR primer used to amplify the 5’ arm. The 3’ arm was released from the TA-vector with PstI and AscI restriction digestion and cloned into PstI/AscI sites of the pFRT-LoxP plasmid; the mid arm was released with Nol and Acc65I, and digested and cloned into Nol/Acc65I sites of the pFRT-LoxP plasmid; the 3’ arm was released from XhoI restriction digestion and cloned into an XhoI site of the pFRT-LoxP plasmid to generate the Porcn-ex3-7Neo-flox targeting construct. This construct was sequenced to confirm the genomic DNA sequence and insertion sites, linearized by PstI restriction digestion and electroporated into mouse ES cells of a 129S5/SvJaeSor-EIIa-Cre (Tg(EIIa-cre)C5379Lmgd) and 129S1/Sv-J females to test for germline transmission of the targeted knockout mice. Male chimeras were bred to C57BL/6J female mice (C57BL/6J) and Hprt-Cre (129S1/Sv-J) and FLP mice were obtained from Jackson Labs. Primers for genotyping Krt14cKO mice were CtcF: TGGCTGTTTCACCTGTT-TATCGGG and CreF: TGCGCCTGTTTACATATGC.

Skeleton preparations, hematoxylin-eosin staining, Oil red O staining, RNA in situ hybridization and immunofluorescence

Skeletons were prepared following the standard Alcian Blue and Alizarin Red staining protocol. Hematoxylin-eosin and immunofluorescence staining were done on 4% PFA-fixed 6–10 μm sections of OCT-frozen dorsal skin or embryos. Oil red O staining was performed on Krt14cKO and control 4% PFA-fixed 6–10 μm sections of OCT-frozen dorsal P9 skin following standard protocol. Automated non-radioactive RNA in situ hybridization with an antisense Porcn riboprobe that is predicted to detect all known transcript variants and a sense control riboprobe was performed on skin at mouse embryos at E16.5 as described [27]. Primers for generating the riboprobe were prepared according to Biechele, et al. [27]. For immunofluorescence analysis, block-diluent solution of 5% normal donkey serum, 2% gelatin, and 0.2% Triton X-100 in PBS was used with primary antibodies to Sox9 (1:100, Santa Cruz Bionotechnology), b4-integrin (1:200, BD Biosciences), P-cadherin (1:200, R&D Systems), and Keratin 5 (1:500, gift from Colin Jamora) and FITC- or RRX-conjugated donkey secondary antibodies (1:150 and 1:200, respectively, Jackson Labs). Nuclei were stained with Hoechst.

WNT3A and WNT1 secretion assay

Human PORCN isoform B (pDONR223-PORCN, ID:7828), WNT3A (pCR-BluntII-4-WNT3A, ID: 40007188) and WNT1 (pCR4-WNT1, ID: 30915309) cDNA clones were purchased from Open Biosystems. All inserts were PCR-amplified and subcloned to pcDNA3.3-topoTA vector (Invitrogen). Since PORCN isotype D is the longest PORCN transcript, we used it to investigate the function of PORCN. Isoform D clones were generated by inserting an 18 bp fragment into the PORCN B sequence using the QuickChange® II Site-Directed Mutagenesis Kit (Agilent Technologies). PORCN isoform D mutants (p.M1I, p.R124X, p.S136F, p.G168R and p. Y359X) were also generated using this kit. Control plasmid pcDNA3.1-GFP was generously provided by Laura W. Burris (San Francisco State University). HEK293T/17 cells (CRL-11268™, ATCC) were cultured in 6-well plates to 50% confluence. For each well, 500 ng of WNT3A or WNT7A was co-transfected with 500 ng of wild type or mutant PORCN isoform D plasmids using the PolyJet™ DNA In Vivo Transfection Reagent (SignaGen). After 48 hours, cells were harvested and cell lysates were prepared for Western blot analysis. The assay was performed at least 4 times with 2 or 3 independent replicates for each. Rabbit polyclonal antibodies to Wnt5a (ab28472, Abcam), Wnt1 (ab15251, Abcam) and β-tubulin (ab6046, Abcam) were used for Western blotting. Quantification was done using ImageJ software (U.S. National Institutes of Health).

Gene expression analysis

RNA from E9.5 and E10.5 Hprt-Cre-driven conditional knockout embryos was extracted with the miRNeasy Mini Kit (217004, Qiagen). Total RNA was reverse transcribed using the qScript cDNA Supermix (Quanta BioSciences, Inc.). RT-qPCR was performed with the PerfeCTa SYBR Green FastMix (Quanta BioSciences, Inc.) on the StepOnePlus Real-Time PCR System.
Deletion of Porcn in Mice Models Human FDH

SUPPORTING INFORMATION

Figure S1 | Generation of the Porcn targeted alleles. (A) "Porcn locus" represents the wild-type (WT) locus; "Targeted Allele" contains loxP sites in introns 2 and 7 and an FRT-flanked neomycin (Neo) gene in intron 7 (Porcn-ex3-7-Neo-fllox); "Floxed Allele" retains only loxP sites after excision of Neo (Porcn-ex3-7-fllox); "Deleted Allele" lacks exons 3 through 7 (Porcn-ex3-7-del). P1–P4 indicate the location of the various genotyping primers; blue boxes represent the probes for southern analysis; E = EcoRI and S = Sall restriction sites; sizes for diagnostic fragments for southern analysis are also shown. (B) Southern analysis of ES-cell genomic DNA digested with EcoRI and hybridized with the 3′probe showing the 4.8-kb WT fragment, the 9.1-kb targeted (TG) Porcn-ex3-7-Neo-fllox fragment, and the 7.2-kb Porcn-ex3-7-fllox fragment, obtained after transplantation of correctly targeted X Porcn-ex3-7-Neo-fllox/Y ES cells with a Pipe-expressing plasmid. (C) Amplification of the targeted Porcn-ex3-7-Neo-fllox allele (439 bp) using primers P1 and P2. (D) Amplification of the Porcn-ex3-7-Neo-fllox allele (407 bp) using primers P3 and P4. (E) Amplification of the Porcn-ex3-7-del deleted allele (365 bp) using primers P1 and P4 (WT: wild type, TG: targeted, Flox: floxed, Del: deleted).

Figure S2 | Genotyping results by PCR of Prx-Cre conditional knockout mice. Amplification of the Prx-Cre (Cre), Porcn-ex3-7-fllox (Flox), Porcn-ex3-7-del (Del), and WT alleles in the Prx-Cre conditional knockout (PrxKO) (M, 1 kb ladder; F, forelimb; H, hindlimb). (B) Genotyping results by PCR of K14-Cre conditional knockout mice. Amplification of the K14-Cre (Cre), Porcn-ex3-7-fllox (Flox), and Porcn-ex3-7-del (Del) alleles in the K14-Cre conditional knockout (K14KO) (M, 1 kb ladder; 1–3, skin samples from 3 mice).

ACKNOWLEDGMENTS

We thank Isabel Lorenzo and Dr. Anthony Rodriguez for help with the generation of the Porcn mutant ES cells and mice, Dr. Roger Price and the Pathology Core of the Baylor College of Medicine Center for Comparative Medicine for performing histological analysis of tissues of the CSD256 mice, and Brooke Middlebrook for editorial assistance. We thank Dr. James Martin for the pFRT-LoxP plasmid, Dr. Laura W. Burrus for the pCDNA3.1-GFP plasmid, and Dr. Elaine Fuchs for the K14-Cre mice.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WL IBVdV HN. Performed the experiments: WI TMS AB MCL XW SW. Analyzed the data: WI TMS AB HN IBVdV. Contributed reagents/materials/analysis tools: MCI HN. Wrote the paper: WL IBVdV HN.

REFERENCES

1. Goltz RW (1992) Focal dermal hypoplasia syndrome. An update. Arch Dermatol 128: 1108–1111.
2. Goltz RW, Peterson WC, Gorlin RJ, Ravnits AG (1962) Focal dermal hypoplasia. Arch Dermatol 96: 708–717.
3. Hall EH, Terranova GT (1993) Focal dermal hypoplasia syndrome. Case report and literature review. J Am Acad Dermatol 9: 443–451.
4. Sutton VR, Van den Veyver IB (2008) Focal Dermal Hypoplasia. In: Pagon RA, Bird TD, Dolan CR, Stephens K, eds. GeneReviews [Internet]. Seattle: University of Washington.
5. Maas SM, Lombrard MP, van Esen A, Waking EL, Castle B, et al. (2009) Phenotype and genotype in 17 patients with Gorlin-Gorlin syndrome. J Med Genet 46: 716–720.
6. Sirous M, Taysir N (2011) A case report of Gorlin-Goltz syndrome as a rare hereditary disorder. J Res Med Sci 16: 836–840.
7. Howell JB, Freeman RG (1989) Cutaneous defects of focal dermal hypoplasia: an ectomesodermal dysplasia syndrome. J Cutan Pathol 16: 237–258.
8. Wang X, Reid Sutton V, Omar Peraza-Llanes J, Yu Z, Rosetta R, et al. (2007) Mutations in X-linked PORCN, a putative regulator of Wnt signaling, cause focal dermal hypoplasia. Nat Genet 39: 836–838.
9. Grzeschik KH, Bornholdt D, Oellner F, Konig A, del Carmen Boruete M, et al. (2007) Deficiency of PORCN, a regulator of Wnt signaling, is associated with focal dermal hypoplasia. Nat Genet 39: 835–835.
10. Clements SE, Mellerio JE, Holden ST, McCusley J, McGrath JA (2009) PORCN gene mutations and the protein nature of focal dermal hypoplasia. Br J Dermatol 160: 1103–1109.
11. Clements SE, Wessagowit V, Lai-Cheong JE, Arita K, McGrath JA (2008) Focal dermal hypoplasia resulting from a novel nonsense mutation, p.E300X, in the PORCN gene. J Med Genet 45: 39–49.
12. Fernandes PH, Wen S, Sutton VR, Ward PA, Van den Veyver IB, et al. (2010) PORCN mutations and variants identified in patients with focal dermal hypoplasia through diagnostic gene sequencing. Genet Test Mol Biomarkers 14: 709–713.
13. Loyvdang P, Suphapatpipat K, Wanamukul S, Shotelersuk V (2008) Three novel mutations in the PORCN gene underlying focal dermal hypoplasia. Clin Genet 73: 573–579.
14. Lasocki AL, Stark Z, Orchard D (2011) A case of mosaic Goltz syndrome (focal dermal hypoplasia) in a male patient. Australas J Dermatol 52: 48–51.
15. Froyen G, Govaerts K, Van Een H, Verbeek J, Tuomi ML, et al. (2009) Novel PORCN mutations in focal dermal hypoplasia. Clin Genet 76: 355–358.
16. Garasole A, Ferraro T, Rimland JM, Tostappen GC (2003) Molecular cloning and initial characterization of the MG61/PORC gene, the human homologue of the Drosophila segment polarity gene Porcupine. Gene 288: 147–157.
17. Hofmann K (2000) A superfAMILY of membrane-bound O-acyltransferases with implications for Wnt signalling. Trends Biochem Sci 25: 111–112.
18. Takada R, Satomi Y, Kurata T, Ueno N, Nioriska S, et al. (2006) Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. Dev Cell 11: 791–801.
19. Dubravskova L, Simova S, Cermak L, Valenta T, Korinek V, et al. (2008) Wnt-expressing rat embryonic fibroblasts suppress Apo2L/TRAIL-induced apoptosis of human leukemia cells. Apoptosis 13: 573–587.
20. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, et al. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423: 466–492.
21. Kadowaki T, Wilder E, Klingensmith J, Zachary K, Perrimon N (1996) The segment polarity gene porcupine regulates Wingless in the Drosophila development. Genes Dev 10: 3156–3167.
22. Takada K, Okabayashi K, Asahina M, Perrimon N, Kadowaki T (2000) The evolutionarily conserved porcupine gene family is involved in the processing of the Wnt protein. Nat Cell Biol 2: 1008–1016.
23. Takata N, Kitagawa Y, Kadowaki T (2002) Drosophila segment polarity gene product porcupine encodes a putative multifunctional membrane protein involved in Wingless processing. Genes Dev 10: 3116–3128.
24. Barrett J, Cash GM, Smith AP, Barrows JB, Murtagh LC (2011) Deletion of mouse Porcn blocks Wnt ligand secretion and reveals an ectodermal role in human focal dermal hypoplasia/Goltz syndrome. Proc Natl Acad Sci U S A 108: 12752–12757.
26. Clements SE (2009) Importance of PORCN and Wnt signaling pathways in embryogenesis. Am J Med Genet A 149A: 2050–2051.
27. Bi, J, Cox JJ, Rossant J (2011) Porcupine homolog is required for canonical Wnt signaling and gastrulation in mouse embryos. Dev Biol 353: 275–285.
28. Gorski JL (1991) Father-to-daughter transmission of focal dermal hypoplasia associated with nonrandom X-inactivation: support for X-linked inheritance and paternal X chromosome mosaicism. Am J Med Genet 40: 332–337.
29. Cox BJ, Vollmer M, Tampin O, Li M, Bechele S, et al. (2010) Phenotypic annotation of the mouse X chromosome. Genome Res 20: 1154–1164.
30. Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, et al. (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 93: 5860–5865.
31. Prakash SK, Cornely TA, McCaff AE, Garcia JJ, Sierra R, et al. (2002) Loss of holocytosine c-type synthase causes the male lethality of X-linked dominant microphthalmia with linear skin defects (MLS) syndrome. Hum Mol Genet 11: 3237–3244.
32. Tang SH, Silva TJ, Tsark WM, Mann JR (2002) A Cre/loxP-deleter transgenic line in mouse strain 129S1/SvImJ. Genesis 32: 199–202.
33. Takada S, Stark KL, Shea MJ, Vassileva G, McMahon JA, et al. (1994) Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev 8: 174–189.
34. Hierholzer A, Kromer R (2010) Beta-catenin-mediated signaling and cell adhesion in postgastrulation mouse embryos. Dev Dyn 239: 191–199.
35. Smigiel R, Jakubiak A, Lombardi MP, Jasovski W, Slezak R, et al. (2011) Co-occurrence of severe Goltz-Gorlin syndrome and pentadology of Cantrell - Case report and review of the literature. Am J Med Genet A 153A: 1102–1105.
36. Lombardi MP, Bulk S, Celli J, Lampe A, Gabbert MT, et al. (2011) Mutation update for the PORCN gene. Hum Mutat 32: 723–728.
37. Etheridge SL, Ray S, Li S, Hamblet NS, Lijam N, et al. (2008) Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. PLoS Genet 4: e1000259.
38. Ohota J, Myers J, Akhtar-Zaidi B, Ruzindik D, Sandesara P, et al. (2008) beta-Catenin has sequential roles in the survival and specification of ventral denticles. Development 135: 2321–2329.
39. Lu B, Ellison D, Zervas M (2011) The lineage contribution and role of Gbx2 in spinal cord development. PLoS One 6: e20940.
40. Li JV, Lao Z, Joyner AL (2002) Changing requirements for Gbx2 in development of the cerebellum and maintenance of the mid/hindbrain organizer. Neuron 36: 31–43.
41. Wassarman KM, Lewandoski M, Campbell K, Joyner AL, Rubenstein JL, et al. (1997) Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. Development 124: 2923–2934.
42. Logan M, Martin JF, Nagy A, Lobe C, Olson EN, et al. (2002) Expression of Cre recombinase in the developing mouse limb bud driven by a Prx enhancer. Genesis 33: 77–80.
43. Yamaguchi TP, Bradley A, McMahon AP, Jones S (1999) A Wnt3a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development 126: 1211–1223.
44. Patel JS, Maher ER, Charles AK (1997) Focal dermal hypoplasia (Goltz syndrome) presenting as a severe fetal malformation syndrome. Clin Dysmorphol 6: 267–272.
45. Vasioukhin V, Degenstein L, Wise B, Fuchs E (1999) The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc Natl Acad Sci U S A 96: 8551–8556.
46. Doubravska L, Krauseva M, Hirad D, Vejcechova M, Tumova L, et al. (2011) Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling. Cell Signal 23: 837–848.
47. Hancock S, Pryde P, Fong C, Bray EZ, Stewart K, et al. (2002) Probable identity of Goltz syndrome and Van Allen-Myhre syndrome: evidence from phenotypic evolution. Am J Med Genet 110: 370–379.
48. Galli LM, Buruts LW (2011) Differential palmitoylation of Wnt1 on C93 and S224 residues has overlapping and distinct consequences. PLoS One 6: e26636.
49. Stryke D, Kawamoto M, Huang CC, Johns SJ, King LA, et al. (2003) BayGenomics: a resource of insertional mutations in mouse embryonic stem cells. Nucleic Acids Res 31: 270–281.