Alopecia in a Viable Phospholipase C Delta 1 and Phospholipase C Delta 3 Double Mutant

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Abstract

Background: Inositol 1,4,5trisphosphate (IP3) and diacylglycerol (DAG) are important intracellular signalling molecules in various tissues. They are generated by the phospholipase C family of enzymes, of which phospholipase C delta (PLCD) forms one class. Studies with functional inactivation of Plcd isoforms encoding genes in mice have revealed that loss of both Plcd1 and Plcd3 causes early embryonic death. Inactivation of Plcd1 alone causes loss of hair (alopecia), whereas inactivation of Plcd3 alone has no apparent phenotypic effect. To investigate a possible synergy of Plcd1 and Plcd3 in postnatal mice, novel mutations of these genes compatible with life after birth need to be found.

Methodology/Principal Findings: We characterise a novel mouse mutant with a spontaneously arisen mutation in Plcd3 (Plcd3mNab) that resulted from the insertion of an intracisternal A particle (IAP) into intron 2 of the Plcd3 gene. This mutation leads to the predominant expression of a truncated PLCD3 protein lacking the N-terminal PH domain. C3H mice that carry one or two mutant Plcd3mNab alleles are phenotypically normal. However, the presence of one Plcd3mNab allele exacerbates the alopecia caused by the loss of functional Plcd1 in Del(9)olt1Pas mutant mice with respect to the number of hair follicles affected and the body region involved. Mice double homozygous for both the Del(9)olt1Pas and the Plcd3mNab mutations survive for several weeks and exhibit total alopecia associated with fragile hair shafts showing altered expression of some structural genes and shortened phases of proliferation in hair follicle matrix cells.

Conclusions/Significance: The Plcd3mNab mutation is a novel hypomorphic mutation of Plcd3. Our investigations suggest that Plcd1 and Plcd3 have synergistic effects on the murine hair follicle in specific regions of the body surface.

Introduction

Phosphoinositide metabolism provides an essential intracellular signalling system involved in a broad spectrum of key events in organ development and function. Phosphoinositol 4,5 bisphosphosphate (PIP2) is converted by phosphoinositide-specific phospholipase C (PLC) to inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG), which function as second messengers in various intracellular compartments. DAG activates protein kinase C, and IP3 causes the release of calcium ions from intracellular stores, which makes PLC isozymes key regulators of intracellular calcium [1–11]. The lack of PLCD4 activity in genetically ablated mice causes disturbances of liver regeneration and interferes with the acrosome reaction in spermatozoa [35,36], while over-expression of Plcd4 in a breast cancer cell line induces anchorage-independent growth [37]. In contrast, the loss of the Plcd3 gene, which is located on mouse chromosome 11, causes no phenotype suggesting that the lack of this enzyme can be compensated for [17,38], although knockdown experiments in vitro and in vivo provided evidence for an involvement of PLCD3 in cortical and cerebellar neuronal

15,17–19]. The PH domain targets PLCD proteins to PIP2 in the plasma membrane and induces activating conformational alterations of the catalytic domain, which is essential for PLCD function [13,20–29]. The activity of PLCD1 is also regulated by the interaction with other proteins such as calmodulin and small GTPases [30–32], and through site specific phosphorylation by protein kinase C alpha (PKC alpha) [29]. Some PLCD proteins can translocate to the nucleus and PIP2 derivatives play important roles in nuclear function [3,5–7,33,34].

The in vivo role of PLCD isozymes has been studied in mice with functional inactivation of Plcd1, Plcd3 and Plcd4, respectively. The lack of PLCD4 activity in genetically ablated mice causes disturbances of liver regeneration and interferes with the acrosome reaction in spermatozoa [35,36], while over-expression of Plcd4 in a breast cancer cell line induces anchorage-independent growth [37]. In contrast, the loss of the Plcd3 gene, which is located on mouse chromosome 11, causes no phenotype suggesting that the lack of this enzyme can be compensated for [17,38], although knockdown experiments in vitro and in vivo provided evidence for an involvement of PLCD3 in cortical and cerebellar neuronal
migration and neurite formation [39]. In humans, down-regulation of PLCδ3 in the right ventricular outflow tract may be associated with idiopathic ventricular arrhythmias [40] and a genomic locus associated with hypertension has been mapped near the PLCδ3 locus [41]. PLCδ3 is expressed in various human tissues and is regulated in permanent cell lines by alterations of intracellular cAMP and calcium levels [42,43].

Studies of mice with spontaneous or engineered ablation of the Pld1 gene, located on mouse chromosome 9, have revealed important roles for this PLC delta isozyme in the normal development and function of the skin and its appendages [44-47]. The Del(9)olt1Pas (synonym Del(9)Cidpl6-Slk22A14) mutation is the spontaneously arisen genetic defect of a recessive mouse mutation formerly called oligotricha (olt), which shows a combination of alopecia and male infertility. Although the Del(9)olt1Pas mutation is a large deletion on chromosome 9 encompassing the genes Cidpl6 (carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase-like), Vill (villin-like), Pld1, Dicel1 (deleted in lung and esophageal cancer 1), Acnab (acyl-Coenzyme A acyltransferase 1B, synonym thiolase B), and a part of Slc22a14 (solute carrier family 22 member 14), the alopecia of the mutant has been attributed to the loss of Pld1 [47]. Mutant mice in which Pld1 expression was disrupted by targeted gene inactivation [45,46] and Del(9)olt1Pas mutant mice show varying degrees of hair loss (alopecia) [47]. However, the disruption of both Pld1 and Plcd3 causes prenatal death in mice due to vasculature defects in the placenta [38], suggesting that both genes may co-operate with each other at least during critical phases of development.

Here, we report on a spontaneous hypomorphic mutation of Pld3 caused by the insertion of an intracisternal A particle (IAP) genome [48,49] into the Pld3 gene, which causes the predominant expression of a truncated PLCδ3 protein lacking the PH domain. Mice homozygous for both the Del(9)olt1Pas deletion and the novel mutant Plcd3mNab gene live for several weeks after birth and show total alopecia.

The hair follicle is a highly complex and dynamic part of the integument which originates from stem cells and undergoes recurring phases of growth (anagen), regression (catagen) and rest (telogen), and produces the hair shaft which contributes to the pelage [50-56]. There are several hundred mutations in mice that cause phenotypic alterations in the pelage [51], which has been pivotal in understanding the molecular mechanisms of hair follicle growth and differentiation. In this report, we show that the phases of growth and regression in hair follicles of the dorsal skin are dramatically altered in all hair follicles of mice homozygous for both the Del(9)olt1Pas deletion and the novel mutant Plcd3mNab gene.

Results

Origin of oltSH and oltNH Mice

A novel pelage phenotype, which we provisionally called oltSH (SH for sparse hair), occurred spontaneously in our stock of Del(9)olt1Pas mutant mice (also see Figure S1). In phenotypic oltSH mice, the loss of pelage by far exceeded the alopecia of homozygous Del(9)olt1Pas mutant mice during hair follicle morphogenesis, which was most pronounced on the ventral surface (Figure 1A,B,E,F) [47]. While in Del(9)olt1Pas mutant mice on postnatal day 14 merely the inguinal and medial femoral region showed a pronounced loss of pelage (arrows in Figure 1F), the oltSH mutant was virtually hairless ventrally (Figure 1F). The loss of dorsal pelage in oltSH mice increased dramatically in the first cycle of hair growth on postnatal day 24, until by postnatal day 28 merely some hairs remained in the dorsal midline and the face (Figure 1C,D,G,H). Crossing of oltSH females with Del(9)olt1Pas heterozygous males yielded phenotypic offspring of both the Del(9)olt1Pas and the oltSH phenotypes in equal numbers, indicating that one dose of the novel mutant allele had caused the exacerbation of the phenotype of Del(9)olt1Pas homozygous mutants.

In order to determine the phenotype of mice being double homozygous for the novel oltSH mutation and the Del(9)olt1Pas mutation, we set up matings of oltSH females with phenotypically normal males descending from oltSH females bred with Del(9)olt1Pas heterozygous males. Among the offspring, we found 10 mice that had not grown any visible pelage and only a few short vibrissae, which we provisionally called oltNH (NH for no hair) (Figure 1, Figure S1).

Apart from the total alopecia, oltNH mice were also smaller and weighed less than their littermates. On postnatal day 8, the body weight of wild-type mice was 5.7 ± 0.6 gr (n = 6), of oltSH mice 4.9 ± 0.3 gr (n = 3), and of oltNH mice 3.8 ± 0.1 gr (n = 3), and on postnatal day 25, wild-type mice weighed 11.5 ± 1.7 gr (n = 4), oltSH mice 9.3 gr ± 0.4 gr (n = 3), and oltNH mice 6.7 gr ± 0.8 gr (n = 3). While Del(9)olt1Pas homozygous mutants and oltSH mice have lived for more than 1 year, almost no oltNH mouse has lived longer than 40 days (n = 21) (with one exception of 64 days). Thus, the combination of the Del(9)olt1Pas mutation with the novel mutation had marked additive effects on the dorsal pelage, the body mass and the longevity.

The oltSH and oltNH Phenotypes are Associated with Altered Plcd3 Transcripts

Since we had previously identified that the Del(9)olt1Pas pelage phenotype was caused by the lack of expression of the Pld1 gene [47], we examined whether the mutation causing the oltSH and oltNH phenotypes might involve other members of the Pld gene family.

Southern blot analyses of BamHI digested genomic DNA from wild-type, oltSH and oltNH mice hybridised with a probe derived from Pld3 intron 1 (Figure 2A) revealed a restriction fragment length polymorphism (RFLP) showing the expected 4.1 kbp fragment in C3H wild-type DNA (based on the Celera mouse genome sequence), and a 4.5 kbp fragment in oltNH genomic DNA (Figure 2A).

As this BamHI fragment in wild-type genomic DNA of C3H mice stretches from intron 1 to intron 3 of Pld3, we attempted to generate short genomic fragments for sequence analyses from this area by PCR using wild-type (C3HeB/FeJ) and mutant oltNH genomic DNA. Using primers 1046 of Pld3 exon 2 and 1049 of exon 3, we amplified the expected 320 bp fragment from wild-type genomic DNA, but a 5.4 kbp fragment from oltNH genomic DNA (Figure 2B). The genomic DNA sequence of this 5.4 kbp fragment revealed the insertion of an intracisternal A particle (IAP) into intron 2 of Pld3 (Figure 2A). The IAP contains flanking LTRs and a gag-pol region, but no env sequences, is oriented opposite to the transcription of the Pld3 gene, and is in its entire length 98% homologous to an IAP recently described (gb|FJ854359.1|) [57]. The RFLP observed in BamHI digested genomic DNA was used to identify oltNH mice.

Based on the sequence data, we designed a genomic PCR screen to identify the Plcd3mNab mutation in mice (Figure 2B). In combination with the PCR screen to identify the Del(9)olt1Pas mutation [47], we have so far analysed 246 mice in 37 litters and consistently found that the genotype of phenotypically oltSH mice...
was (Del(9)olt1Pas) +/+ , Plcd3mNab +/+ (n = 21), while that of phenotypically oltSH mouse was (Del(9)olt1Pas) −/− , Plcd3mNab +/+ (n = 24), with + referring to the respective wild-type allele. We also identified 8 phenotypically normal Plcd3mNab −/− mice that had at least one wild-type allele of Plcd1.

Northern blot analyses of total RNA obtained from dorsal skin demonstrated that mice of the oltSH or oltNH phenotype lacked expression of Plcd1 mRNA (Figure 2C) and expressed truncated Plcd3 transcripts (Figure 2C). While oltSH mutants expressed both the wild-type and the truncated Plcd3 transcripts, oltNH mutants expressed only the truncated Plcd3 transcript.

We also performed Western Blot analyses on protein lysates obtained from dorsal skin using monoclonal antibodies binding specifically either to the N-terminal PH domain of PLCδ3 (4H 5–9) or to the more centrally localised catalytic domain (IF12–15) (Figure 2D). The antibody directed against the PH domain detected the 88 kDa full-length PLCδ3 protein in wild-type mice, the catalytic domain detected the 88 kDa band in wild-type mice, and a band of approximately 75 kDa in oltNH mice. In the oltSH mutant, both the 88 kDa and the 75 kDa protein were found (Figure 2B). These experiments suggest that the Plcd3mNab mutation contributed to the oltSH and oltNH phenotypes by expressing a truncated PLCδ3 protein variant that lacks the PH domain.

Altered Plcd3 transcripts and PLCδ3 protein were also found in phenotypically wild-type mice (example in lane 2, Figure 2C and D) that expressed Plcd1 normally, confirming that the expression of an altered Plcd3 transcript is not sufficient to cause a phenotype by itself, but only in combination with the loss of Plcd1 expression [38].

Thus, Plcd3 is not required for normal hair follicle morphogenesis in dorsal skin in the presence of at least one wild-type allele of Plcd1, but truncation of PLCδ3 in Plcd1-defective mice aggravates the loss of dorsal pelage suggesting that PLCδ3 expression may compensate at least partially for the loss of Plcd1 expression in dorsal hair follicles.

Hair Follicle Morphology of oltSH and oltNH Mutant Mice

The absence of visible pelage in oltNH mice and its sparseness in oltSH mutants is not caused by the absence of hair follicles, but the disability of the hair shafts to penetrate to the surface. On postnatal day 9, wild-type (Wt) hair shafts are straight and penetrate through the pilary canal to the surface, irrespective of being homozygous (arrows in Figure 3A) or oltNH mice during hair follicle morphogenesis (P11 and P14) and the first hair cycle (P24 and P28). The + and − indicate the wild-type and mutant allele, respectively. The genotypes given below the images were determined by genomic PCR assays as described in Figure 2 B. Mutants of the oltSH phenotype have a reduced dorsal pelage on day P11 and P14 (A and B), which becomes very sparse during the first hair cycle (C and D). Ventrally, oltSH mutants show total alopecia (E to H) compared to homozygous Del(9)olt1Pas mice (F to H), in which the coat is predominantly reduced in the medial femoral and inguinal region (arrows in F, G, and H). oltNH mutants have no pelage.

Histological Examination of the Hair Follicle Morphogenesis and First Cycle in oltNH Mutants

To analyse the stages of hair follicle morphogenesis and the first hair cycle, we investigated biopsies of dorsal skin in wild-type and oltNH mice histologically from postnatal day 2 to day 37. The wild-type specimens showed hair follicles in anagen stages from day 2 to day 12 (Figure 4A to E), in the catagen phase on day 17 (Figure 4F), followed by the resting phase, telogen, on day 19

![Figure 1. The phenotypes of wild-type, Del(9)olt1Pas, oltSH and oltNH mutant mice.](image-url)
In oltNH mutants, however, the growth phase lasted only from day 2 to day 8 (Figure 4G to I), when the hair bulbs became visibly narrower (Figure 4I) and the follicles were shorter than in the wild-type (compare Figure 4I to 4C). On postnatal days 11 and 12, the mutant hair follicles were further shortened (Figure 4J and K) and the dermal papilla had been excluded from the trailing epithelial end of the regressing follicle (Figure 4M).

Figure 2. Molecular analysis of the Plcd3<sup>mNab</sup> mutation. A. Schematic representation of the genomic context of the Plcd3<sup>mNab</sup> mutation. The Plcd3<sup>mNab</sup> mutation is caused by the insertion of 5.4 kbp of IAP sequences in intron 2 of Plcd3. Hybridisation with a probe (P) derived from intron 1 using primers 572 and 573 (Table S1) reveals a restriction fragment length polymorphism in BamHI digested genomic DNA of wild-type (+/+), oltSH (+/−) and oltNH (−/−) mice, respectively. The wild-type allele shows a 4.1 kbp fragment and the mutant allele a fragment of 4.5 kbp. The increased length of the mutant fragment is caused by the BamHI site (B) within the inserted IAP. The Plcd3 locus is shown in reverse orientation with respect to the chromosomal DNA, with Plcd3 exon 1 to the left. The intracisternal A particle (IAP) has no env sequences and is inserted in intron 2 in reverse orientation with respect to the transcription of the Plcd3 gene. B. Location of the primers used to analyse the Plcd3<sup>mNab</sup> mutant and the wild-type Plcd3 locus (f, forward; r, reverse). On the left, electrophoresis of amplified genomic DNA fragments that are indicative of the wild-type Plcd3 and the Plcd3<sup>mNab</sup> allele using the primers indicated. On the right, the PCR products from genomic DNA around Plcd3 intron 2 obtained from wild-type and oltNH mice using primers f1046 and r1049 (Table S1) are shown. The 5.4 kbp long fragment in the oltNH mutant contains the inserted IAP sequence.

cNorthern blot analysis of mutant dorsal skin. A DIG-labelled probe derived from the 5′ region of Plcd1 hybridises to transcripts only in phenotypically wild-type animals. A DIG-labelled RNA probe derived from the 3′ region of Plcd3 hybridises to two transcripts of 3 kb and 2.6 kb, respectively (marked by arrowheads). oltSH mice express both transcripts, while oltNH mice show only the 2.6 kb transcript. Note that the wild-type mouse in lane 2 shows the mutant Plcd3 transcript and expresses Plcd1. Hybridisation with a Gapdh-specific probe as loading control is also shown.

D. Western blot analysis of mutant dorsal skin using antibody IF12–15 directed against the catalytic region of PLCD3 protein and 4H 5–9 directed against the PH domain of PLCD3. The phenotypes of the mice (Wt, oltNH, oltSH), from which the lysates were obtained, are given on top of each lane. The 2.6 kb mutant Plcd3 transcript is translated to a truncated protein of 75 kDa, which is detected by the antibody IF12–15, but not the PH domain-specific antibody 4H 5–9 (lanes 2 and 3). This antibody also bound to an unknown protein of 110 kDa in all samples. Arrowheads indicate the wild-type protein. An immunoblot using antibody against actin is given below as a control.
Figure 3. Histology of the infundibular region and distorted hair shafts. Methacrylate (Technovit 7100) sections of representative areas in the dorsal skin on postnatal day 9, HE stain. The phenotype (Wt, olt (i.e. Del(9)olt1Pas), oltSH or oltNH, respectively) and genotype with respect to the Pld1 (Pld1* "-" refers to the Del(9)olt1Pas mutation) and Pld3 (Pld3** "-" refers to the Pld3** allele) gene is indicated for each image. At least 4 biopsies of each genotype have been investigated. In E, Bar = 25 μm. These is no hair loss and are no distorted hair shafts neither in wild-type mice

Wt Pld1* +/- Pld3** +/-  

Wt Pld1* +/- Pld3** -/-  

Wt Pld1* +/- Pld3** +/-  

olt Pld1* -/- Pld3** +/-  

oltSH Pld1* -/- Pld3** +/-  

oltNH Pld1* -/- Pld3** -/-  

Pld1 and Pld3 in the Murine Hair Follicle

Figure 3. Histology of the infundibular region and distorted hair shafts. Methacrylate (Technovit 7100) sections of representative areas in the dorsal skin on postnatal day 9, HE stain. The phenotype (Wt, olt (i.e. Del(9)olt1Pas), oltSH or oltNH, respectively) and genotype with respect to the Pld1 (Pld1* "-" refers to the Del(9)olt1Pas mutation) and Pld3 (Pld3** "-" refers to the Pld3** allele) gene is indicated for each image. At least 4 biopsies of each genotype have been investigated. In E, Bar = 25 μm. These is no hair loss and are no distorted hair shafts neither in wild-type mice.
heterozygous for both mutant alleles (A), nor those heterozygous for the Del(9)olt1Pas mutation and homozygous for the Plcd3mNab allele (B), nor others being wild-type for Plcd1 and homozygous for the Plcd3mNab mutant allele (C). Arrows indicate normal hair shafts. Arrowheads mark distorted hair shafts in Del(9)olt1Pas (olt), oltSH and oltNH mice. The alterations of the hair shafts appear histologically similar in all three mutant specimens.

doi:10.1371/journal.pone.0039203.g003

(Figure 4J* and K*). This premature regression of the mutant hair follicles was accompanied by the appearance of numerous granulocytes in the adjacent subcutaneous tissue (marked as G in Figure 4I* and J*). By day 17, oltNH mutants had re-entered a growth phase and showed anagen hair follicles still on day 19 (Figure 4L and S). After a further regression phase on days 22 and 24 (Figure 4T and U), in which the dermal papilla had been excluded again from the lower end of the hair follicle (Figure 4T* and U*), the mutant hair follicles entered into another growth phase by day 30 (Figure 4V, V*, W and W*), which ended in a telogen phase by day 37 (Figure 4X and X*).

We also quantified these observations measuring the hair follicle length (Figure 5A) and the width of the hair bulbs (Figure 5B). These measurements of hair follicle length corroborate that oltNH mutant hair follicles have completed three growth phases by day 37, when wild-type hair follicles are still in the middle of the second growth phase, i.e. the first cycle anagen (Figure 5A). They also show that postnatal day 6, the width of the oltNH hair follicle bulb is significantly reduced compared to the wild-type (Figure 5B).

Thus, all hair follicles in oltNH mutants lacking functional Plcd1 and expressing only the mutant Plcd3mNab allele show shortened phases of growth and regression that are not synchronous with the morphogenesis and cycle stages of hair follicles in wild-type mice.

We also examined oltSH mutants histologically on postnatal day 12. When wild-type hair follicles were in anagen and oltNH mutant hair follicles were in regression (Figure 4E and K), oltSH exhibited hair follicles in anagen and regression side by side (Figure 6A to C). Inflammatory infiltrates consisting of neutrophilic granulocytes were found at the border between dermis and in the subcutaneous layer in the vicinity of regressing hair follicles (Figure 6D). As Plcd3 is expressed in all hair follicles during anagen, the heterogeneity of hair follicle stages in oltSH mutants on day 12 compared to wild-type and oltNH mutants may suggest that the isolated premature entry into regression could possibly be caused by the limited concentration of unknown stimulating or negative factors exceeding a threshold for some, but not all hair follicles.

Proliferation and Apoptosis in oltNH Hair Follicles

As the histological investigation had suggested that oltNH hair follicles enter a phase of regression by postnatal day 8, we examined proliferation (Figure 7) and apoptosis (Figure 8) in mutant and wild-type hair follicles during this critical period.

Using PCNA immunoreactivity as a marker for proliferation, we found that cells in the matrix of wild-type hair bulbs proliferate from days 2 to 12 (Figure 7A to E). Similar PCNA immunoreactivity was detected in oltNH mutant hair bulbs from day 2 to day 8 (Figure 7F to H). While there was still some faint PCNA immunoreactivity in the histologically regressing hair follicles in the oltNH mutant on day 11 (arrowheads in Figure 7I), there was none left on day 12 (arrowheads Figure 7J). Thus, proliferation of precursor cells in the hair bulb of oltNH mutants does not continue beyond day 11.

Using the TUNEL assay as an indicator of apoptosis, we found no sign of apoptosis in the oltNH hair bulb during the early anagen stage of hair follicle morphogenesis on day 2 (Figure 8A) and the following anagen phase on day 25 (Figure 8G), while numerous TUNEL positive cells were found in the bulb region of mutant hair follicles on day 6 to 13 (arrowheads in Figure 8B to F).

Throughout these stages examined in the oltNH mutants, hair bulbs of wild-type hair follicles showed no TUNEL positive cells (not shown), but some TUNEL positive cells in the inner root sheath (arrowheads in Figure 8H), which has been observed before [58]. Thus, in the oltNH hair matrix, cell proliferation and apoptosis co-exist from postnatal days 6 to 11, which may explain why the hair bulbs of the mutant are not the same size as those of the wild-type after day 6. These observations suggest that expression of Plcd1 and Plcd3 may be required for proliferation and survival of matrix cells in dorsal hair follicles.

Expression of Plcd3 During Hair Follicle Morphogenesis

To identify cell types and developmental stages possibly affected by the Plcd3mNab mutation in oltSH and oltNH mutants, we examined the expression of Plcd3 during hair follicle morphogenesis in wild-type C57BL/6J mice using a probe covering Plcd3 sequences from exon 3 to exon 5. Plcd3 expression was found from postnatal days 2 to 14 in the hair bulb (arrows in Figure 9A to D), as well as in the trichal bulbs during catagen on day 17 (arrows in Figure 9E) and the cells surrounding the club hair during telogen (arrow in Figure 9F). Plcd3 is also expressed in the inner root sheath and cortex (arrowheads in Figure 9B to D), the medulla (insert in Figure 9C) and the epidermis (double arrowhead in Figure 9A,B,E,F). We also detected expression in the dermis on day 2, day 4 and day 17 (Figure 9A,B,E,F). The expression of Plcd3 in the hair matrix supports a possible involvement of Plcd3 in regulating the proliferation and survival of these progenitor cells.

Expression of Structural and Regulatory Genes in oltNH Mutants

To elucidate which cellular mechanisms might underlie the histological characteristics associated with the oltSH and oltNH mutations, we also investigated the expression of some genes involved in the growth and differentiation of the hair follicle as well as other genes encoding structural proteins of the outer and inner root sheath, and the hair shaft (Table S2). Semi-quantitative RT-PCR analysis on postnatal day 8 revealed no striking differences between wild-type, oltSH and oltNH dorsal skin hair follicles with respect to the expression of genes encoding several structural proteins like epidermal and outer root sheath keratin Krt5, hair shaft keratins Krt85 and Krt55, IRS keratin Krt71 and keratin associated proteins (Ktap11-1, Ktap3-3, Ktap4-7, Ktap9-1) [59–63]. Three important transcription factors involved in the transcription of hair keratin and keratin associated protein encoding genes in mice and humans [51,64–74], Foxn1, Mnx2 and Hoxc13, showed unaltered expression between wild-type and oltSH and oltNH mutants. The expression levels of genes encoding for secreted signalling proteins Pdgfa, Pdgfb, Shh, Bmp2 and Bmp4 were also unchanged (Figure 10) [75–81]. However, Ktap12-1 (in the hair cuticle) and Crisp1 (in the hair medulla) [82] were clearly expressed at lower levels (Figure 10). In situ hybridisations using a gene-specific probe revealed that Crisp1 is expressed in the medulla of the hair shaft in wild-type, but not oltNH mice on postnatal days 6 and 8 (Figure 11) confirming our RT-PCR data.

Discussion

In this report, we demonstrate that mice lacking expression of both functional Plcd1 due to a genomic deletion in the Del(9)olt1Pas
Figure 4. Histology of wild-type and oltnH hair follicles during hair follicle morphogenesis and the beginning of the first cycle. Paraffin and methacrylate (Technovit 7100) sections, HE stain. Postnatal days examined are indicated. In wild-type animals, the hair follicles increase in length during anagen from P2 to P12 and show active melanogenesis in their hair bulbs during this period (arrows in A to E). The diameter of the hair bulb decreased from P6 to P11 and remained like this until catagen sets in on postnatal day 17 (F). In oltnH mice, however, the hair follicles decrease in length after postnatal day 6 (G to K) and exclude the dermal papilla from the bulb on days 11 and 12 (arrows P in J* and K*). Numerous granulocytes (G) are found in the vicinity of the mutant hair bulbs at this time (marked as G in I* and J*). The diameter of the hair bulb decreases remarkably after postnatal day 6 (arrows in G to K). While wild-type mice have entered catagen by day 17 as shown by the long epithelial strand and reduced hair follicle length (arrows in F), oltnH hair follicles re-enter an anagen phase on days 17 to 19 (L and S) exhibiting a broad hair matrix (marked as M in L* and S*), a large dermal papilla (marked as P arrow in S*) and active melanogenesis (L* and S*), which is followed by a regression on postnatal days 22 (T and T*) and 24 (U and U*). While wild-type hair follicles proceed through the first cycle anagen from day 22 to day 37 (arrows in N to R), oltnH hair follicles re-enter anagen on postnatal day 25 (V, marked as P arrow in V*) and show continued increase in hair follicle length by day 30 (W and W*). This growth phase of the mutant follicle ends in a telogen phase on day 37 (X and X*), when wild-type follicles are still in the growth phase (R). Three biopsies from different animals were used for each time point investigated. P, dermal papilla; SG, sebaceous gland; M, matrix; G, neutrophilic granulocytes. Bar = 100 µm in images A to X, and 50 µm in images G* to L* and S* to X*.

doi:10.1371/journal.pone.0039203.g004
mutant and functional Plcd3 due to a genomic insertion of an IAP show total alopecia, weight loss and die during the first two months of life. While Del(9)olt1Pas homozygous mutant mice show a mild predominantly ventral alopecia due to the deletion of the Plcd1 gene [47], homozygotes of the Plcd3mNab mutant allele have no obvious phenotype, which has also been described for mice in which Plcd3 was inactivated by homologous recombination [38]. As experimental inactivation of both genes by homologous

Figure 5. Histomorphometric analysis of hair follicle length and hair bulb diameter. Measurements were taken from a sample of 150 hair follicles in three different biopsies using the Image J software. Statistical analysis using the paired t-Test was performed employing the GraphPad Prism4 software. Data are expressed as mean ± SEM. *** signifies p<0.001. White columns depict data from wild-type mice and chequered columns those from oltNH mice. The age of the mice is given on the X axis. Hair follicle length represents the distance from the infundibulum to the most distal part of the hair follicle. The widest diameter of the hair bulb or distal end of the hair follicle in catagen and telogen stages is shown as “hair bulb diameter”. Both parameters indicate that oltNH mice terminate their first postnatal anagen by day 14 and re-initiate a growth phase thereafter, which in turn ends by day 24. The second cyclic growth phase of oltNH hair follicles ends by day 37.

doi:10.1371/journal.pone.0039203.g005
recombination causes early prenatal death with defects in the placenta [38], the novel Plcd3mNab allele described in this report behaves like a hypomorphic mutation of the Plcd3 gene allowing for a limited period of postnatal life. While in oltNH mutants the bulk of Plcd3 transcripts are shorter and immunoblots predominantly show a truncated PLCD3 protein in oltNH mice, apparently normal transcripts of Plcd3 can also be amplified by RT-PCR using primers in exon 1 and exon 5 (not shown). Therefore, we cannot exclude that trace amounts of normal transcript, too little to be detected in Northern blots, are expressed from the mutant Plcd3mNab allele, just sufficient to overcome the placental defects of the double knockout mice. Still, the oltNH mutant offers the unique opportunity to study postnatal functions of Plcd1 and Plcd3.

Many spontaneous mouse mutations are the result of insertions of retroviral elements, recently reviewed in [48,49,83]. Subtypes of IAPs are particularly active in the C3H/HeJ strain of mice [84], which is related to the genomic background of the Del(9)olt1Pas mutant mice, in which the Plcd3m1Nab mutation occurred. Similar IAP insertions with 98% sequence homology to the Plcd3m1Nab IAP have recently been described in various mutant mice [57]. Due to the promoter and enhancer elements in the viral LTRs, such insertions into the genome can either increase or decrease the expression of adjacent host genes, or enhance the expression from endogenous promoters, as well as cause aberrant splicing [48,49]. As in the case of the Plcd3m1Nab mutation, the IAP is integrated in reverse orientation with respect to the transcription of the Plcd3 gene, it is most likely that it causes its effects by enhancing transcription from cryptic promoters. The Plcd3 transcript variant ENSMUST00000128650 starting in exon 5 would be predicted to have a molecular weight well within the range of the oltNH mutant.
PLCD3 protein detected in our immunoblots. This truncated PLCD3 protein would have no PH domain, which is important for the targeting of the enzyme to the substrate in the plasma membrane [23,25].

Mutant mice homozygous for the Del(9)olt1Pas mutation [47], oltSH and oltNH mutants (this report) exhibit a similar histological aspect of fragile hair shafts, but differ with respect to the distribution of this defect over the body surface. Similar fragility of hair shafts has been found in mice with altered expression of Foxn1 and Hoxc13, which are directly involved in the expression of hair keratins and keratin associated proteins [60,61,64–73,85,86]. The co-expression of Foxn1 and Plcd1 in the pre-cortical zone of the hair bulb and the down-regulation of Plcd1 in nude mice suggested that Plcd1 has some function downstream of Foxn1 [46,68,73]. We found that the expression domain of Plcd3 encompasses the entire hair bulb during anagen including and exceeding the domain of Plcd1. It is therefore possible that Plcd3 can compensate for some aspect of Plcd1 function. Since we demonstrated that on postnatal day 8 the medulla-specific, Hoxc13-regulated gene Cnp1 is down-regulated in oltNH mice, but not in oltSH mice, some hair shaft-specific genes may depend strictly on the expression of Plcd3. This may imply that in the absence of normal Plcd1 expression, Plcd3 could possibly play a role in the Hoxc13–Foxp1 axis of gene regulation in the hair medulla [82,86].

It has previously been suggested that the alopecia of mice with functional inactivation of Plcd1 develops in the context of an inflammatory response [44]. We also observed neutrophilic granulocytes in oltNH skin, but also in oltSH mutants, in which at least some hair follicles were apparently in anagen. As the infiltrates in day 12 oltSH skin were associated with hair follicles in premature catagen, the influence of the inflammatory response is possibly very locally elicited and operative, but may contribute to the sustenance of the abnormal hair follicle regression in the mutant.

Recently, Plcd1 was shown to exert direct effects on adipocytes. Knockdown of Plcd1 in an adipocyte cell line interfered with lipid accumulation during differentiation, which was also observed in primary cells obtained from Plcd1 defective mice [87]. This serves to explain the reduced body mass in mice lacking Plcd1 expression. Further studies will reveal whether Plcd3 is also involved in adipocyte differentiation and function in order to explain, why oltNH mice show an even more dramatic reduction in body mass than mice lacking functional Plcd1 alone. Inflammatory infiltrates in oltNH mice were usually found in the subcutaneous adipose tissue near the dermis, where adipocyte precursors are located. Most recently, the stimulatory activity of BMP (bone morphogenetic protein) and PDGFA (platelet-derived growth factor A) secreted by subcutaneous fat cells and their precursors, respectively, has been highlighted with respect to their stimulating activity for hair follicle stem cells and the sustenance of anagen [88]. The inflammatory response in the subcutaneous adipose layer of oltNH mice could possibly negatively interfere with the stimulatory activity of this tissue, which could contribute to the curtailment of the growth phase in the mutant hair follicle.

Phospholipase C delta isozymes are associated with signal transduction processes involved in cell cycle regulation and cell proliferation [2,7,30,31,35,89–92], and are altered in various
tumours and tumour cell lines [93–97]. PLCD1 protein can also translocate to the nucleus and exert direct effects in the nuclear compartment [6,7,33,34,98]. We found that growth in the hair follicle bulb is uniformly short-phased in the oltNH mutant after birth and ceases on postnatal day 12, while anagen was less shortened in the lower ventral body region of Plcd1 defective Del(9)olt1Pas mutants, where the alopecia of this mutant is most pronounced [47]. Since Plcd3 is expressed in the hair bulb during this critical period, Plcd1 and Plcd3 may possibly play additive roles in the sustained growth during hair follicles morphogenesis after birth.

Materials and Methods

Ethics Statement

The animals were sacrificed according to §4.3 of the German law for the protection of animals (Tierschutzgesetz) (file number 50.203.2-BN 6/02). The mice were killed by cervical dislocation avoiding unnecessary pain.

Mice

We have previously described the origin of Del(9)olt1Pas mutant mice (synonym Del(9Ctdspl-Slc22a14)1Pas, formerly called oligotriche, olt) [47]. The oltSH and oltNH mice were in a mixed C3He/Orl, C3H/HeJ and C3Heb/FeJ background, which we collectively refer to as C3H in this report. The animals were kept in a 12 hour light/dark cycle with food and water ad lib.

Histology, Immunohistochemistry and In Situ Hybridisation

Skin biopsies were taken from the dorsal mid-thoracic region of mice after killing them by cervical dislocation. The skin biopsies were fixed for 4 hours in Bouin’s solution, dehydrated in increasing alcohol concentrations and embedded in paraffin as described [47]. Histological staining with haematoxylin/eosin and immunohistochemistry with mouse monoclonal antibodies against PCNA (proliferating cell nuclear antigen) (Bio-Genex, MU252-KL), diluted 1:200 and Cy3-conjugated goat anti mouse IgG F(ab)2

Figure 8. Apoptosis in oltNH hair follicles. Apoptosis was investigated by performing the TUNEL assay on paraffin sections. Apoptosis is visualised by Cy3 fluorescence (red signal). DAPI was used as a nuclear counterstain (blue signal). Sections were taken from skin biopsies of oltNH mice (A to G) from postnatal day 2 to 25. A wild-type skin section on postnatal day 13 is shown as control (H). Three biopsies from different animals were used for each time point investigated. Arrows mark the hair bulb (B) or the trailing ends (T) of regressing hair follicles. Arrowheads point at TUNEL positive cells. There are numerous TUNEL positive cells in the matrix of oltNH hair follicles beginning on days 6 and 8 (arrow heads in B and C) and the regressing follicles on day 11 to 13. On day 25, the oltNH hair follicle re-enters anagen and shows no TUNEL positive cells (arrow in G). Throughout this period, there were no TUNEL positive cells in the hair bulbs of wild-type mice (not shown), only some cells in the inner root sheath exhibited a TUNEL positive signal (arrowheads in H), while the medulla showed unspecific autofluorescence. E, epidermis. Bar in E = 50 μm for all images.

doi:10.1371/journal.pone.0039203.g008
The TUNEL assay was performed on paraffin sections using the Apoptag Red In Situ Apoptosis Detection Kit (Millipore Chemicon, S7165) according to the manufacturer's recommendations. Nuclei were stained with DAPI.

Tissue sections of three different biopsies of dorsal skin of wild-type and oltNHmice were analysed histomorphometrically with respect to the length of the hair follicle (from the distal end to the infundibulum) and the width of the hair bulb at its widest diameter. 50 measurements of both parameters were taken from each biopsy using the ImageJ software and statistically analysed with the paired t test using PrismGraphPad 4 software.

PCR and RT-PCR

PCR experiments were performed in a TProfessional Basic Thermocycler (Biometra). Usually 35 cycles were run with the optimal annealing temperature chosen according to the recommendation of the supplier of Phusion high fidelity DNA polymerase (Thermo Scientific, Finnzymes, Finland). All primers (Tables S1 and S2) were purchased from Eurofins MWG BIOTECH AG (Ebersberg, Germany). PCR products were sequenced by Eurofins MWG BIOTECH AG, Ebersberg, Germany.

For RT-PCR experiments, the cDNAs were synthesised from total RNA (RNasey Midi Kit, QIAGEN, Hilden, Germany) or mRNA (Oligotex mRNA mini kit, QIAGEN) from skin biopsies using PowerScript™ reverse transcriptase from CLONTECH (Palo Alto, CA) with an Oligo (dT)15 primer. PCR was performed on templates of cDNA or genomic DNA prepared from tail biopsies (DNeasy Mini Kit, QIAGEN, Hilden, Germany) using Phusion high fidelity DNA polymerase (Finnzymes, Finland) according to the manufacturer’s recommendations.

The cDNA preparations synthesised from mRNA were used to amplify expressed sequences of genes that have only one exon, e.g., some Krtap genes. Gene-specific fragments were amplified from 1 μg of cDNA with the optimal annealing temperature chosen according to the recommendation of the supplier of Phusion high fidelity DNA polymerase (Thermo Scientific, Finnzymes, Finland). The number of PCR cycles run was usually 35, or is otherwise given in the Figure legend.
**RNA Blot Hybridisation**

To synthesize DIG labelled cRNA probes, RT-PCR products were re-amplified using a modified antisense primer, to which the sequence of the T7 RNA polymerase binding site (GGATCC-TAATACGACTCACTATAGGG) had been added at its 5’ end. Antisense cRNA probes were then derived from these PCR products by in vitro transcription in the presence of DIG-labelled dUTPs with T7 RNA polymerase from Roche (Mannheim, Germany) [47].

The cRNA probe for Northern blot hybridisations to *Plcd3* mRNA was generated from skin cDNA using primers 511 (exon 11) and 512 (3’UTR) (Table S1). DIG labelled probes for RNA blot hybridisations were synthesised by PCR using the PCR DIG probe synthesis kit (Roche). The cRNA probe for *Plcd1* has been described [47].

**In Situ Hybridisation**

The 350 bp *Plcd3*-specific cRNA probe was generated as described above from the RT-PCR product synthesised with forward primer 527 (exon 3) and reverse primer 1532 (exon 5) (Table S1) and the 400 bp Crisp1 probe was generated in the same fashion using forward primer FR634 and reverse primer FR635 (Table S2). In situ hybridisations were performed as described [47].

Because the histochemical stain for alkaline phosphatase activity in Bouin-fixed tissue sections gives a rather brownish colour and is difficult to distinguish from pigmented areas, skin biopsies were taken from C57BL/6J mice that have mostly black eumelanin and the sections were photographed and analysed using a Nuance RX multispectral camera (obtained from INTAS, Goettingen, Germany) with the manufacturer’s software. Areas displaying spectral characteristics of eumelanin were pseudo-coloured in black, those displaying spectral characteristics of the specific in situ signal in red.

**DNA Blot Hybridisation**

Genomic DNA was digested with BamHI (Fermentas), the fragments separated by agarose gel electrophoresis and transferred to positively charged Nylon membrane (Roche) by vacuum blotting using a Model 785 Vacuum Blotter (BIORAD). Hybridisation was carried out in Dig Easy Hyb (Roche) using 100 ng/ml of DIG labelled probe. Washes and detection of bound DIG were carried out as described in the Roche DIG Application Manual for Filter Hybridisations (https://www.roche-applied-science.com/PROD_INF/MANUALS/DIG_MAN/dig_toc.htm).

DIG labelled probes for DNA blot hybridisations were synthesised by amplifying genomic fragments using the PCR DIG probe synthesis kit (Roche). For hybridisations to *Plcd3*-specific genomic fragments, we amplified a 900 bp fragment of intron 1, 800 bp upstream of exon 2, using primers 572 and 573 (Table S1) (marked as P in Figure 2A).}

**Western Blot**

Mouse monoclonal antibodies binding specifically to the catalytic domain of the PLCD3 protein (IF12–15) or the PH domain of PLCD3 (4H 5–9), respectively, were supplied by K. Fukami. The hybridoma supernatant was used undiluted. The secondary antibody, (peroxidase-conjugated goat anti mouse Ig, Dianova) was used at a dilution of 1:30.000. Blotting and signal detection were performed as described [47].

**Supporting Information**

**Figure S1 Origin of *oltNH* mice.** Pedigree showing the breeding scheme that led to the discovery of *oltNH* mice. White symbols represent wild-type mice and blue symbols mice with the phenotype of Del(9)olt1Pas homozygotes. Red symbols represent mice of the *oltSH* phenotype and yellow symbols are mice of the *oltIH* phenotype. The symbols merely show the presence of such phenotype in a litter, but do not represent the relative proportion of phenotypes in each litter. The numbers designate the crosses.
referred to in the following text. All mice in the F1 generation of a Del(9)olt1Pas female mated with a wild-type C3HeB/FeJ male (cross 1) were phenotypically normal. In the F2 generation of cross 2, (56 mice in 7 litters), we found 10 phenotypically Del(9)olt1Pas homozygotes and one female that showed a greater extent of alopecia, which we termed oltSH (for sparse hair). This female was backcrossed with her father (cross 3) resulting in one litter of six, in which 2 of 6 mice showed the oltSH phenotype. When one of these oltSH females was crossed with a known Del(9)olt1Pas heterozygous male (cross 4), the phenotypically altered offspring showed in equal parts the oltSH and the Del(9)olt1Pas homozygous phenotype, suggesting that one dose of a novel mutation could exacerbate the

Figure 11. Crisp1 expression in wild-type and oltNH hair follicles. In situ hybridisation with a gene-specific probe for Crisp1 on skin sections of wild-type (A and C) and oltNH (B and D) mice on postnatal days 6 (A and B) and 8 (C and D). The DIG-labelled probe was visualised using alkaline phosphatase-conjugated anti DIG antibody. Images were taken in brightfield mode with a Nuance VX camera and further processed by spectral analysis using the accompanying software. The specific in situ signal spectrum was pseudo-coloured in red and the eumelanin spectral signal in black. Three biopsies from different animals were used for each time point investigated. Bar = 100 μm. Crisp1 expression is detected in the medulla of wild-type hair follicles (arrows in A and C, red signal), but not in comparable sections of oltNH mice (arrows in B and D). doi:10.1371/journal.pone.0039203.g011
alopecia in Del(9)oltPas homozygous mice. When the olSH founder female of the F2 generation was crossed with one of the phenotypically normal sons in cross 5, we found altogether 52 mice with different phenotypic alterations in the offspring (n = 121 in 21 litters): the Del(9)oltPas mutant phenotype, the olSH phenotype, but all mice that did not develop any visible pelage and only a few short vibrissae, which we termed olNVH. Further brother-sister matings of this offspring (cross 6) again produced all three mutant phenotypes.

Table S1 List of Plcd3-specific primers. The oligonucleotide sequences are given in 5' to 3' orientation; genome coordinates are given according to NCBI37 mm9 July 2007; “Plcd3 genomic” indicates the location of the oligonucleotide sequence in the Plcd3 locus.

| Primer Name | Sequence | Genome Location |
|-------------|----------|-----------------|
| Fw1         | TCTGCTAGCCTCAGAGAGACAGGGGCTTGGG | 41309828-41309838 |
| Rev1        | GCTTGGTGGGTTTTTGGTTTCTTTTCTTTT | 41309828-41309838 |

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